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PROTEOMICS

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Proteomic Profile of the Bacterium *Sinorhizobium meliloti* Depends on Its Life Form and Host Plant Species

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Abstract—The importance of root nodule bacteria in biotechnology is determined by their distinctive feature: symbiotic nitrogen fixation resulting in the production of organic nitrogen-containing compounds. While interacting with host legume plants, the cells of these bacteria undergo global changes at all levels of expression of genetic information leading to the formation in root nodules of so-called bacteroids functioning as nitrogen fixation factories. The molecular mechanisms underlying plant-microbial symbiosis are actively investigated, and one of the most interesting and poorly studied aspects of this problem is the species-specificity of interaction between root nodule bacteria and host plants. In this work we have performed the proteomic analysis of the *Sinorhizobium meliloti* bacteroids isolated from two legume species: alfalfa (*Medicago sativa* L.) and yellow sweet clover (*Melilotus officinalis* L.). It has been shown that the *S. meliloti* bacteroids produce a lot of proteins (many of them associated with symbiosis) in a host-specific manner, i.e., only in certain host plant species. It has been demonstrated for the first time that the levels of expression in bacteroids of the genes encoding the ExoZ and MscL proteins responsible for the synthesis of surface lipopolysaccharides and formation of a large conductance mechanosensitive channel, respectively, depend on a host plant species that confirms the results of proteomic analysis. Overall, our data show that the regulation of bacteroid development by the host plant has species-specific features.

Keywords: root nodule bacteria, *Sinorhizobium meliloti*, *Medicago*, *Melilotus*, bacteroid, symbiotic nitrogen fixation, mass spectrometry, chromatography, MALDI, HPLC, MscL, ExoZ

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The root nodule bacteria of the order Rhizobiales, class Alphaproteobacteria, are a unique group of microorganisms capable of fixing atmospheric nitrogen in symbiosis with legumes [1, 2]. During the formation of the symbiotic interactions, in plant root nodules these bacteria form bacteroids, large immobile branched cells that have lost reproduction ability and are specialized for atmospheric nitrogen fixation [3, 4]. The formation of bacteroids is accompanied by the rearrangement of gene expression profiles leading to global changes at the proteomic level [5, 6].

It is known that the symbiotic interactions between root nodule bacteria and plants are under complex genetic control [2]. Symbiosis is induced by a flavonoid complex secreted by a leguminous plant [5], root nodule bacteria respond to it by producing LysR-type transcriptional regulators, which are encoded by the *nod* operon [2], and mediate the synthesis of lipochitooligosaccharides, so-called Nod factors (from nodulation) [7]. The proteins encoded by the *nod* operon, play the key role in host selection by root nodule bacteria [8–10]. It has also been shown that the levels of production of some other proteins by the *Bradyrhizobium japonicum* bacteroids, such as ABC transporters, dehydrogenases and transketolases, significantly depend on a host plant species [11]. Nevertheless, diversity of proteins and molecular mechanisms determining the species specificity of interactions between bacteria and host plants remain to be poorly studied [1, 2], in spite of significant progress in the transcriptomics [12–14] and proteomics [15] of root nodule bacteria in recent years.

In this work we attempted to reveal the key participants and potential mechanisms of bacteroid regulation by a host plant. To identify bacterial proteins that are host plant-specific, we compared the proteomes of bacteroids of the root nodule bacteria *Sinorhizobium meliloti* isolated from the root nodules of legumes from two different genera: *Medicago* (alfalfa, *M. sativa* L.) and *Melilotus* (yellow sweet clover, *M. officinalis* L.).

EXPERIMENTALS

Cultivation of microorganisms. The *S. meliloti* bacteroids were obtained as follows: the plants of alfalfa (*M. sativa* L. no. 45969, the Catalog of Vavilov Research Institute of Plant Industry (VRIPI)) and yellow sweet clover (*M. officinalis* L. no. 44565, the Catalog of VRIPI) were inoculated with the efficient nitrogen-fixing strain RCAM04491 of *S. meliloti* and grown in vessels with sterile vermiculite for five weeks in a green house under standard lighting conditions [16]. The bacteroids were isolated from plant root nodules according to the previously published protocol [17]. The free-living culture of *S. meliloti* used as a control was grown on a solid TY medium for two days at 28°C.

Protein assay. Total proteins were isolated from the bacteria with the Complete protease inhibitor (Roche, Switzerland); the proteins were denatured through boiling in sodium phosphate buffer with 1% sodium dodecyl sulfate; salts and detergents were removed in HiPPR and Zeba Spin Desalting Columns (Thermo-Scientific, United States) according to the manufacturer's protocol. The concentrations of isolated proteins were aligned using the Qubit (Thermo Scientific) fluorescent assay.

The trypsinolysis of proteins was performed according to [18]. The peptide mixtures (1 μL) were applied to an Acclaim™ PepMap 300 HPLC column for reversed-phase chromatography (150 mm × 75 μm; particle size, 5 μm (Thermo Scientific)) and separated according to [18] in the UltiMate 3000 UHPLC+ RSLC nano high-performance liquid chromatography system (Dionex, United States). The proteins were identified with the Ultraflextrime tandem time-of-flight mass spectrometer (BrukerDaltonics, United States). The mass spectra of each fraction were determined. The spectra were analyzed with the WARP-LC software, taking into account their production as a result of liquid chromatography. The MS/MS analysis of unique peptides was performed in the fractions with maximum peak intensity of these peptides. The correspondence of spectra to proteins was determined by Mascot version 2.4.2 (MatrixScience, http://www.matrixscience.com) in the UniProt database (http://www.uniprot.org/) with the limitation for *S. meliloti*. The α-cyano-4-hydroxycinnamic acid was used as a matrix. Mass tolerance settings were as follows: precursor mass tolerance, 100 ppm; fragment mass tolerance, 0.9 Da. Carboxymethylation of cysteine, partical oxidation of methionine and one missed site of trypsinolysis were indicated as permissible modifications.

Real-time polymerase chain reaction (qPCR). The total RNA for qPCR was isolated with Trizol (Invitrogen, United States). Reverse transcription was performed with the SuperScript III reverse transcriptase (Invitrogen) according to the producer's protocol. The amounts of DNA templates were controlled by the reference *SMc00128* gene with the previously published primers [19]. The levels of expression of the *exoZ* and *mscL* genes were analyzed using primers F—5'-ATCATGTGGGTCATCAGCGA and R—5'-TCCAGTGTCAGCACCAGATT; F—5'-ATCATGTGGGTCATCAGCGA and R—5'-TCCAGTGTCAGCACCAGATT, respectively. qPCR was performed with SYBR GreenMasterMix (BioRad, United States). The results were processed by the method of Livak and Schmittgen [20]. Statistical significance of the differences was assessed by the Mann–Whitney U test in Statistica version 6.0 (StatSoft).

RESULTS AND DISCUSSION

The Proteomic Profiles of the S. meliloti Bacteroids Isolated from Plants of the Genera Medicago and Melilotus Are Substantially Different

We have identified 417 proteins of the free-living culture, 684 proteins of the bacteroids isolated from the *M. sativa* L. nodules, and 634 proteins of the bacteroids isolated from the *M. officinalis* L. nodules (Fig. 1). This result might be an evidence in favor of a slightly limited resolving power of proteomic methodology compared to transcriptomics, because according to the data of transcriptomic analysis, more genes are expressed in free-living cells compared to bacteroids [13].

The genome of *S. meliloti* contains a ring chromosome and two megaplasmids [21]. The comparison of localizations of the genes encoding the identified proteins showed in bacteroids the activation of production of the proteins encoded by the genes located on megaplasmids (Fig. 2), which is in agreement with the ideas of significance of the products of such genes in plant-microbial interactions [21].

The sets of proteins identified in the two variants of bacteroids from the *Medicago* and *Melilotus* root nodules demonstrate a high similarity: 597 common proteins, 223 of them being identified only in the bacteroids but not in the free-living culture (Fig. 1). As expected, the highest mass-spectrometric score (Mascot Score) in the protein samples of the bacteroids was shown for nitrogenase (NifH), chaperones and chaperonins (Tig, DnaK, GroEL), and the proteins encoded by the *fix* operon that play an important role in symbiosis (Table 1). The comparison of the proteomes of bacteroids showed 87 proteins that were present in the bacteroids from *Medicago* nodules and not in the bacteroids from *Melilotus* nodules (Fig. 1b, Table 1) and 38 proteins identified in the bacteroids from *Melilotus* but not from *Medicago* (Fig. 1b, Table 1). All of these proteins were detected with a low score (no more than 114), which might reflect the minor level of their production compared to the major bacteroid proteins with a score up to several thousands (Table 1). Most of these host-specific bacterial proteins are metabolic enzymes, transcriptional regulators, components of the translational machinery, and factors

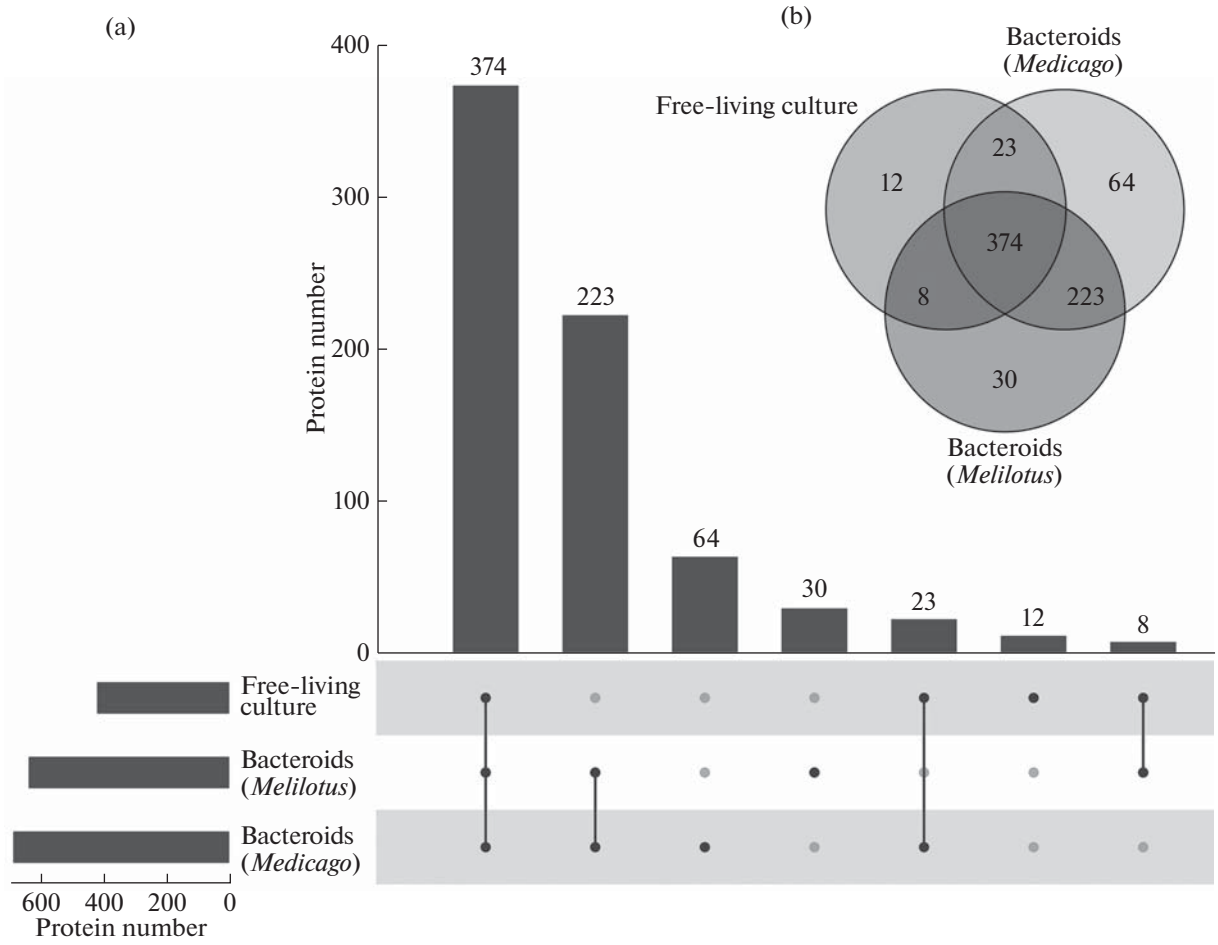

Fig. 1. The Venn diagram (a) and column histogram (b) illustrating the number of identified proteins of *S. meliloti*. The numbers of identified proteins are shown for the free-living culture, the bacteroids isolated from *Medicago sativa* L. nodules, and the bacteroids isolated from *Melilotus officinalis* L. nodules. The number of proteins in each of the overlapping samples is indicated.

responsible for the synthesis and transport of polysaccharides and siderophores.

It is noteworthy that some operons had a high content of the genes encoding the host-specific proteins of *S. meliloti* bacteroids (Fig. 2). For example, the *exo* operon encoding the machinery for surface lipopolysaccharide production, which plays a key role in plant-microbial interactions [22], carries the genes encoding six proteins identified in our work as host-specific (Table 1) and is located on the pSymB plasmid in the region of 1169–1185 kbp. (Fig. 2). The *rhb* operon responsible for the siderophore transport system and probably involved in the regulation of nitrogen fixation efficiency [23], carries genes of three host-specific proteins (Table 1) and is located on the pSymA plasmid in the region of 1306–1318 kbp. (Fig. 2). Generally, we may conclude that many of the host-specific proteins of *S. meliloti* bacteroids are involved in the regulation of plant–microbial interactions.

The Levels of Expression of the exoZ and mscL Genes Significantly Vary in Bacteroids Isolated from Different Plant Species

qPCR was used for the quantitative analysis of expression levels of the genes encoding some host-specific proteins of *S. meliloti*. The statistically significant changes in expression levels were revealed for two (*exoZ* and *mscL*) out of five (*exoZ*, *mscL*, *rnc*, *tam*, *tal*) genes under study, which partially correspond to the proteomic data (Fig. 2b).

The similarity of modifications at the mRNA and protein levels is substantial evidence for the dependence of the gene expression regulation in bacteroids on a host plant organism. Thus, the level of expression of the *exoZ* gene encoding acetyltransferase, which is involved in the synthesis of surface lipopolysaccharides and detected only in the proteome of *Medicago* bacteroids, was higher in the *Medicago* bacteroids than in the *Melilotus* bacteroids and free-living culture ($p < 0.01$) (Fig. 2b). It is known that the strains with mutations in this gene form bacteroids in *Medicago* root

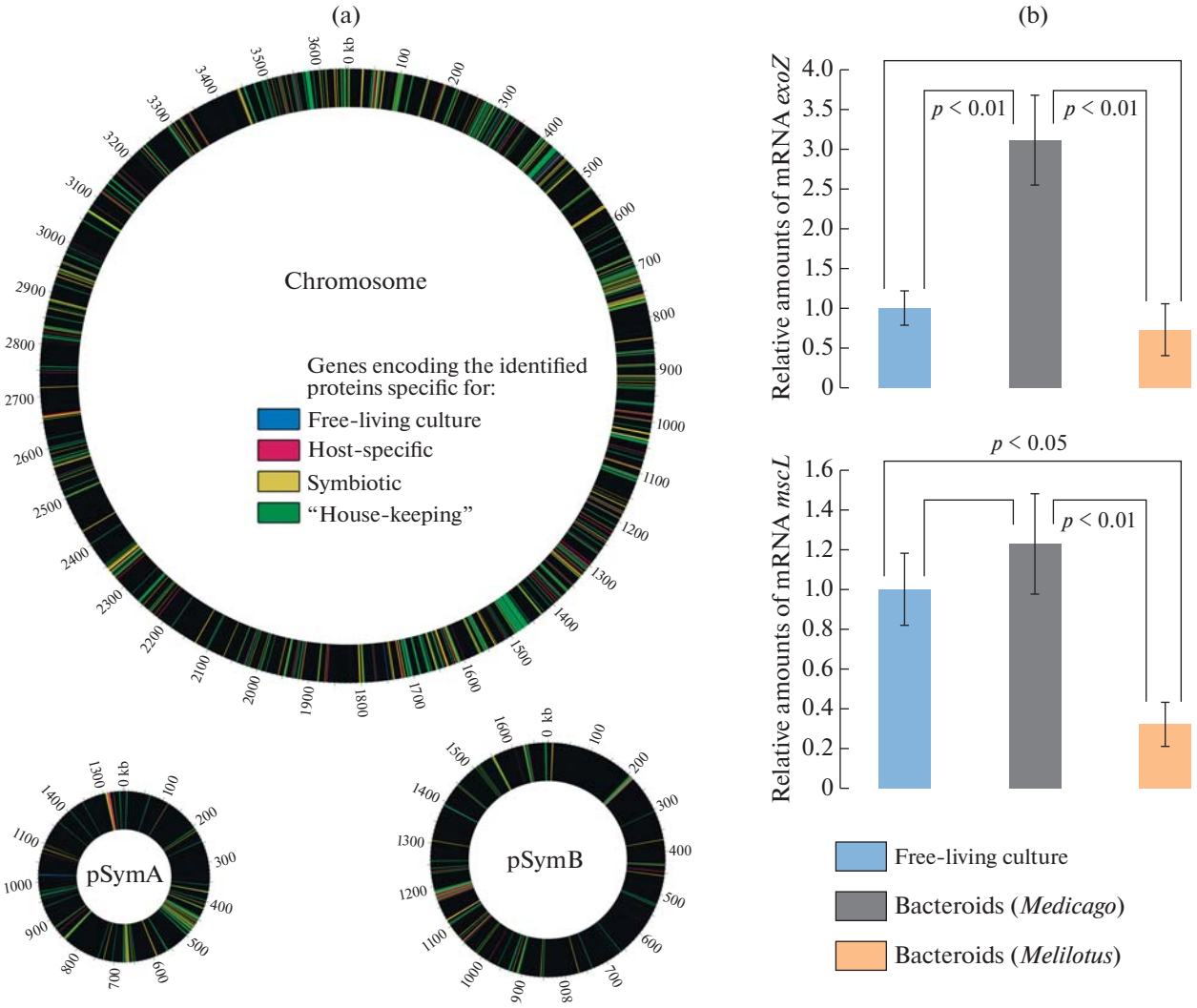

Fig. 2. The localization of genes encoding the identified proteins on the chromosome and the pSymA and pSymB megaplasmids (a) and the results of comparative analysis of the *exoZ* and *mscL* gene expression in the free-living culture of *S. meliloti*, the bacteroids from *Medicago* and the bacteroids from *Melilotus* by qPCR (b). (a) The genes encoding the proteins identified only in the free-living culture (blue); in both variants of bacteroids isolated from the nodules of *Medicago sativa* L. and *Melilotus officinalis* L. (yellow); in bacteroids from *Medicago* or from *Melilotus* (species-specific) (red); identified both in the free culture and in bacteroids isolated from both plant species (house-keeping proteins) (green). The distances on the genetic map are given in kilobases (kb). (b) The cDNA amount of the respective gene in the control (free-living culture) is set as 1. The observed differences were assessed by the nonparameteric Mann–Whitney test. *p* is the level of significance.

nodules but have the lower ability to form infection threads [24]. These and our data suggest that ExoZ is important for the formation of infection threads during the symbiotic interactions with *Medicago* but not with *Melilotus*; therefore, the levels of the *exoZ* gene expression (Fig. 2b) and ExoZ protein production (Table 1) in bacteroids isolated from *Medicago* is much higher than in bacteroids isolated from *Melilotus.*

The gene encoding the mechanosensitive channel protein (MscL), which we have found in the free-living culture and bacteroids from *Medicago* but not from *Melilotus*, shows a higher level of expression in the bacteroids from *Medicago* nodules compared to the bacteroids from *Melilotus* ($p < 0.01$) (Fig. 2b), confirming our proteomic data (Table 1). It should be ascertained that having no MscL in the list of proteins found in the bacteroids from *Melilotus* does not suggest having no production of this protein but rather demonstrates a drastic decrease in its synthesis, probably due to the reduced expression of *mscL* that we have revealed by qPCR (Fig. 2b). Mechanosensitive channels are important osmotic regulators responding to the changes in plasma membrane tension [25]. It is notable that the genes encoding the small mechanosensitive channel of root nodule bacteria (MscS) are differentially expressed in symbiosis with legumes [25]. Our data also contribute to the involvement of mechanosensitive channels in the regulation of species

Table 1. Bacteroid-specific proteins of *S. meliloti*

ID*	Protein	Protein function	Gene location**	Score***		
				bacteroids from *Medicago*	bacteroids from *Melilotus*	free-living culture
Proteins detected with the highest score in bacteroids****						
P35469	GroEL	Chaperonin GroEL	Chromosome	4179	2446	2233
P00460	NifH	Nitrogenase	pSymA	3721	3199	0
P09820	FixC	Oxidoreductase FixC	pSymA	2400	1527	40
O33915	GltA	Type II citrate synthase	Chromosome	1748	909	218
Q92LK8	AtpD	ATP synthase subunit	Chromosome	1242	1760	877
P95631	KatA	Catalase HPII(III)	Chromosome	1040	538	0
P09818	FixA	Electron transfer flavoprotein	pSymA	958	553	69
Q9R9N3	PdhB	Dihydrolipoamide-S-acetyl transferase	Chromosome	911	865	65
P42374	DnaK	Chaperone DnaK	Chromosome	790	661	49
Q92Q12	Tig	Trigger factor	Chromosome	772	947	537
The full list of proteins specific for bacteroids isolated from the *Medicago* nodules*****						
AglK, AnmK, Apt, ArgC, AroC, AroE, AtpH, ClpS, Csp1, CyaB, CycL, Ddl, Def, ExoL, ExoR, ExoV, ExoY, ExoZ, FabA, FabZ, FlaD, FlgA, FtsZ2, GlxC, GpsA, Gpt, GreA, GroES5, HisH, HmrR2, IhfB, KsgA, Kup1, LpsL, LpxA, MaiA, MoaE, MscL, MsrA3, MucR, NadA, NodD2, NrdR, NuoI, NuoN, NusB, PckA, PdxJ, PhrR, PqqB, PqqD, QueA, RhbE, RhbF, RhtA, RibH, RplU, RpmD, RpmI, RpsK, SM_b20363, SM_b20590, SMc00118, SMc00294, SMc01940, SMc02042, SMc02046, SMc02475, SMc02495, SMc02715, SMc03110, SMc03159, SMc03871, SMc04009, ThiG, TrpF, UbiG, Ung, UreA, WggR						
Proteins specific for bacteroids isolated from *Medicago* detected with the highest score						
Q92LK3	Tal	Transaldolase	Chromosome	114	0	37
Q92S72	MscL	Mechanosensitive channel	Chromosome	109	0	66
Q92RH3	AroC	Chorismate synthase	Chromosome	91	0	0
P58330	FlaD	Flagellin D	Chromosome	63	0	149
P26502	ExoZ	Acetyl transferase	pSymB	62	0	0
The full list of proteins specific for the bacteroids isolated from the nodules of *Melilotus*****						
Alr, AspS, BacA, CbbP, CheB2, CycJ, ExoH, ExsB, FdxN, FixM, FlaA, FolD2, Glk, GuaA, HisC2, HisG, IlvB2, IspH, MobA, MurI, NadD, NuoC1, Pal, PrmA, PurA, PurQ, RimM, RncS, RplQ, RpsU, SM_b20433, SMc03781, SyrM, Tam, TrmB, Upp, UreD						
Proteins specific for bacteroids from *Melilotus* detected with the highest score						
Q92R47	Rnc	Ribonuclease III	Chromosome	0	86	37
Q92RC5	Tam	*trans*-Aconitate 2-methyl-transferase	Chromosome	0	68	0
Q92MA5	PurA	Adenylosuccinate synthetase	Chromosome	0	62	51
Q92QV4	AspS	Aspartyl-tRNA synthetase	Chromosome	0	60	0
Q92RR6	HisG	Phosphoribosyltransferase	Chromosome	0	58	0

* Protein identifier in the UniProt database (http://www.uniprot.org/).
** Gene localization on the chromosome or on plasmids pSymA or pSymB.
*** The "Score" parameter of mass-spectrometric identification in the Mascot database.
**** The list is ranked by the proteins identified in bacteroids from *Medicago*.
***** The proteins identified not only in bacteroids from plants of the respective species but also in the free-living culture.

specificity of symbiosis. The role of these proteins can be associated with the adaptation of bacteroids to an osmotically aggressive internal medium of root nodules of different legume species.

Overall, the comparative proteomic analysis of the free-living culture of *S. meliloti* and the bacteroids isolated from two host plant species, *Medicago sativa* L. and *Melilotus officinalis* L., has shown that the proteomes of bacteroids formed in the root nodules of different host plant species are substantially different. This result suggests for the specificity of regulation of bacteroids by different plant species. The revealed host-specific proteins of *S. meliloti* bacteroids are characterized by the low level of production and great diversity of functions (Table 1). These proteins should be considered candidates that require additional verification by targeted methods, including the comparative analysis of the level of expression of the corresponding genes by qPCR. We have used this technique to show host-specific changes in the level of expression of the genes encoding the MscL large conductance mechanosensitive channel and the ExoZ protein responsible for the synthesis of surface lipopolysaccharides, confirming that these genes and their products are associated with the regulation of species-specificity of plant-microbial interactions.

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