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REVIEWS

Root Nodule Bacteria *Sinorhizobium meliloti*: Tolerance to Salinity and Bacterial Genetic Determinants

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Abstract—The theoretical and experimental data on salt tolerance of root nodule bacteria *Sinorhizobium meliloti* (*Ensifer meliloti*), an alfalfa symbiont, and on genetic determination of this feature are reviewed. Extensive data are provided on the genes affecting adaptation of proteobacteria and on the groups of genes with activity depending on the osmolarity of the medium. Structural and functional polymorphism of the *bet* genes involved in betaine synthesis and transport in *S. meliloti* is discussed. The phenotypic and genotypic polymorphism in 282 native rhizobial strains isolated from the centers of alfalfa diversity affected by aridity and salinity is discussed. The isolates from the Aral Sea area and northern Caucasus were shown to possess the *bet*C gene represented by two types of alleles: the dominant A-type allele found in Rm1021 and the less common divergent E-type allele, which was revealed in regions at the frequencies of 0.35 and 0.48, respectively. In the isolates with the salt-tolerant phenotype, which were isolated from root nodules and subsequently formed less effective symbioses with alfalfa, the frequency of E-type alleles was 2.5 times higher. Analysis of the nucleotide and amino acid sequences of the E-type allele of the *bet*C gene revealed that establishment of this allele in the population was a result of positive selection. It is concluded that diversification of the functionally diverse *bet* genes occurring in *S. meliloti* affects the salt tolerance and symbiotic effectiveness of rhizobia.

Keywords: *Sinorhizobium meliloti*, *bet*C, *bet*B, and *bet*T genes, *Medicago* spp., salt tolerance, symbiotic effectivity, root nodule and soil isolates

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Salinity, aridity, and extreme temperatures are the most common climatic and edaphotopic environmental stress factors limiting the growth and productivity of the economically valuable plant species [1, 2]. Ionic and osmotic stresses affect the structure of the membrane complexes in soil bacteria [3]. Intense introduction of *Rhizobium*–legume nitrogen-fixing symbioses, as well as of mixed legume–grass crops, is an efficient and environmentally friendly way to increase the quality and quantity of fodder and restore the vegetation on degraded soils [4, 5].

Root nodule bacteria *S. meliloti* form a nitrogen-fixing symbiosis with the plants of the genus *Medicago*, which includes 83 species and 18 interspecies forms of alfalfa plants [6, 7] differing significantly in their response to drought, cold, and soil acidity. For example, *M. varia* grows on slightly saline soils of the arid climate (Kazakhstan), whereas black medic (*M. lupulina*) or sickle alfalfa (*M. falcata*) grow on acidic soils of humid temperate continental (Northwestern region of the Russian Federation) or marine temperate arctic types of climate (Murmansk, Arkhangel'sk region), respectively [8, 9]. While new species and biovars of root nodule bacteria have been recently revealed (owing to the interest in rare forms of alfalfa), the species *S. meliloti* bv. *meliloti* remains the most widespread and well-studied. The practical significance of alfalfa-based legume–rhizobium symbioses has been shown for the temperate and subtropical climatic zones of the Mediterranean coastline, North Africa, Morocco, Mexico, as well as for Russia [4, 5, 10–16]. At the same time, while native symbioses adapted to temperate continental and temperate arctic northern water-logged acid soils may also be of no small interest due to the current global climatic changes, they remain to be studied.

The genotypic characteristics of bacteria (micro-) and plants (macrosymbionts) forming nitrogen-fixing symbioses are very important [17]. Matching of selected strains of root nodule bacteria substantially increase the viability and seed production of alfalfa on degraded soils [9, 18, 19]. The influence of the genotypes of inoculant strains and host plants on symbiosis formation is different under controlled conditions and on exposure to stress. For example, under salinity stress, the role of the inoculant strain increases, and the effect of the interaction between both symbionts increases almost sevenfold (Fig. 1). Root nodule bacteria are therefore a promising subject for agricultural biotechnology [20, 21], while investigation of genetic

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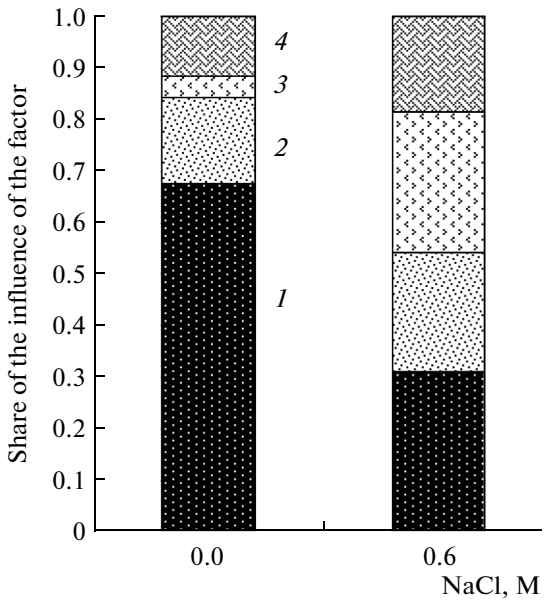

Fig. 1. Effect of abiotic and biotic factors on formation of the symbiotic systems under the standard and stress conditions (according to the two-factor analysis of 10 symbiotic systems). Ordinate: shares of the influence of biotic factors. Abscissa: NaCl concentration, in M. 0.0 M, the standard conditions; 0.6 M, stress conditions [49]. The "*M. varia* and *M. truncatula*" factor (*1*); "the five *S. meliloti* strains" factor (*2*); "the species—strain" factor (*3*); and "uncontrolled" factors (*4*). The experiment was made in 10 replicates.

regulation of tolerance to stress factors is a prerequisite for directed construction of symbiotic systems with a predetermined adaptive potential.

DIVERSITY AND SALT TOLERANCE OF RHIZOBIA

Investigation of the native populations of root nodule bacteria has always been of interest, specifically for the purpose of studying the native tolerance of rhizobia to different abiotic stress factors [22–26]. A considerable genotypic diversity of rhizobia was shown in the centers of origin of host plants, where germ plasma polymorphism is very high [5, 27, 28]. The diversity centers, described in the works of N.I. Vavilov and his followers [29, 30] and located in different soil and climatic zones with drastic daily and seasonal temperature differences, are liable to aridity and/or salinization and other factors. Therefore, analysis of native populations of rhizobial strains from such centers in order to search for the symbiotically effective stress-tolerant isolates is promising [21]. Even in the salinity-affected area (area adjacent to the Aral Sea), the probability of isolation of symbiotically effective *S. meliloti* strains from the root nodules of wild-growing host plants exceeds 5% [18]. Positive correlation between salt tolerance and symbiotic effectivity was shown for the strains isolated from root nodules of wild plants ($r = 0.76$) [25]. Available data also indicate that, unlike salt-sensitive isolates, the strains with a high level of salt tolerance predominantly form symbioses with a higher efficiency (comparative assessment of the mass of inoculated and uninoculated plants) [18, 26, 31].

Alfalfa rhizobia are characterized by native tolerance to relatively high sodium ion (Na^+) concentrations. Thus, *S. meliloti* strain 102F34 is resistant to 0.3 M NaCl, which is considerably higher than the tolerance of the host plant (*M. varia*) [22]. A similar level of tolerance was shown for *Agrobacterium tumefaciens* GMI 9023, whereas the type strains of other species of rhizobia, e.g., *R. tropici* (strain IIB), *S. fredii*, and *Mezorhizobium huakuii*, are tolerant to 0.2 M, and the representatives of the species *R. leguminosarum* (all biovars), *R. etli, A. rhizogenes*, and *Bradyrhizobium japonicum* are sensitive to 0.1 M [3, 32].

The high level of salt tolerance of *S. meliloti* is probably a result of coevolution of rhizobia and their host plants, since the osmolality level in alfalfa root nodules and bacteroids is 600 mOsm/L (or 0.3 M NaCl), which corresponds to hyperosmotic conditions [33]. At the same time, rhizobia, being the typical representatives of soil biota, are able to retain viability in the absence of a host plant under high salinity conditions, at 3.85 mSm/cm (0.36 M NaCl) [34]. The predominant majority (71.4%) of native *S. meliloti* strains are capable of growing under laboratory conditions at 0.6 M NaCl. This is true of the rhizobia isolated from root nodules (hereinafter N-isolates) and soils (hereinafter S-isolates); the trapping method was used to isolate the latter [35, 36], according to analysis of 650 isolates from five geographically different regions (Figs. 2a, 2b) [according to 18, 25, 28, 37]). However, among the isolates from saline soils adjacent to the area of the Aral Sea, those with salt tolerance lower than the indicated level (0.6 M) occurred significantly more often [28]. It was suggested that adaptation of rhizobia to natural hyperosmotic conditions caused their level of salt tolerance to decrease to 0.3 M, which is the level minimally required for the symbiotic system to be formed (see above) but sufficient for root nodule bacteria to exist in the saprophytic form [38], which agrees with [39]. However, this implies further research on the mechanisms of salt tolerance, a feature, which, according to [10], can be of evolutionary significance to the survival of bacteria.

GENOMIC ORGANIZATION OF *S. meliloti*

The genome of alfalfa root nodule bacteria consists of the chromosome (SMc; 3650 kb) and two high-molecular megaplasmids, the so-called symbiotic ones (SMa and SMb), which are 2.7 and 2.2 times smaller than SMc [40]. About 80% of natural *S. meliloti* strains contain one to four cryptic 40–440-kb plasmids, which are normally not transmissible [38]. Apparently, the presence of the compos-

ite genome in *S. meliloti* is due to the fact that rhizobia are saprophytes entering into symbiosis with legumes.

The structure of SMb is relatively conservative and similar to that of the chromosome; on the contrary, the structure of SMa is highly polymorphic. Horizontal gene transfer has been shown between the chromosome and megaplasmids [41, 42] and, possibly, between the megaplasmids, since a third copy of the *nod*PQ genes, localized on SMa in the type strain and involved in the synthesis of species-specific Nod-factors, was detected in SMb [43]. Involvement of the SMb genes in the control of salt tolerance was shown by research on the mutants in cluster-3 genes participating in the synthesis and transport of methylated cell surface polysaccharides, which had the salt-sensitive phenotype. One of the cluster genes, SMb21071, tentatively encodes glycosyl transferase and has an osmolarity-dependent transcriptional regulation [44].

Cryptic plasmids, irrespective of their molecular mass, may contain homologous sequences, and the plasmid composition may vary under the influence of the host plant [45–47]. The strains containing two or three plasmids were shown to have a lower level of salt tolerance significantly more often [38]. Analysis of the symbiotic, cultural, and biochemical properties of the native strains made it possible to predict the genes affecting the adaptive properties and symbiotic effectivity of rhizobia to be localized on *S. meliloti* 140–200 kb cryptic plasmids [38]. The functional significance of cryptic plasmids was brilliantly demonstrated in the study of pSmeSM11a and pSmeSM11b of strain *S. meliloti* SM11, which frequently occurs in different regions of Germany [45, 46]. Analysis of the plasmid nucleotide sequences revealed the presence of typical and not typical genes for rhizobia; it was also suggested that the plasmids were probably formed as a result of the combination of parts of the replicons from different bacterial hosts [45, 46]. The composition of both plasmids revealed the *rep*ABC replication system typical of the *Alphaproteobacteria* (the first segregation system), whereas pSmeSM11a also has the second A(II) replication system described for the plasmids of strain *S. meliloti* GR4.

The 144-kb accessory plasmid pSmeSM11a was found to contain six loci that included two to three genes and a region (42.4 kb long) which were homologous to the SMa sequences of the type strain *S. meliloti* Rm1021 [45]. Nevertheless, deletions and insertions of single nucleotides, deletions of single genes, and the presence of the gene encoding the hypothetical protein and lacking on SMa were revealed at these regions. In the opinion of the authors, such structural changes suggest genetic adaptability and the divergent type of evolution, which results in modification of the products of the genes determining their functioning under changed conditions. The genes encoding the TauABC transport system involved in sulfur metabolism, as well as the above-mentioned genes *nod*P and *nod*Q, and the genes not typical of *S. meliloti* genome, e.g., *tau*D (taurine

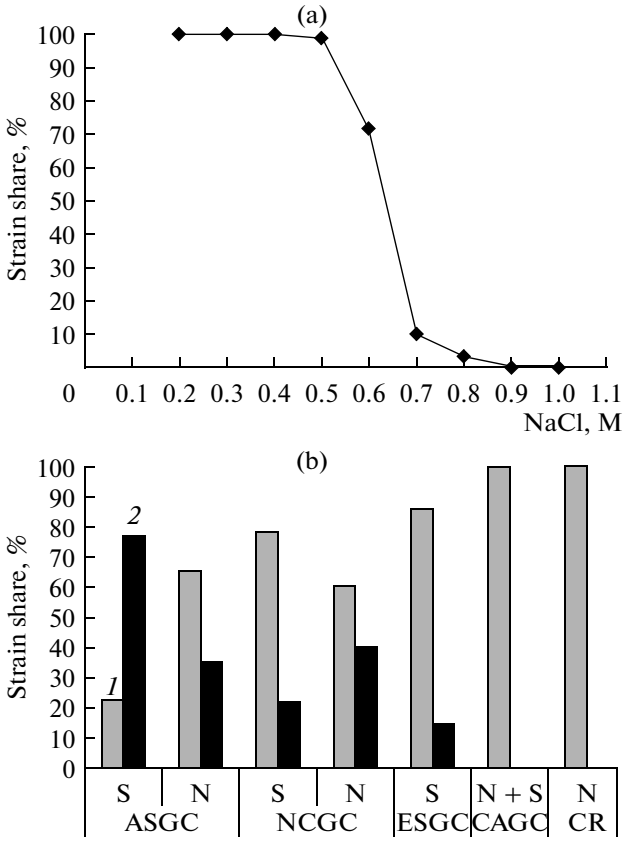

Fig. 2. Survival curve of 650 native *S. meliloti* strains under salinity conditions (a) and the salt-tolerant to salt-sensitive ratio of the root nodule and soil isolates in the gene centers (b). Ordinate: the strain shares, % are plotted. Abscissa (Fig. 2b): ASGC, Aral Sea Area gene center; NCGC, Northern Caucasus gene center; ESGC, European-Siberian gene center; CAGC, Central Asia gene center; CR, Chinese regions, a group of strains isolated in Chinese regions (the strains were provided by Prof. K. Lindstroem, Helsinki University, Finland); N, root nodule isolates; S, soil isolates. N + S, the summary data on N and S isolates [23, 33, 36, 50]. The isolates tolerant of 0.6 M NaCl (*1*) and the isolates sensitive to 0.6 M NaCl (*2*).

metabolism) and *acd*S (regulation of the level of the phytohormone ethylene, which plays a certain role in stress tolerance, amino acid metabolism, and other processes), were revealed on plasmid pSmeSM11a. The sequences homologous to pSmeSM11a often occurred in the composition of cryptic plasmids in geographically remote strains [45]. A locus of six genes involved in sugar metabolism or in polysaccharide catabolism, which is homologous to that on SMb of the type strain *S. meliloti* Rm1021, and which is flanked by transposons was revealed in the composition of the second 181-kb accessory plasmid pSmeSM11b, which indicates that it could have been transferred between the replicons [46]. Surprisingly, the sequences containing the genes encoding nonribosomal peptide synthesis and the proteins homologous to the products of nitrogen fixation genes of *Frankia* sp. strain CcI3 appeared

to be the most extensive regions (a total of 20 kb) in the structure of pSmeSM11b [46].

All the features of the structure of cryptic plasmids, including the fact that the replicons are saturated with IS-elements and transposons, give evidence of their active involvement in horizontal gene transfer—which increases the possibility of introduction of foreign genes, in particular those affecting the stress tolerance of rhizobia—with the subsequent change in their adaptation potential.

THE GENES AFFECTING SALT TOLERANCE OF *S. meliloti* AND OTHER PROTEOBACTERIA

Similar to *Escherichia coli* and other species of bacteria, rhizobia accumulate K^+, glutamate, and/or trehalose ions in response to salt stress [48]. However, different species of rhizobia have differences in regulating the biosynthesis of trehalose, which is required for desiccation tolerance. For example, in *R. leguminosarum* bv. *trifolii*, the activity of the gene *ots*A involved in trehalose biosynthesis does not depend on ionic strength of the medium, whereas in *S. meliloti*, on the contrary, the corresponding gene SMa0233 is activated when osmolarity increases [49, 50].

Identification of the genes involved in the processes of stress tolerance was carried out actively enough using the method of Tn5-mutagenesis or site-directed insertion of transposons. The *gfp*-type molecular marker, the *lac*Z reporter gene, or GUS-based constructs were often used for studying the mutants [26, 50–56]. In a number of cases, the Tn5-mutants of *S. fredii, R. leguminosarum* bv. *viciae* (previously *R. leguminosarum* bv. *faba*), or *M. ciceri* acquired the salt-sensitive phenotype and simultaneously lost the ability to form nitrogen-fixing symbioses with the relevant host plant species [26, 57, 58]. A valuable contribution to the study of the functional role of the genes of alfalfa root nodule bacteria was made by our colleagues N. Pobigaylo and A. Becker [59, 60]. These authors developed the method for obtaining a collection of transposants based on *en masse* insertion of the mini-Tn5 containing short genome-specific oligonucleotide sequences (the method of **s**ignature-**t**arget-**m**utagenesis, STM-mutants), which subsequently made it possible to pinpoint the site of the mini-transposon insertion and to reveal the target gene. A library of 10000 STM Rm2011::mTn5 mutants was constructed, and 5000 genes were analyzed for their involvement in the control of the cultural and symbiotic properties [60]. Analysis of 50 transposants from this collection under salinity stress *ex planta* and *in planta* showed that mini-Tn5 inserts led with similar frequencies (0.2 average) either to a change in the level of salt tolerance of rhizobia or to a significant increase in dry mass of inoculated *M. varia* plants grown under saline conditions; however, the level of effectivity under nonsaline conditions did not change (V.S. Muntyan, personal data). Our findings lead us to the conclusion that, in *S. meliloti*, different groups of genes control the feature of salt tolerance in the free-living state and affect the adaptability of host plants to salinity under symbiosis conditions.

In *S. meliloti*, the genes determining the formation and functioning of symbiosis under stress-free conditions have been studied best of all. These are the families of the *exo, exp*, and *lps* genes involved in exo- and lipopolysaccharide synthesis and affecting the process of symbiosis formation; the families of the *nod*, *noe*, *nol* and *hsn* genes determining the capacity of rhizobia for nodule formation on the roots of host plants; and numerous *nif* and *fix* genes responsible for nitrogen-fixing activity and symbiotic efficiency. However, *nod* and *lps* have been shown to be able to affect the stress tolerance of rhizobia [61].

The genes with the products involved in membrane transport play an important role in tolerance to stress factors. The ABC transporters involved in the processes of virulence and pathogenicity efficiently transport various compounds acting as osmoprotectors. For example, in *S. meliloti*, the proline betaine transporter Prb, which has an affinity to glycine betaine, belongs to the family of oligopeptide permeases (Opp) [39]. Along with this, the products of the genes of oligopeptide transport systems may be also involved in a variety of processes, for example, in biofilm synthesis in *Vibrio fluvialis* and in growth control under hyperosmotic conditions in *R. etli* [26]. Moreover, the activity of the gene SMb20316 encoding the sugar ABC sugar transporter or of SMc02871 determining the permease of the protein ABC transporter in *S. meliloti* depend on the variations in the ionic strength of the medium [62].

The genes of the BCCT transport system (**b**etaine-**c**holine-**c**arnitine **t**ransport), also belonging to the ABC type, have been studied in detail. These are the genes of the *pro*U and *bet*T transport systems described in *E. coli*; the latter of which is also present in *Sinorhizobium* ssp., and the gene *opu*C of the soil gram-positive bacterium *Bacillus subtilis* is involved in the transfer of betaines or of such compounds as choline or carnitine, which may be converted to betaines [63]. The structure, substrate specificity, the functional role, and, in a number of cases, regulation of the genes of these transport systems have been studied in some detail. Despite the fact that *opu*A and *pro*U have increased affinity to glycine betaine, the structural and functional similarity of *opu*A and *opu*C is greater than that to the gene *pro*U [32]. It was recently shown that transporters with an affinity to osmolytics can actively be involved in the processes of tolerance to high- and low-temperature stresses [64, 65].

Apart from the genes discussed, the gene *bsr*A encoding oxidoreductase in *Burkholderia* spp. (class *Betaproteobacteria*) also deserves attention. Under hyperosmotic conditions, a tenfold increase in oxidoreductase activity was shown, whereas a mutation in gene *bsr*A, on the contrary, resulted in the salt-sensitive phenotype of the strains [66]. The role of oxi-

doreductase in the formation of the salt-tolerant phenotype in bacteria remains, however, unknown. The *asn*O–*ngg* locus revealed recently in *S. meliloti*, with activity depending on ambient osmolarity, is also of strong interest [67]. The genes of this locus control nonribosomal synthesis of the dipeptide *N*-acetylglutamylglutamate (NAGGN) from *N*-acetylglutamine and glutamine. It is suggested that NAGGN determines the osmotolerance of rhizobia in the absence of other osmolytics. At the same time, the proteins AsnO and Ngg are supposedly highly conservative peptidases of human and animal pathogens, such as *M. avium, Pseudomonas aeruginosa, P. mendocina*, and *Ochrobactrum anthropi*, but not of *Mesorhizobium* spp. or eukaryotes. In the authors' opinion, NAGGN can be an efficient latent factor of the virulence of pathogenic bacteria, which could be of interest in the development of medications [67].

Thus, while accumulation of the data on the genes which can determine tolerance of proteobacteria to abiotic factors is intense, their incompleteness and the fragmentary character are obvious. Investigation of genetic regulation of salt tolerance is complicated by the fact that unrelated and taxonomically remote representatives of bacteria are studied; moreover, microorganisms normally utilize different osmoprotectors during different growth phases [48]. Nevertheless, the data described suggest that the property of salt tolerance in rhizobia is functionally controlled by different genes whose regulatory interrelationships remain unclear, except for a number of the transport systems mentioned above.

THE GROUPS OF *S. meliloti* GENES INVOLVED IN DIFFERENT TYPES OF SALT STRESS

Extensive gene groups involved in the processes of salt tolerance were revealed as a result of analysis of expression of rhizobial genes using the biochip technique. In 2003, involvement of 137 *S. meliloti* genes was first shown in response of the rhizobial culture to long-term exposure in salt stress (cultivation for 49 h at 0.38 M NaCl; [68]). At the same time, participation of 1003 genes of differential (changing) expression was revealed in response to a short-term (4 h) salt shock of 0.3 or 0.4 M NaCl in the early lag-phase cells [62]. These studies were conducted using the DNA-biochips constructed on the basis of strain Rm1021 being tolerant to 0.55 M NaCl. However, in the former case, the biochip SM6kPCR constructed on the basis of PCR-amplified 350-bp oligonucleotides (6225 triplicate spots on a biochip; [68]) was used; in the latter, SM6kOligo with synthesized 70-bp oligonucleotides (6205 triplicate spots on a biochip; [62]) was used.

We compared the frequencies of occurrence of the genes of different localization in the genome, which had increased (hereinafter URG-group, **u**p-**r**egulated **g**enes) or decreased expression (hereinafter DRG-group, **d**own-**r**egulated **g**enes) in response to long-term stress and shock caused by similar osmolarity values (Table 1). The DRG genes revealed in both types of stress were predominantly localized on SMc, whereas the URG genes occurred more often on SMc in the case of long-term stress and on SMb during shock. According to the authors, active involvement of the SMb genes in response to shock is due to the fact that a large number of genes encoding different transporters are present on the replicon [62, 69]. However, in the case of long-term stress, the genes revealed on SMb were represented by similar shares in the DRG- and URG-groups (Table 1), whereas the activity of identified genes on SMa directly depended on the type of salt stress: under long-term stress, the genes with decreased activity prevailed, and on the contrary, in response to shock, the genes with increased activity predominated (the DRG- and URG-groups, respectively) (Table 1).

Hence, the salt stress induced by the same osmolarity, but with different duration of exposure, exerted a different effect on metabolism of rhizobia. In the case of long-term stress, a decrease in gene activity was mostly noted, while in the case of shock, the gene activity was both increased and repressed at a similar frequency. Interestingly, the genes with increased activity were predominantly foreign, i.e., introduced as a result of horizontal transfer from the genomes of α-, γ-, and δ-proteobacteria, as well as from cyanobacteria, whereas the genes with repressed activity were characteristic of the genus *Sinorhizobium* and were assigned to the core genome ($\chi^2 = 6.007$, $P = 0.01425$; [70]).

However, the effect of salt shock depends not only on the duration of stress but also on ionic strength of the salt solution. For example, at ionic strength of 0.4 M, the number of differential expression genes after 30 min was five times less than at 0.3 M, but became similar after 60 min, whereas at 0.3 M, the number of differential expression genes after 30 and 60 min did not substantially change [62]. According to other studies, in *S. meliloti* cells the maximal amount of the osmoprotectant (glycine betaine) in response to 0.3 M NaCl shock was accumulated by the 30th min [63]. With this in mind, we singled out two groups of differential expression genes in response to 0.3 M salt shock, in which the level of expression after 30 min was either increased or decreased in relation to the tester genes (expressed constitutively) [according to 62], and analyzed the activity of these genes in both a later period (60 min) and at higher osmolarity (0.4 M). There were a total of 25 URG and 20 DRG such genes (Fig. 3).

DRG genes were predominantly localized on SMc and in single cases on the megaplasmids (Fig. 3, a-1). All the three DRG genes on SMa remained repressed under all the shock conditions. At the same time, the SMb and SMc DRG genes repressed at 0.3 M could exhibit an expression at a level of the tester genes in

Table 1. Rm1021 differential expression genes under long-term salt exposure and salinity shock conditions localized on different replicons [according to 68 and 62]

Experiment no.	Conditions of cultivation	Level of gene expression	Frequency of genes of particular group in a replicon			Genes, total
			SMa	SMb	SMc	
I[1]	Long-term cultivation at 0.38 M NaCl	URG[3]	0.04	0.12	0.22	52
		DRG[4]	0.12	0.11	0.39	85
						137
II[2]	Salt shock at 0.4 M NaCl	URG[3]	0.12	0.22	0.19	538
		DRG[4]	0.03	0.04	0.38	445
		Variable[5]	0.00	0.01	0.01	20
						1003

Designations: [1] according to the data [94]; [2] according to the data [68]; [3] and [4] the genes with increased (URG) or repressed activity (DRG); [5] the genes with activity changing at different time points.

response to 0.4 M shock. Of 20 DRG genes initially repressed after 30 min in response to 0.3 M shock, 60% remained inactive after 60 min, whereas at 30 min after exposure to 0.4 M shock, only half of the genes from this group was repressed; by the 60th min, 75% of the genes were repressed (Fig. 3, a-1). The DRG genes included the genes that determined *gro*ESL2 chaperone, the protein supposedly involved in the degradation of xenobiotics, the LacI-type transcription regulator of the adenosine metabolic pathway, the ABC transporter with affinity to spermidine or putrescine, and conserved hypothetical proteins. Thus, analysis of the same group of genes showed that, in response to 0.3 M shock, the number of genes with derepressed activity increased with time, and, vice versa: as the duration of the 0.4 M shock increased, the number of repressed genes increased accordingly.

The URG genes, whose expression was increased after 30 min in response to 0.3 M shock, were localized on all the three replicons in similar amounts (8 genes on SMc and SMa and 9 genes on SMb). Of 25 URG genes with enhanced expression at 30 min in response to 0.3 M shock, 44% retained their activity at 60 min (Fig. 3, a-2). After exposure to shock of a higher osmolarity (0.4 M), only 24 and 68% of the genes had increased activity after 30 and 60 min, respectively.

Thus, under 0.3 M shock, the number of genes of the DRG and URG groups decreased with time, which gives evidence of metabolic stabilization of the cells under hyperosmotic conditions. However, as shown above, such an osmolarity level is typical of rhizobia and cannot be considered as a shock level. In the case when the ionic strength of the medium was increased by 0.1 M, the situation was reverse: after 30 min, the number of repressed genes exceeded twofold the number of those with increased activity; however, after 60 min, the shares of the genes with decreased and increased expression leveled out (Fig. 3b). The shares of differential expression genes in response to shock of different ionic strength were significantly different ($\chi^2 = 11.9$, $P < 0.001$). Consequently, the shock induced by 0.4 M NaCl should be considered as hyperosmotic for rhizobia.

Thus, analysis of the group of genes with increased expression in response to the action of 0.3 M shock showed that their number decreased with a time increase, whereas, after 0.4 M shock, the number of genes with increased expression increased with time. Since the shares of the genes with activities constantly repressed or increased were low (0.04 and 0.2, respectively), it is evident that the differential activity genes play the key role in the adaptability processes.

Six genes with increased activity in response to hyperosmotic shock (0.4 M NaCl; according to [62]) were identified in the genome of Rm1021. Among them are the *ngg* gene (SMb20482) encoding *N*-acetylglutamylglutamate synthase involved in *N*-acetylation of aminoglycosides, peptides, polyamines, and proteins (see above; [67]). The *ngg* gene has increased expression at a lower osmolarity (0.3 M), whereas activity of the second gene of the locus, *asn*O (SMb20481), remained constant at the level of tester genes [62]. Two genes (SMc03959 and SMc01959) localized on the chromosome encode a conserved hypothetical protein, presumably DNA ligase and ISRm1 transposase, respectively. Another chromosomal gene (Smc03201) supposedly determines the α-subunit of 2-oxoisovalerate dehydrogenase that possesses oxidoreductase activity in *M. loti* and *R. etli*. This protein is homologous to a similar protein of the mitochondrial dehydrogenase complex (BCKDH) involved in conversion of α-keto acids. Interestingly, the gene Smc03201 has 41% homology with the human mitochondrial gene encoding the α-subunit of the dehydrogenase/decarboxylase of α-keto acids. Mutations in this gene result in dysfunction of

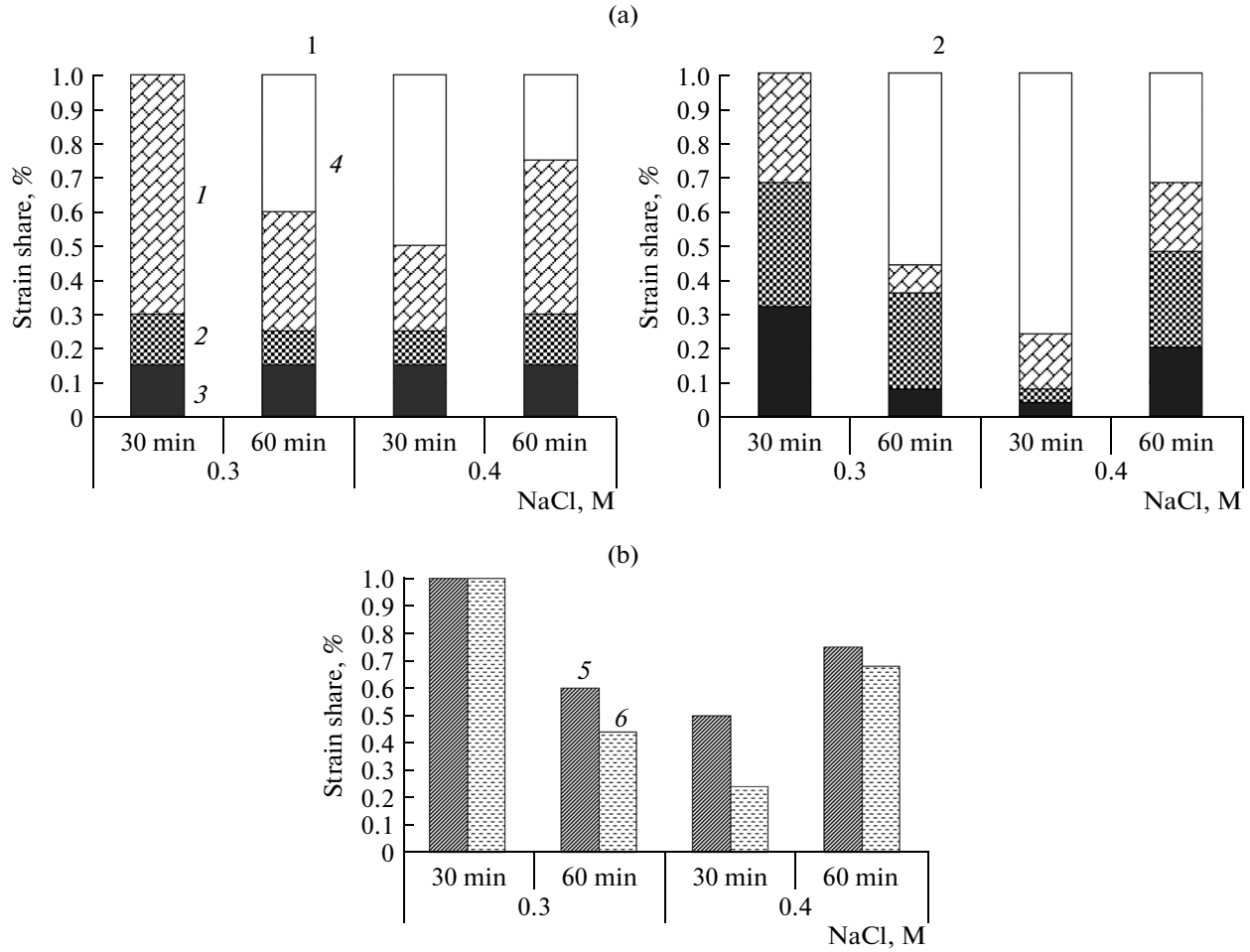

Fig. 3. Distribution of the RM1021 differential expression genes under salt shock of different ionic strength (Na^+) by localization (a) and the level of activity (b) [according to 87]. Designations: groups of down-regulated genes (DRG) (1); groups of up-regulated genes (URG) (2). Ordinate: strain ratios. Abscissa: 30 and 60 min after the shock. NaCl concentrations (0.3 and 0.4) are given in mol. The genes localized on the SMc chromosome (*1*); on SMb (*2*); on SMa (*3*); the genes with an activity at the level of tester genes (*4*); the groups of DRG genes (*5*); and the groups of URG genes (*6*).

the BCKDH complex and determine the maple syrup urine disease (MSUD) (leucinosis), which is characterized by mental and physical handicap and is often the cause of death in newborns [71]. The last two genes (SMa0130 and SMc02227) supposedly encode fatty acid desaturase and the α-subunit of the fatty acid oxidation complex, respectively. Desaturases are known to control the synthesis of unsaturated fatty acids required to maintain the physical properties of the membrane lipids [72]. The biosynthesis of these acids is regulated in response to the environmental changes and, accordingly, influences transmembrane signaling [73]. Hence, in response to hyperosmotic shock, the genes with the functions yet unstudied are activated in *S. meliloti*.

However, the overwhelming majority of native *S. meliloti* strains have earlier been shown to be tolerant to 0.6 M NaCl, the osmolarity level that remains almost unstudied. One of the approaches to detecting the groups of genes involved in alfalfa rhizobial tolerance to higher concentrations could be development of a biochip based on the full-genome sequence data of a strain capable of growing, e.g., at 0.7 M NaCl. We attempted to reveal the genes involved in adaptive processes using comparative analysis of genomes (CGH, **c**omparative **ge**nome **h**ybridization) of the strains widely differing in salt tolerance using the DNA of the biochip SM6kOligo. Pairwise *in silico* analysis of five strains of a salt-tolerant strain and of two strains with the salt-sensitive phenotype enabled us to predict participation of the group of 178 genes in determining the salt-tolerant phenotype (V.S. Muntyan, personal data). Since the studies of gene expression were conducted using the biochip based on strain Rm1021 with a "moderate level" of salinity tolerance, the functional significance of the genes involved in the control of salt tolerance in environmental strains is addressed to future studies.

Table 2. Homology of the *bet* gene nucleotide sequences in *S. meliloti* strains

S. meliloti strain	Gene homology, %						
	*bet*I	*bet*A	*bet*B	*bet*C	*bet*S	*bet*B2	SMa1726
Rm1021	100	100	100	100	100	100	100
Rm2011	100	100	100	100	100	100	100
102F34	100	99	99	99	100	ND	ND
SM11	100	100	99	100	99	99	99
GR4	100	100	99	100	99	99	98
BL225C	99	100	99	100	99	99	99
AK83	100	99	99	99	–[1]	99	100
Rm41	98	99	99	99	–[1]	99	100
E. coli	–[1]	67[2]	66	–[1]	64	73[3]	–[1]

Designations: [1] the gene is absent according to the BLASTn data; [2] the homology level at 23%; [3] 17% overlapping of the sequences; ND, no data.

THE FAMILY OF THE *bet* GENES IN *Sinorhizobium*

The genome of *S. meliloti* contains *bet* genes responsible for the synthesis and exogenous transport of glycine betaine, the main osmoprotectant in the early exponential-phase cells at 0.4 M (2.5%) NaCl [63, 74, 75]. However, the expression of all *bet* genes at 0.3 and 0.4 M NaCl is at a level of the test genes [62, 68]. The role of glycine betaine as an osmoprotectant was also observed at higher osmolarity values (0.6 M NaCl) [67]. However, in *S. meliloti*, this osmoprotectant can also be used as a carbon and nitrogen source, which was also shown for *P. aeruginosa* and *Xanthomonas translucens* but not found in other microbial species [32, 76]. Rhizobia synthesize glycine betaine from choline presented in root exudates. However, choline is also required for the synthesis of phosphatidyl choline, a membrane component of gram-negative bacteria.

The *bet*ICBA in *Alphaproteobacteria* of the genus *Sinorhizobium* has a similar organization and is localized on the chromosome. It has been studied equally well as *E. coli* operon *bet*TIBA [77]. The composition of both operons includes the *bet*B and *bet*A genes encoding betaine-aldehyde dehydrogenase and oxygen-dependent choline dehydrogenase, respectively. Although both genes are quite common in many species of bacteria, as well as in higher plants and animals, the gene sequences are divergent even at the species level (Table 2). It is evident that the *bet* genes have both structural and functional plasticity, since BetB and BetA exhibit 54 and 50% of identity and 71 and 68% of similarity in *S. meliloti* and *E. coli*, respectively. An interesting feature of both dehydrogenases is the presence of the glycine box sequence containing a conservative motif (GXGXXG) and a number of amino acids and the N-terminal end, which form the FAD-binding center characteristic of flavoproteins [74]. Aldehyde dehydrogenase (ALDH), a gene *bet*B product, is involved in choline metabolism and plays a dual role. The enzymes of this class may supply NADH as well as NADPH for ATP synthesis via oxidative phosphorylation or for protection against oxidative stress. ALDHs have also uncertain substrate specificity to quaternary amines, tertiary sulfonium components, and to some amino aldehydes [78]. The second dehydrogenase, the product of the *bet*A gene, is choline-specific and may utilize oxygen in the catalytic reaction in the absence of other electron acceptors. This oxygen-dependent enzyme belongs to the family of glucose-methanol-choline flavin-dependent oxidoreductases. However, under saline stress conditions, an increase in the oxidase activity characteristic of *E. coli* was not revealed in *S. meliloti* strain 102F34 [79]. It was suggested that BetA can substitute part of the functions of the BetB product, participating in choline and betaine aldehyde conversion, according to analysis of the substrate specificity of the deletion mutant Δ*bet*B (gene deletion) and the corresponding transposon mutants *bet*C::Tn5 and *bet*A::Tn5 [75]. Involvement of BetA in choline and betaine aldehyde conversion was previously shown for halophilic bacteria [80, 81]. It is highly probable that BetA is the key enzyme in *S. meliloti* which switches choline catabolism to synthesis of osmoprotectant or to maintenance of metabolism.

The second copy of the *bet*B gene localized on the megaplasmid SMa, *bet*B2 (SMa1731), was revealed in the genome of *S. meliloti*. The protein products BetB and BetB2 have 63% of identity and 76% of similarity. We established that, unlike *bet*B, *bet*B2 was neither expressed at 0.3 M (2%) NaCl nor involved in the transformation of betaine aldehyde into glycine betaine [75]. However, the deletions of *bet*B, of *bet*B2, or of both genes in Rm2011 had almost no effect on tolerance to 0.4 M NaCl and the ability to catabolize glycine betaine in mutants [75]. Hence, in *S. meliloti*

BetB and BetB2 do not play the key role in the process of conversion of choline to glycine betaine. Nevertheless, the function of the *bet*B2 gene remains to be understood, because the high level of expression under nonsaline conditions and repression of activity under saline conditions were observed for one-third of the structural portion of gene *bet*B2-144 after deleting 70% of the initial sequence [75]. Mutant *bet*B2-144 utilized glycine betaine for growth but did not accumulate it as an osmoprotectant, and in the presence of 3% NaCl, accumulation of glutamate and trehalose, the osmolytes usually present in the stationary phase, occcurred [75]. The activity of *bet*B2-144 seems to depend on the activity of the *bet* operon, because the transposon mutant *bet*C::Tn5 could actively utilize glycine betaine but not choline for growth.

The third gene of the *bet* operon gene, *bet*C, encodes choline sulfatase catalyzing the conversion of choline-O-sulfate, a typical component of root exudates, to choline with its subsequent conversion to glycine betaine. However, such a role of BetC in the mechanisms of salt tolerance is specific only for *Rhizobiaceae*, whereas in other microorganisms and fungi, this enzyme is involved solely in constructive sulfur (S) metabolism, and the degree of homology of the corresponding amino acid sequences is 42–100% at the level of genus. It has recently been shown that the protein BetC has at least two divergent structures in different species of microorganisms that differ in six motifs predominantly localized at the N-terminal end [77]. One type of BetC is a highly conservative protein determined by the *bet*C gene, which is a part of the *bet*-operon, for example, as in *S. meliloti*. The structure of the other type of BetC is determined by *bet*C, which is a part of the structure of the cassette located on the chromosome or plasmid and incorporating two more genes encoding the sulfate-transporter and the LysR-type transcriptional regulator. This type of BetC is characteristic of different species of microbes (including the archaeal group of halobacteria, as well as γ-, β-, and δ-proteobacteria) and has a high degree of convergence with the same gene in the ascomycetes. In rhizobia, both types of BetC may occur: for example, the presence of two cassettes with *bet*C genes on two plasmids and of the *bet*ABI operon on the chromosome was revealed in the genome of *R. leguminosarum* bv. *trifolii* strain WSW 2304. Another example is *R. leguminosarum* bv. *viciae* strain 3841, whose similar *bet*-operon is located on the chromosome, while the second gene *bet*A copy and the locus containing *bet*CI genes are localized on plasmid pRL11. It is evident that the structural organization of the *bet* genes varies considerably even within the family *Rhizobiaceae*. The BetC type described in *S. meliloti* was suggested to be involved in the processes of adaptation of bacteria to stress conditions in soil, with *bet*C controlling the switching of glycine betaine metabolic pathways, thus resulting in its being used as an osmoprotector or as a carbon and nitrogen source [77]. Consequently, one of the key *bet* genes encoding choline sulfatase underwent the structural and regulatory diversification caused by the process of coevolution of rhizobia with the legume host plant and established in the process of vertical evolution. The bifunctional activity of the *bet*-encoded enzymes probably determines the metabolic plasticity that rhizobia require for adaptation to the ever-changing environmental conditions.

The activity of the *S. meliloti bet* operon located on the chromosome is regulated by the transcriptional repressor gene *bet*I. The mechanism of negative regulation of BetI consists in its binding to the *bet*I-box-sequence in the promoter only at a certain choline concentration, which results in operon activation [82]. A similar regulatory system was shown for the *E. coli* operon *bet*TIBA also localized on the chromosome. However, in *E. coli*, the activity of BetI depends on the osmolarity and oxygen concentration, whereas in *S. meliloti* the repressor activity does not depend on either these parameters or the glycine betaine concentration, but strictly depends on choline concentration and on the presence of a number of less specific substrates: acetylcholine, choline-*O*-sulfate, and, to a lesser degree, on phosphorylcholine [79, 82, 83]. The gene *bet*T determining glycine betaine transport is under the negative regulation of *bet*I. In *E. coli*, this gene is an operon constituent but has a reverse orientation. In *S. meliloti*, the same transporter gene *bet*S (*bet*T) is located on the other replicon, the SMb megaplasmid. The homology between *S. meliloti bet*T and similar transport systems of the BCCT family (for example, *E. coli bet*T or *B. subtilis opu*D) is 30 and 34%, respectively [84]. *S. meliloti bet*T is constitutive, and BetT is regulated at the posttranslational level [33], which allows rhizobia to instantaneously provide a rapid response to salt shock carrying out the membrane transport of the betaines present in root exudates, as well as from the rhizosphere or soil.

Summarizing the above material, it should be concluded that the activity of *S. meliloti bet* genes in the saprophytic state is at the level of activity of test genes and does not depend on the osmolarity or stress duration (49 h or shock from 15 min to 4 h) [33, 62, 68, 79]. Active expression of genes *bet*A, *bet*B, *bet*I, and *bet*T, but not *bet*B2, was shown in infection threads, in zones II and III of bacteriods in root nodules where the osmolality level attains high values (see above; [33, 75, 82]). However, in bacteriods, glycine–betaine transport is 30 times lower than in free-living cells [33]. Choline concentration in alfalfa root nodules—where it is present in various nodule compartments, cytosol, and the peribacteroid space, and is probably transported into symbiosomes and bacteroids—is doubled under the salt stress conditions [82, 85]. In all probability, the structural and functional role of *bet* genes was evolutionarily determined in the course of establishment of plant–microbial interactions.

We attempted to assess the role of *bet* genes in the formation of symbiosis with alfalfa under model con-

ditions. It appeared that the mutants lacking BetB or with impaired BetB structure (Δ*bet*B or *bet*B::mTn5, respectively) formed an effective symbiosis with alfalfa under the standard conditions or in the presence of 0.6% NaCl (Muntyan, personal data). The effectivity of the symbioses formed by the mutants were the same as those obtained upon inoculation with strain Rm1021. Similar results were obtained when shock conditions were tested in Gibson tube experiments when salt solution was added to the seedlings two weeks after inoculation to attain an end concentration of 0.5% (or 0.086 M NaCl; [75]). The mutants with impaired *bet*B2 structure or lacking it (*bet*B2::mTn5 and Δ*bet*B2, respectively) also formed effective symbioses under salt-free conditions. However, under salinity conditions (at 0.6% or 0.103 M NaCl), as well as in the shock variant, the biomass of the plants inoculated with *bet*B2-mutants (*bet*B2::mTn5 or Δ*bet*B2 or *bet*B2-144) was reliably lower than that of Rm2011-inoculated plants. Survivability of the plants inoculated with mutant *bet*B2-144 was 20% lower under saline shock conditions [75].

Thus, the *bet*B gene does not influence significantly the symbiotic effectivity under either the standard conditions or under stress, whereas *bet*B2 localized on a symbiotic plasmid affects plant adaptability and symbiotic effectivity under saline stress conditions. Hence, the *bet* gene copies localized on different replicons undergo a functional diversification, which, undoubtedly, affects the adaptive potentialities of rhizobia. Nevertheless, the above results were obtained on strains 102F34 and Rm1021, which appeared to differ contrastingly in the catabolic pathways of the osmoprotectant glycine betaine [75]. Strain 102F34 utilizes it for methionine synthesis or catabolizes it to form serine and pyruvate as a source of carbon and nitrogen [78, 79], while Rm1021, similar to *E. coli*, does not metabolize glycine betaine [75]. It is possible to assess the level of structural diversification of the *bet* genes at the species level of *S. meliloti* and to acquire primary knowledge of the involvement of these genes in the process of the evolution of plant–microbial interaction based on the analysis of the representative sample of native strains from the geographically remote centers of their host plants' origin and exposed to different abiotic stress-factors, which will be discussed hereinafter.

POLYMORPHISM OF THE *bet* GENES OF NATIVE STRAINS

High level of the *bet* gene polymorphism is quite expected in native populations of *S. meliloti*, considering that the genes are localized not only on the chromosome but also on plasmids; this can also be expected from the data on polymorphism of the nucleotide sequences in known strains (Table 2) and from the differences in metabolism between the test strains (see above). Moreover, the structure of *S. meliloti bet* operon contains non-encoding sequences of 167 and 211 bp between the *bet*I-*bet*C and *bet*B-*bet*A genes, respectively, whereas there is only one nucleotide between the unique *bet*C gene and the *bet*B gene [86]. The intergenic sequences have a mosaic structure, which probably indicates them as the recombination "hot points."

We studied the structural polymorphisms of the *bet* genes in 282 native strains from two distant regions of host plant diversity belonging to the gene centers in northern Caucasus (hereinafter NCGC) and in the Aral Sea area (hereinafter ASGC), which is subject to salinization [according to 25]. The geographically different *S. meliloti* populations appeared to have low and close values of the heterogeneity coefficient ($H = 0.65$ and 0.6, respectively) determined for the site of the *bet*ICBA operon incorporating parts of the *bet*C (1401 bp) and *bet*B (hereinafter *bet*B*; 143 bp) sequences. The RELP-type of *bet*CB* locus characteristic of Rm1021 (hereinafter the A-type or the typical one; frequency 0.52), as well as the co-dominant E-type (frequency 0.48), were predominant in both populations. The frequencies of occurrence of the typical and divergent *bet*CB* structures had inverse distribution in the isolates from ASGC and NCGC ($\chi^2 = 5.56$, $P < 0.02$). The A-type prevailed in the ASGC population, as well as in the subpopulations of the N- and S-isolates of the same region ($\chi^2 = 4.49$, $P < 0.05$). The divergent E-type dominated in NCGC isolates ($\chi^2 = 8.67$, $p < 0.01$; Fig. 4, a-1) and predominantly among N-isolates (frequency 0.53; $\chi^2 = 6.19$, $P < 0.05$, respectively; Fig. 4, a-2). In the NCGC and ASGC, the isolates (the respective frequencies 0.03 and 0.09), which had no *bet*CB* sequence or had significant structural changes (no PCR amplicon was obtained; Fig. 4, a-1), were revealed. Such isolates were symbiotically ineffective (no significant differences in the masses of inoculated and uninoculated *M. varia* plants). This finding suggested the conclusion that the *bet*C gene plays a key role in metabolism of *S. meliloti*, which agrees with the suggestion of the group of French researchers [77]; however, we were the first to reveal the influence of gene *bet*C on symbiotic effectiveness.

In the overwhelming number of isolates (59%) of both gene centers, the structure of the *bet*T transporter gene was represented by the A-type (Rm1021 RELP-type), whereas the sequences differing from the A-type occurred with a frequency of no higher than 0.015 (Fig. 4b). In the remaining cases, the strains (over 40%), which were predominantly S-isolates, lost this gene (no PCR amplicon was obtained; $\chi^2 = 17.2$, $P < 0.001$). The ASGC S-isolates (frequency 0.71) lost the *bet*T gene more often than the same NCGC isolates (0.38; $\chi^2 = 8.57$, $P < 0.01$), as well as the N-isolates from ASGC (0.29; $\chi^2 = 31.39$, $P < 0.001$) and NCGC (0.38; $\chi^2 = 12.6$, $P < 0.001$). Consequently, the *bet*T sequence was either represented by the A-type or was

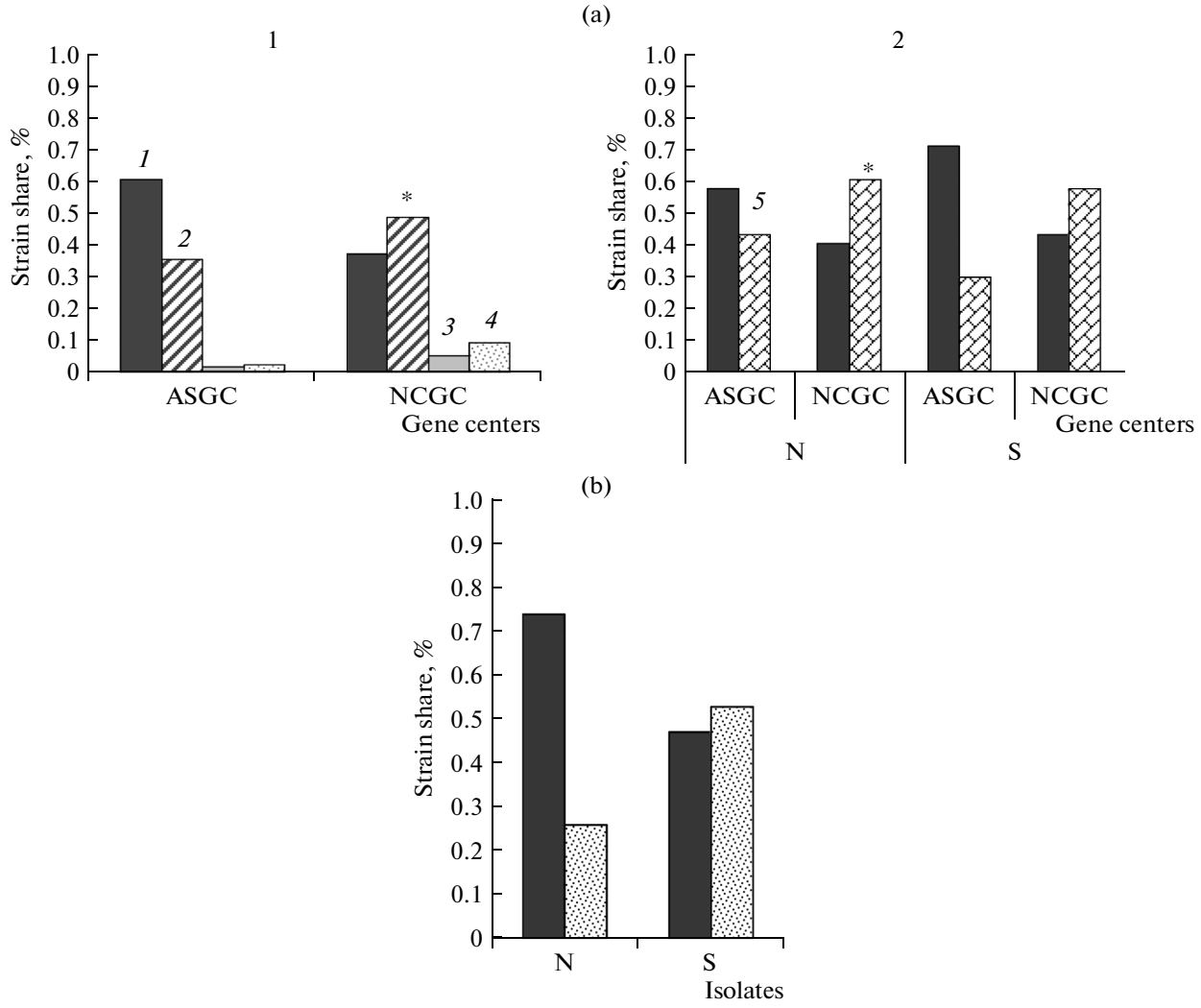

Fig. 4. Polymorphism of the locus *bet*CB* (a) and the *bet*T gene (b) in *S. meliloti* root nodule and soil isolates from geographically different alfalfa diversity centers. Designations: (1) the ratio of all RELP-types in the gene centers; (2) the ratio of the A to divergent types depending on the source of the isolate; the strain shares are plotted along the vertical axis. ASGC and NCGC are the Aral Sea Area gene center and the Northern Caucasus gene center, respectively. N and S, nodule and soil isolates, respectively. The A or typical RELP-type (*1*); the E RELP-type (*2*); the RELP-types revealed in single isolates (unique) (*3*); the absence of amplification (*4*); and the RELP-types different from the A-type (divergent) (*5*). * The differences are significant at $P < 0.05$ (see text) [according to 33].

absent from the genomes of native strains. As a rule, the *bet*T gene was lost by the S-isolates surviving in saline soils on which host plants did not grow. Therefore, it was concluded that inheritance of the transporter gene localized in the accessory genome was influenced by an abiotic stress factor. It is possible that the transport systems of these strains, which carry out membrane transport under higher osmolarity conditions and have an optimal level of activity of 0.3 M [62], differ from those of BetT.

Thus, four types of combinations of the A- and E-types of the *bet*CB* locus (the corresponding frequencies 0.35 and 0.39) with the presence or absence of the *bet*T gene predominated in native strains (Figs. 5a, 5b [according to 25]). The isolates having the typical *bet*CB* and *bet*T sequences had the salt-tolerant or the salt-sensitive phenotype with an equal probability and mostly formed effective symbioses with *M. varia* ($\chi^2 = 1.8$, $P > 0.1$; Figs. 5a, 5b). The groups of isolates with the E-type of the *bet*CB* locus and the *bet*T gene, or which lack the latter, mostly formed inefficient symbioses ($\chi^2 = 4.77$, $P < 0.05$ and $\chi^2 = 3.77$, $P < 0.1$, respectively; groups II and IV in Fig. 5b). A decreased level of symbiotic effectivity with *M. varia* in the isolates with the E-type of the *bet*CB* locus and the *bet*T gene could be determined by their preferences in host specificity, because the strains were isolated from the root nodules of *M. falcata* and because *Melilotus officinalis* were prevalent in the region where the material was collected. Thus, *S. meliloti* strains,

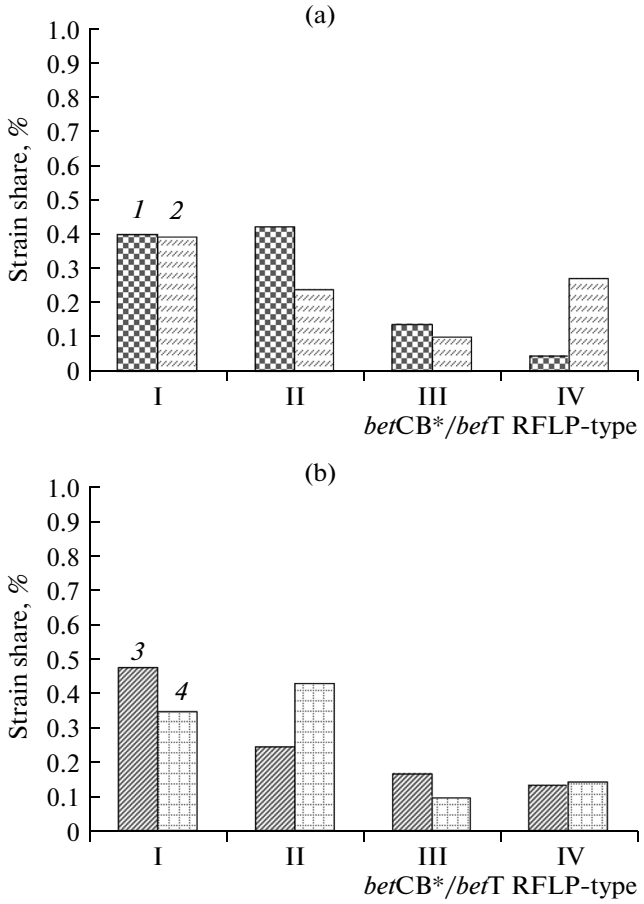

Fig. 5. Frequency of occurrence of combinations of the dominant RELP-types (alleles) of *bet*CB*/*bet*T in *S. meliloti* root nodule isolates differing in salt tolerance (a) and symbiotic effectivity (b) [according to 33]. Designations: I, *bet*CB*/*bet*T A-type; II, *bet*CB* E-type and *bet*T A-type; III and IV, *bet*CB* A- or divergent types, respectively, in the absence of gene *bet*T. Salt-tolerant isolates (*1*); salt-sensitive isolates (*2*); effective isolates (*3*); and ineffective isolates (*4*).

which had modified the *bet*C sequences or lost this gene, formed poorly effective symbioses, whereas the isolates lacking *bet*T had a low level of salt tolerance ($\chi^2 = 16.29$, $P < 0.001$). These data led us to the conclusion that polymorphism of the *bet* genes localized on the chromosome is controlled by the host plant, which predetermines the importance of further study of these genes.

Analysis of the nucleotide sequences of the *bet*C gene in the dominant A- and E-types and in one of the rarely occurring types (or alleles) showed that the central part of the E-type sequence had 10 nucleotide replacements leading to substitution of four amino acids—and in the case of the rarely occurring allele, 13 asynonymous replacements in the central part of the gene—resulting in impaired functioning of one of the structural choline-sulfatase domains (V.S. Muntyan, M.L. Roumiantseva, unpublished data). Interestingly, no insertions or deletions were revealed in sequences of these alleles. The ratio of the number of asynonymous to synonymous substitutions (Ka/Ks) between the A- and E-types was 4.37, indicating that the E-type allele was under the positive pressure of evolution. Hence, the divergent *bet*C gene sequences revealed by PCR-RELP analysis had significant structural changes that had to result in the changed functional activity of BetC, which, in turn, could determine the changes in the symbiotic properties observed in the strains.

Thus, wide occurrence of the divergent E-type allele of *bet*C in the geographically different populations is the result of positive selection of the core genome regions evolving adaptively under the action of natural selection. This type of the divergent *bet*C allele was revealed in geographically different populations and occurred 2.5–2.9 times as frequently in the isolates from the root nodules that had the salt-tolerant phenotype and formed low-effective symbioses with alfalfa. These structural changes evidently did not affect the viability of bacteria in the saprophytic form or their capacity for symbiosis formation. Moreover, a decreased level of symbiotic effectivity with alfalfa could be the result of the fact that the strains had enhanced specificity towards the host plants of the genus *Melilotus*, which is especially widespread in northern Caucasus, in the region where the material was collected. The activity of rhizobial *bet* genes is important for plant bacteroids under both nonstress and stress conditions, whereas the *bet* genes do not seem to have considerable a functional load in the rhizobia of the soil subpopulation and undergo structural changes or are "washed out" of the population, as was shown for the *bet*T gene in the isolates with a decreased level of salt tolerance.

Analysis of the native strains of root nodule bacteria from the centers of diversity of host plants revealed clearly the active process of diversification of the functionally diverse *bet* genes represented in both the core and accessory parts of the genome.

Summing up our results and the data published in the literature, we should conclude that salt tolerance of alfalfa nodule bacteria is not only a physiological and a physicochemical process enabling a bacterial cell to withstand the conditions of hyperosmotic environment (the general nonspecific cell response to salt stress [87]), but is also a genetically determined process, the investigation of which is only in its initial stage. The genetic and biochemical systems responsible for *S. meliloti* tolerance to salinity are evolutionarily labile. This is due to the presence in their genome of functionally different groups of genes with predominantly differential activity depending on ambient osmolarity, from the functional complementarity of the gene products of one group (which is possible for *bet*A and *bet*B) and from localization of the genes of one group on different replicons. As we have already shown, the presence of all the *bet* genes, as well as their specific alleles, exhibited a significant correlation with

the symbiotic efficiency in *S. meliloti*. It is important that the *bet* genes, in contrast to many other genes, retain the constitutive level of expression in bacteroids under hyperosmotic conditions. All this indicates that the role of the *bet* genes and, especially, their influence on the carbohydrate and nitrogen metabolism, should be addressed in future investigations. Nevertheless, the *bet* genes discussed have a high degree of substrate affinity to glycine betaine, while the most widespread betaine in root tissues and root exudates is proline, whose role has yet to be understood.

Thus, the feature that appeared under the influence of the macroevolutionary vector of external effects significantly affected the evolution of the development of legume–rhizobial symbioses, and the root nodule bacteria of the species *S. meliloti* are a unique model subject for the study and detection of new genes determining the stress tolerance of rhizobia, as well as for influencing the adaptability of host plants.

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