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EXPERIMENTAL ARTICLES

Symbiosis between the Root-Nodule Bacterium *Sinorhizobium meliloti* and Alfalfa (*Medicago sativa*) under Salinization Conditions

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Abstract—Two hundred forty-three isolates of alfalfa root-nodule bacteria (*Sinorhizobium meliloti*) were obtained from nodules and soils sampled in the northern Aral region, experiencing secondary salinization. Isolates obtained from nodules (N isolates) were significantly more salt-tolerant than those from soils (T isolates) when grown in a liquid medium with 3.5% NaCl. It was found that wild species of alfalfa, melilot, and trigonella preferably formed symbioses with salt-tolerant root-nodule bacteria in both salinized and nonsalinized soils. Only two alfalfa species, *Medicago falcata* and *M. trautvetteri*, formed efficient symbioses in soils contrasting in salinity. The formation of efficient symbiosis with alfalfa in the presence of 0.6% NaCl was studied in 36 isolates (N and T) differing in salt tolerance and symbiotic efficiency. Fifteen isolates formed efficient symbioses in the presence of salt. The increase in the dry weight of the plants was 25–68% higher than in the control group. The efficiency of symbiotic interaction under salinization conditions depended on the symbiotic efficiency of the isolates under standard conditions but did not correlate with the source of root-nodule bacteria (soil or nodule) or their salt tolerance. The results indicate that the strains of root-nodule bacteria forming efficient symbioses under salinization conditions can be found.

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Key words: natural isolates, *Sinorhizobium meliloti*, *Medicago sativa*, cryptic plasmids, symbiosis efficiency, symbiosis under salinization conditions.

Soil salinization is a great threat to agriculture. At present, about 40% of lands, including irrigated lands, contain elevated concentrations of mineral salts [1]. Use of leguminous crops, forming symbioses with root-nodule bacteria (rhizobia), is a promising method of soil improvement, as they are stress-resistant and capable of improving soils by fixation of atmospheric nitrogen. Cultivated alfalfa (*Medicago sativa* L.) is a candidate for bioremediation. It is highly resistant to drought and frost and moderately tolerant to salinity [2].

Numerous papers have been dedicated to the formation of symbioses between root-nodule bacteria and various legume species under salinization conditions [3–6]. In legumes, high salinity causes suppression of photosynthesis and reduces the yield of dry mass of stems, roots, and nodules [7]. The survival of root-nodule bacteria in soil and rhizosphere decreases, the time of cell generation increases, and the cell ultrastructure is disrupted [3, 4, 8]. Nevertheless, it is still unknown whether root-nodule bacteria can affect the tolerance of nitrogen-fixing symbioses with regard to salt, an abiotic adverse factor.

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In this work, we studied the influence of salt tolerance and symbiotic properties of root-nodule bacteria on their symbioses with alfalfa at high salinity. We used a collection of natural isolates of the nodule bacterium *Sinorhizobium meliloti* from soils and nodules of wild species of alfalfa, melilot, and trigonella, belonging to one cross-inoculated group which had been sampled in areas of the northern Aral region (Kazakhstan) differing in salinity.

MATERIALS AND METHODS

Soils were sampled in the southwestern foreland of the Mugodzhar Hills (Chelkar Region, Kazakhstan), where salinization is dominated by chlorides. The ion compositions of soil suspensions were determined in extracts from 1-g soil samples (each suspended in 10 ml of distilled water) prepared by a standard method [9]. The salinity of the soil extract was determined from its electric conductivity [10] using a DIST-3 Total Dissolved Solids Tester (Hanna Instruments, United States). The results were converted to percentages according to the equation 0.18 *dS/m* = 1.0% NaCl [2].

Table 1. Isolation of natural isolates of alfalfa root-nodule bacteria from areas differing in salinity

Soil salinity* (conductivity, dS/m)	N isolates			T isolates		
	Number of sampling locations	Wild host plant species	Number of isolates	Number of soil sampling sites	Inoculated host plant species	Number of isolates
Nonsalinized (0–0.7)	35	*Medicago varia*, *M. falcata*, *M. lupulina*, *M. trautvetter*, *Melilotus* sp., *Mel. album*, *Trigonella orthoceras*	107	31	*M. sativa*, *M. truncatula*, *M. polymorpha*	43
Weak salinity (0.7–2.0)	6	*M. falcata*, *M. trautvetteri*, *Melilotus* sp.	20	25	*M. sativa*, *M. truncatula*	38
Moderate salinity (2.0–10.0)	4	*M. falcata*, *M. trautvetteri*	7	9	*M. sativa*, *M. truncatula*	13
Strong salinity (10.0–25.0)**	3	*M. trautvetteri*, *M. falcata*	7	6	*M. sativa*	8
Total:	48	–	141	71	–	102

* Soil classification according to [2, 14].
** The maximum conductivity value of the soil extract in this experiment was 10.5 dS/m.

Root-nodule bacteria were isolated from soil extracts prepared as described above. The extracts were incubated overnight at 28°C (on a shaking platform) and used for inoculation of two-day-old seedlings of alfalfa (*Medicago sativa* L. cv. Vega, *M. truncatula*, and *M. polymorpha*) under sterile microvegetation conditions [11]. After six weeks of vegetation, one nodule was taken from each plant for isolating root-nodule bacteria by conventional methods [12]. The same methods were used for isolating bacteria from nodules of wild-host plants. Species identification of the natural isolates was performed by amplified ribosomal DNA restriction analysis (ARDRA) and randomly amplified polymorphic DNA (RAPD) analysis as in [13].

Plasmids were identified by the Eckhardt method [12]. Plasmid sizes were calculated from the logarithms of their electrophoretic mobilities in comparison with data for the plasmids of the test strain *Sinorhizobium meliloti* MVII.

Salt tolerance was estimated from the changes in the optical densities of the cultures (A_{600}), measured with an ULTROSPEC II spectrometer after three days of cultivation of the bacteria in liquid TY medium supplemented with 0.0, 3.0, 3.5, and 4.1% NaCl. The background content of NaCl in TY was assumed to be 0.0% NaCl.

The germination of *M. sativa* was studied in petri dishes (50 seeds per dish), in water solutions with 0.0–1.0% NaCl. The percentages of germinating seed were recorded on the third day.

The symbiotic performance of isolates was studied under sterile microvegetation conditions [11]. The growth substrate was 0.7% agar with Krasil'nikov–Korenyako medium devoid of nitrogen and NaCl (standard conditions [11]). Two *M. sativa* cv. Vega plants were grown in each test tube. Data from the two plants were combined for further analysis. The symbiotic efficiencies (Eff) of isolates were determined by weighing the dry matter of inoculated plants and comparing the value with the dry weight of either control noninoculated plants or plants inoculated with the efficient *S. meliloti* test strain 1021 [11]. The isolates were considered highly efficient (Eff^{++}) if they showed a significant (≥15%) increment of dry weight (compared to the result obtained for the test strain 1021), and efficient (Eff^{+}), if the increment was insignificant. Nodules were counted 14 and 42 days after the plant inoculation. Plant tests with salt was performed in the same way, salt solutions (final concentration, 0.6% NaCl) being added with the Krasil'nikov–Korenyako medium. The experiments were performed in triplicate.

Statistical evaluation of the results was performed using Statistica 6.0 and Microsoft Excel 2000.

RESULTS AND DISCUSSION

1. Isolation and Identification of Alfalfa Root-Nodule Bacteria

Natural bacterial isolates were obtained from soil samples and nodules collected in 74 locations of the northern Aral region (Kazakhstan), differing in salinity. One hundred two isolates (referred to as T isolates) were isolated from 71 soil samples. Most of them (58%) were obtained from salinized soils (Table 1). Most of the N isolates, 107 of 141 (76%), were isolated from nodules of different host plants collected from nonsalinized areas (Table 1). Ten percent of the isolates were obtained from salinized areas with two predominant alfalfa species, *M. falcata* and *M. trautvetteri*. Thus, a total of 243 natural isolates of alfalfa root-nodule bacteria were collected. They were assigned to the species *S. meliloti* according to ARDRA (see MATERIALS AND METHODS).

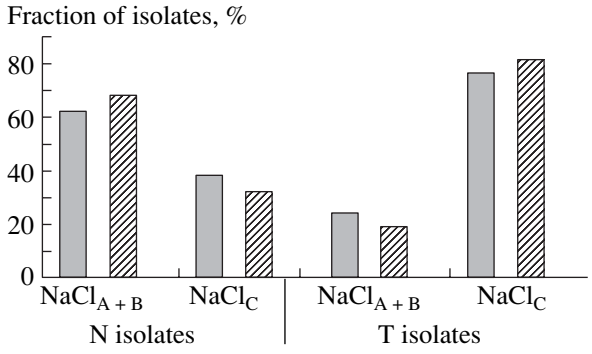

Fig. 1. Isolation of salt-tolerant and salt-sensitive *S. meliloti* N and T isolates from soils and nodules sampled in locations differing in salinity; $NaCl_{A+B}$ and $NaCl_C$ are salt-tolerance groups; ▫, salinized areas; ▨, nonsalinized areas.

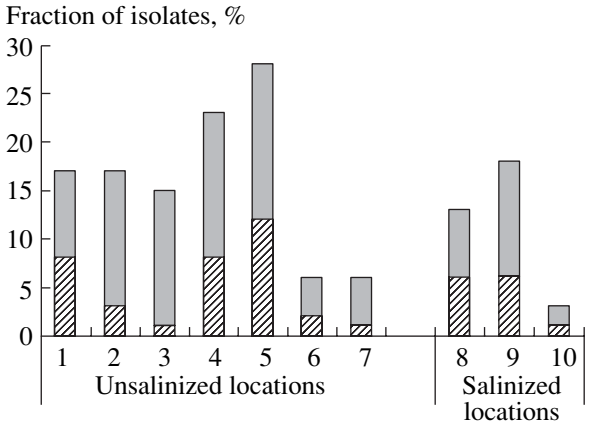

Fig. 2. Isolation of salt-tolerant and salt-sensitive *S. meliloti* N and T isolates from various wild host plant species: 1, *M. lupulina*; 2, *M. varia*; 3, *T. orthoceras*; 4, *M. falcata*; 5, *M. trautvetteri*; 6, *Melilotus* sp.; 7, *Melilotus albus*; 8, *M. falcata*; 9, *M. trautvetteri*; 10, *Melilotus* sp.; ▫, $NaCl_{A+B}$ isolates; ▨, $NaCl_C$ isolates.

2. Study of Salt Tolerance in Natural S. meliloti Isolates

Sinorhizobium meliloti isolates were cultivated in liquid TY medium at variable NaCl concentrations (see MATERIALS AND METHODS). The isolates were divided into three groups according to their growth at 3.5% NaCl: $NaCl_A$, the isolates showing increases in optical density in excess of 100 times; $NaCl_B$, isolates showing increases by a factor of 10–100; and $NaCl_C$, isolates showing increases of no more than 10 times. Isolates from groups $NaCl_A$ and $NaCl_B$ were considered to be salt-tolerant (the combination of these groups is designated $NaCl_{A+B}$), and group $NaCl_C$ was considered to be salt-sensitive. The test strain Rm1021 belonged to group $NaCl_C$.

Most T isolates (78.4%) were found to be salt-sensitive ($NaCl_C$), and their frequency of occurrence did not depend on the salinity of soil samples ($r = 0.02$, $P > 0.05$). In contrast, most N isolates (66.7%) were salt-tolerant ($NaCl_{A+B}$). They occurred at equal frequencies in nodules of wild host species from both salinized and nonsalinized soils (Fig. 1).

We found that in nonsalinized soils, salt-tolerant and salt-sensitive N isolates formed symbioses with *M. lupulina* and *M. falcata* with equal frequency. Nevertheless, most salt-tolerant N isolates were obtained from nodules of *Medicago varia*, *Melilotus albus*, and *Trigonella orthoceras* (Table 1, Fig. 2). Note that pink nodules (efficient symbiosis) were found only on the roots of *M. falcata*, *M. trautvetteri*, and *Melilotus* sp., and they largely provided salt-tolerant rhizobia (Fig. 2). The fact that a host plant prefers symbiosis with salt-tolerant root-nodule bacteria revealed in our study calls for further studies on the specificity of symbiosis formation under conditions of abiotic stresses.

3. Plasmid Content of Natural S. meliloti Isolates

Adaptive traits of root-nodule bacteria can be determined by genes located on additional nonsymbiotic (cryptic) plasmids. Their sizes reach 800 kb [15]. Analysis of plasmids of our N and T isolates revealed one to three cryptic plasmids, with sizes in the range 60–440 kb. Isolates with single cryptic plasmids of various molecular weight constituted about 65% in all salt-tolerance groups (Fig. 3). The most frequent plasmid sizes were about 150 kb (21.6% of $NaCl_{A+B}$ N isolates) and 200 kb (18.1% of $NaCl_{A+B}$ N isolates and 47.1% of $NaCl_C$ T isolates). The majority of isolates without cryptic plasmids belonged to group $NaCl_A$ (Fig. 3; a statistically significant result), whereas root-nodule bacteria with two or three cryptic plasmids showed reduced salt tolerance (groups $NaCl_B$ and $NaCl_C$). Therefore, we suggest that genes of cryptic plasmids could be involved in the regulation of salt tolerance in rhizobia. However, the increase in the amount of plasmid DNA is an additional metabolic load on the cell, which can hamper its adaptation to salt stress.

4. Determination of the Efficiency of the S. meliloti/M. sativa Symbiose in the Presence of NaCl

First, we studied the germination of *M. sativa* cv. Vega in the presence of NaCl (see MATERIALS AND METHODS) and found that it decreased to 27.7% at 0.6% NaCl and to 3.6% at 1.0% NaCl. The salt tolerance of the *S. meliloti/M. sativa* symbiotic system was studied by adding 0.1–1.0% NaCl to the growth substrate. The maximum NaCl concentration at which efficient symbiosis (pink nodules) was formed by the test strain 1021 with alfalfa was 0.6%. Two of N isolates formed pink nodules at 0.7 and 0.8% NaCl, respectively. The symbiosis became inefficient at 1.0% NaCl. The isolates formed white nodules, apparently because of impaired nitrogenase synthesis or disruption of nodule structure, resulting in the termination of symbiotic nitrogen fixation [4]. Thus, 1.0% is a threshold NaCl

Table 2. Efficiency of symbiosis between *S. meliloti* isolates and alfalfa *M. sativa* under microvegetation conditions

Isolate type[1]	Salt tolerance groups[2]	Number of isolates in salt tolerance groups	Number of isolates of the phenotype[2]			
			under standard conditions		+ 0.6% NaCl	
			Eff^{++}	Eff^{+}	Eff^{+}	Eff^{-}
N	$NaCl_{A+B}$	10	6	4	5	5
	$NaCl_{C}$	5	2	3	2	3
T	$NaCl_{A+B}$	3	0	3	0	3
	$NaCl_{C}$	18	5	13	8	10
Total		36	13	23	15	21

[1] N, nodule isolates; T, soil isolates.
[2] For designations of salt tolerance groups and phenotypes see the text.

concentration for both the germination of alfalfa and the formation of the efficient *S. meliloti*/*M. sativa* symbiotic system. Under the natural conditions of the northern Aral region, the maximum concentration at which pink nodules could be detected on some alfalfa species was 0.58% NaCl (10.5 dS/m) (Table 1). For this reason, we chose the concentration of 0.6% NaCl as the limit for studying the effect of salt on symbiosis between root-nodule bacteria and alfalfa under microvegetation conditions.

Salt tolerance and symbiotic performance were studied in 36 (15 N and 21 T) isolates differing in salt tolerance and symbiosis efficiency under standard conditions (Table 2). We found 15 isolates (7 N + 8 T) that formed efficient symbioses with alfalfa under salinization conditions. The increase in dry weight of plants varied from 25 to 68% compared to the control without inoculation, because the test strain 1021 and other isolates formed inefficient symbioses with alfalfa. The weights of inoculated plants did not differ significantly from those in the noninoculated control group. Most of the isolates forming efficient symbioses with alfalfa in the presence of salt had the phenotype Eff^{++} (7 N and 3 T isolates) or Eff^{+} (5 S isolates, Table 2). This result is the first demonstration that inoculation of alfalfa with root-nodule bacteria under salinization conditions can significantly increase its dry weight and thereby favor its adaptation to stress conditions. Note, however, that only 40% of the symbiotically efficient isolates formed efficient symbiosis in the presence of salt. It follows that studies on constructing symbiotic pairs (nodule bacterium strain–host plant variety) for particular areas are promising and highly important.

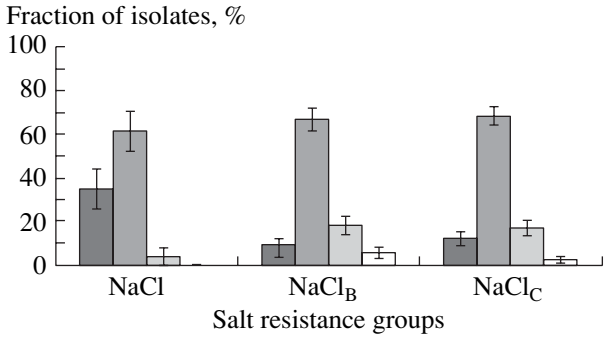

Fig. 3. Number of plasmids in *S. meliloti* isolates belonging to different salt-tolerance groups: ■, no plasmids; ▩, one plasmid; ▨, two plasmids; □, three plasmids.

Another problem was the correlation between the salt tolerance of the N and S isolates and their symbiotic efficiency in the presence of salt. Out of the 15 isolates selected, 5 belonged to group $NaCl_{A+B}$ and 10, to $NaCl_{C}$ (33.3 and 66.7%, respectively). We conclude that the salt tolerance of *S. meliloti* has no significant effect on symbiosis efficiency in the presence of salt (Table 2). This fact appears explicable, because, according to our data, salt tolerance of root-nodule bacteria exceeds that of alfalfa plants no less than eightfold, as estimated from 50% survival rates of root-nodule bacteria and alfalfa seeds.

It is worth noting that 12 out of the 15 isolates forming efficient symbioses in the presence of NaCl had one cryptic plasmid each, 200 kb in size. According to the literature, plasmids of this size may contain genes affecting the adaptability of rhizobia and determine nodulation efficiency [15, 16]. However, this fact requires further studies on the detection of genes involved in symbiosis formation under abiotic stress conditions.

The formation of nodules on roots of the host plant is an important symbiosis index. There are conflicting data on variations of nodule numbers and weights during symbiosis formation under salinization conditions [5, 17]. Studies of symbiosis between root-nodule bacteria and chickpea in the presence of salt showed that the symbiotic efficiency positively correlated with the number of nodule but not their weight [17]. The other authors who analyzed the same symbiotic system in the presence of salt have demonstrated that efficient isolates reliably formed the nodules of higher mass than the inefficient ones [5]. We showed that under salinization conditions, efficient symbioses on alfalfa roots were associated with the formation of significantly greater numbers of nodules than those recorded for inefficient symbioses (Table 3). An increase in nodule

Table 3. Numbers and weights of nodules formed by *S. meliloti* isolates on *M. sativa* roots under standard and salinization conditions

Isolate type[1]	Symbiotic efficiency (0.6% NaCl)[2]	Number of isolates	Number of nodules[3]		Nodule weight (μg)[4]	
			under standard conditions	+ 0.6% NaCl	under standard conditions	+ 0.6% NaCl
N	Eff^+	8	4.7 ± 0.3*	0.9 ± 0.2*	0.9 ± 0.05*	1.4 ± 0.19*
	Eff^-	13	3.1 ± 0.7*	0.2 ± 0.2*	1.0 ± 0.08*	1.8 ± 0.43*
T	Eff^+	7	3.4 ± 0.5	1.6 ± 0.1*	0.8 ± 0.07	0.8 ± 0.13
	Eff^-	8	2.8 ± 0.3	1.0 ± 0.2*	0.8 ± 0.05	0.9 ± 0.06

[1, 2], see Table 2; [3], counted on day 14 after inoculation; [4], counted on day 42 after inoculation.
* Differences significant at $P < 0.05$.

weight was detected only with N isolates, probably as a result of nonspecific adaptive responses to salt stress.

Thus, root-nodule bacteria contribute considerably to the tolerance of the symbiotic system to abiotic stresses and increase alfalfa yield. The collection of natural isolates of *S. meliloti* obtained has a high scientific value for understanding the mechanisms of stress resistance of bacteria and their symbioses with leguminous host plants.

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