UDC 633.31:631.461.5

doi: 10.15389/agrobiology.2015.3.339rus

doi: 10.15389/agrobiology.2015.3.339eng

POPULATION POLYMORPHISM OF THE ALFALFA NODULE BACTERIA (*Sinorhizobium meliloti*) FOR THE GENES ENCODING FOR SYMBIOTIC EFFICIENCY AND COMPETITIVENESS

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Acknowledgements:

Supported by Russian Foundations for Fundamental Research (grant № 14-04-01441), EurAsEC Program (Contract 14.M04.12.0011) and Russian Science Foundation (grant № 14-26-00094)

Received March 30, 2015

Abstract

The most important traits of nodule bacteria (rhizobia), which are tested for selection of commercially valuable strains are the symbiotic efficiency (ability to increase the mass of the inoculated host plants due to intensive fixation of molecular nitrogen) and competitiveness (ability to inoculate the plants in the presence of other rhizobia strains of the same species). By PCR analysis of alfalfa rhizobia (*Sinorhizobium meliloti*) of various origins we for the first time showed polymorphism of natural populations for genes *Smc03879* or *phbA* (negative regulator of the effective symbiosis, encodes for synthesis of poly-β-hydroxybutyrate) and *Sma0907* or *cmp-107* (involved in the competition for nodulation). The population of the Uralsk region of Kazakhstan living in the impoverished saline soil, *phbA* gene was detected in 100 % of the strains, while population of the rich Chernozem soil of the Ternopil region of Ukraine in only 82.2 % of the strains. Even lower occurrence of *phbA* (30.8 %) is characteristic of the strains isolated from alfalfa nodules collected in the Ternopil region. This polymorphism may reflect a low adaptive value for conversion of C-compounds into poly-β-hydroxybutyrate for rhizobia strains from the Ternopil region in comparison with the strains of the Uralsk region. Participation of gene *phbA* in the control symbiotic efficiency is confirmed by the fact that the gene *nifH*, which encodes for the structure of the small subunit of nitrogenase, but does not affect the intensity of its synthesis, differences in polymorphism between strains of two populations were not identified. The absence of polymorphism for gene *Smb21375* or *eff-798* in all studied populations may be due to the fact that it encodes an ABC transporter required for the uptake by bacteria the broad range of essential nutrients obtained from both the host plant or from the soil. An increased incidence of effective symbionts among strains lacking gene *phbA*, indicates the prospects to use the molecular probes containing this gene for the selection of practically important rhizobia genotypes that can provide the plant host with the significant amounts of fixed nitrogen.

Keywords: legume-rhizobia symbiosis, nodule bacteria, nitrogen fixation, symbiotic efficiency, competitiveness, population polymorphism, PCR analysis.

Nodule bacteria (rhizobia) that form N₂-fixing symbioses with legumes are the most widely used in agriculture microorganisms: the scope of their annual production as part of biological products for legumes inoculation is measured by hundreds of millions of hectares [1]. Until recently, rhizobia strains for biological preparations were obtained by analytical selection methods, by their isolation from soil and plant nodules. Using transposon (Tn5) mutagenesis in alfalfa rhizobia (*Sinorhizobium meliloti*), a collection of *eff*-gene mutants that increase bacterial symbiotic efficacy (SE), i.e. their ability to increase productivity (biomass, the amount of accumulated nitrogen, seed yield) in inoculated plants, was obtained [2]. Many of these genes identified as negative symbiosis regulators encode plant C-compounds metabolism including their transport into cells (*eff-798*), storage in the form of poly-β-hydroxybutyrate (*phbA*) and spending for

polysaccharide synthesis (*exoZ*, *rkpC*, *eglC*). These genes are located in different parts of *S. meliloti* (chromosome megaplasmidy) genome which is a prerequisite for their use in the construction of strains with increased SE [3]. The *cmp*-genes that determine the ability of rhizobia to compete for nodulation with less active native soil strains are also of great interest for strain construction [4]. In this case, the promising sources are natural *eff*- and *cmp*-rhizobia populations.

In this article, we demonstrate *S. meliloti* population polymorphism for genes controlling symbiotic efficiency and competitiveness, which can be used for selection of commercially valuable rhizobia strains.

Technique. Using the standard method [5], alfalfa nodule bacteria strains (*Sinorhizobium meliloti*) were isolated from saline soils of Uralsk Region (Kazakhstan), from the chernozem soil of Ternopil Region (Ukraine), and from the nodules of various alfalfa species (*Medicago lupulina*, *M. sativa*) growing in these areas. The genes controlling economically valuable symbiotic features have been identified earlier by analyzing the Tn5-mutant effective SKhM1-105 and SKhM1-188 strains with enhanced symbiotic efficiency (*eff*-798 and *phbA*) or decreased competitiveness (*cmp*-107) [2, 6, 7].

The following primers were used for PCR amplification of DNA fragments containing the genes studied:

Smb21375 (*eff*-798) — 5'-GAGAACAGCCGGGAGGAAA-3' and 5'-CAGAACACAGCAGCCAAAGC-3'

phbA (*eff*-152) — 5'-CCTTCGGCAATACTCTCGC-3' and 5'-GTTGAGACCCGAAGCGTTG-3'

Sma0907 (*cmp*-107) — 5'-CCACCCTCCAGATCCAAGGT-3' and 5'-CCTACGAGCTTTCGGTCTC-3'

PCR protocol was as follows: preliminary denaturation — 3 min at 95 °C; 30 cycles — denaturation 30 sec at 94 °C, primer annealing 30 sec at 54 °C, DNA synthesis 1 min at 72 °C; final DNA synthesis — 5 min at 72 °C (Thermocycler C1000™, Bio-Rad, USA; CCU Genomic technology and cellular biology of the All-Russian Research Institute for Agricultural Microbiology). The resulting PCR products were separated on 1% agarose gel according to the standard method [8]. The lysates of rhizobia strains studied were used in PCR as DNA matrices. For this purpose, fresh bacterial culture was inoculated into 20 ml of lysis buffer (0.25 mM NaOH; 0,25 % SDS), denatured in PCR machine (95 °C, 15 min), then 180 ml of double distilled water was added, the resulting liquid was centrifuged for 1 min and stored at -20 °C. Chromosomal IGS locus (region between the 16S and 23S rRNA genes), and the plasmid locus containing the *nifH* gene (encoding the small nitrogenase subunit) were analyzed as previously described [9].

Symbiotic efficacy of *S. meliloti* strains was studied in sterile microvegetation experiments in alfalfa (*M. sativa*) var. Vega [5].

Student's *t*-test was used for statistical data processing [10]

Results. To study population variability of alfalfa (*S. meliloti*) rhizobia for the genes of efficacy and competitiveness depending on the environmental conditions (soil type, possibility of symbiosis), *eff*-798, *phbA* and *cmp*-107 polymorphism was analyzed in isolates from contrast different soils: the poor, saline soil of Uralsk Region of Kazakhstan without vegetation and the rich chernozem soil of Ternopil Region of Ukraine where numerous populations of black medick and alfalfa (*M. lupulina* and *M. sativa*) grow.

Data analysis (Table 1) demonstrated the presence of *eff*-798 (encodes ABC-transporter that provides the C-compounds entry into bacterial cells) in all *S. meliloti* strains regardless of the source of their isolation. The *phbA*-

containing DNA fragment (encodes β -ketotiolase involved in bacterial accumulation of poly- β -hydroxybutyrate) is present in all strains of Uralsk Region soil but only in 82.2 % of Ternopil Region soil strains. Even lower occurrence of *phbA* (30.8 %) is characteristic for the strains isolated from alfalfa nodules collected in Ternopil Region.

1. Occurrence of efficacy and competitiveness genes among *Sinorhizobium meliloti* strains of different origin

Strain origin (their number)	Proportion of strains with identified gene, %*		
	<i>eff-798</i>	<i>phbA</i>	<i>cmp-107</i>
Uralsk region, soil (24)	100	100 ^a	91.7 $\pm$ 5.6 ^a
Ternopil region, soil (46)	100	82.2 $\pm$ 5.7 ^b	84.8 $\pm$ 5.3 ^a
Ternopil region, nodules (13)	100	30.8 $\pm$ 12.8 ^c	52.8 $\pm$ 13.8 ^b

* In each column, the same letters represent the values that are not significantly different ($P_0 > 0.05$).

Logically, the rhizobia *phbA* polymorphism in Ternopil Region population may reflect a reduced bacteria ability to accumulate carbon in the form of poly-hydroxybutyrate due to the switching on symbiotrophic carbon nutrition, carbon excessively supplied by plants. Probably, bacteria circulation among carbon-rich chernozem soil and plants contributes to selection weakening in favor of the strains actively accumulating reserve constituents. At the same time, the selection in favor of this feature which determines the *phbA* homogeneity of Uralsk Region population can provide its maximum survival in nutrient-poor saline soil devoid of vegetation. The absence of polymorphism for gene *eff-798* in studied populations may be due to the fact that it encodes an ABC transporter required for the bacteria uptake of the broad range of essential nutrients obtained both from the host plant and the soil.

Analysis of *S. meliloti* SE (symbiotic efficacy) in microvegetation experiments demonstrated the efficacy of all strains in which gene *phbA* has not been detected (inoculation significantly increased the dry weight of alfalfa var. Vega), while 29 $\pm$ 8.1 % of strains containing this gene were ineffective. However, SE extent does not depend on the presence of gene *phbA* in efficient strains, which may be associated with the varying of the genetic background for this gene expression.

In both *S. meliloti* soil populations, equally high polymorphism in gene *cmp-107* was identified in which Tn5-mutation reduces bacteria competitiveness (see Table 1). At the same time, the gene is less common among the nodule isolates collected in Ternopil Region than among soil isolates. This suggests the participation of gene *cmp-107* encoding a protein with unknown functions in bacteria competition not only for bacterial but also for soil ecological niches.

2. Parameters of the structure of *igs* and *nifH* *Sinorhizobium meliloti* soil and nodule subpopulations loci (Ternopil Region)

Locus	Parameter	Strain origin (number)	
		soil (40)	nodules (13)
IGS	Genotype number (per strain number, %)	5 (12.5 %)	6 (46.1 %)
	Diversity index	0.384	0.798
	Proportion of dominant genotype, %	77 $\pm$ 6	27 $\pm$ 13*
<i>nifH</i>	Genotype number (per strain number, %)	3 (7.5 %)	4 (30.8 %)
	Diversity index	0.486	0.489
	Proportion of dominant genotype, %	63 $\pm$ 8	69 $\pm$ 13

* Difference between soil and nodule population is significant ($P_0 < 0.05$).

During experimental tests on rhizobia polymorphism analysis using the markers not related to the quantitative symbiosis activity expression directly, it was found (Table 2) that, first, the variation in locus IGS for nodule isolates is higher than for the rhizosphere which reflects the previously described general increase in rhizobia population polymorphisms in the transition from soil to

the nodule niche [9]; second, no interpopulation differences were found in locus *nifH* which encodes the small nitrogenase subunit but is not involved in the quantification of nodule nitrogen-fixing activity. Thus, the difference between soil and nodule subpopulations in the presence of genes *phbA* and *cmp-107* are determined precisely by their participation in the control of quantitative symbiosis features.

Earlier, a number of genes controlling symbiotic efficacy [11-17] and competitiveness [18-22] have been described, but the studies of these genes population polymorphisms have not been performed.

Thus, we for the first time showed population polymorphism of alfalfa nodule bacteria (*Sinorhizobium meliloti*) in the genes controlling economically valuable features of symbiotic efficacy (*phbA*) and competitiveness (*cmp-107*). High variability of the population of the nutrient-rich Ternopil Region chernozem soil in gene *phbA* which controls poly- β -hydroxybutyrate synthesis may be related to high availability of carbon obtained due to heterotrophic or symbiotrophic nutrition which reduces the selection intensity in favor of the strains accumulating C-compounds actively. The polymorphisms analysis for the genes that act as negative regulators of symbiotic efficacy can be used for the selection of perspective strains for legumes inoculation.

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