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THE DIVERSITY OF *RHIZOBIUM LEGUMINOSARUM* BV. *TRIFOLII* LOCAL POPULATIONS: GENETIC AND PHYSIOLOGICAL TRAITS CONNECTION

Genetic and metabolic variability within local population of Rhizobium leguminosarum bv. trifolii in context of possible interconnections between metabolic-physiological properties of rhizobia was studied. Considerable genetic diversity was observed in 16-23S rDNA regions and in the plasmid profiles of studied strains. Furthermore, the strains within defined PCR-RFLP group were greatly different. Metabolic profiles of rhizobia studied by Biolog test showed large diversity, even within one genetic group. PCA analysis of metabolic traits revealed differences in utilization of two large groups of the substrates: (a) saccharides, and (b) organic acids, amino acids and modified sugars, named here "non-sugars". Utilization of chemically various saccharides by rhizobia was more commonly observed than "non-sugars". Moreover, utilization of "sugars" and "non-sugars" were positively correlated in the strains belonging to PCR-RFLP Groups 1, 2 and 3. The "sugars" were utilized differently than "non-sugars" by the strains of Group 4. Finally, the significant differences in the growth rate of the strains belonging to particular PCR-RFLP groups in different media were found. We concluded that the local population of Rhizobium leguminosarum bv. trifolii was not uniform. It was composed of physiologically different strains and high metabolic diversity reflected the level of genetic diversity of population.

K e y w o r d s: Rhizobium leguminosarum, metabolic and genetic diversity.

The metabolic and adaptive potential of a bacterium is correlated with a degree of its genome complexity. The soil bacterium *Rhizobium leguminosarum* bv. *trifolii (Rlt)* occupies highly challenging soil habitats and induces complex symbiotic interaction with the host plant clover (*Trifolium* spp.) [11]. A common feature of the *Rlt* and other rhizobial genomes is the complexity of genomic organization, with a single chromosome and several large plasmids (megaplasmids). The genes encoding symbiotic functions usually constitute independent replicons, known as symbiotic plasmids (pSym), or symbiotic islands when incorporated into the chromosome [10]. The cryptic plasmids constitute a pool of accessory genetic information [15] and even though they are in general dispensable for bacterial survival. Functional interactions among different replicons are often required for successful completion of both the symbiotic and saprophytic lifestyle of rhizobia [1, 8]. The large, complex and dynamic rhizobial genomes, which encode many potentially useful metabolic traits, play a role in their high metabolic diversity and adaptive potential [9, 14].

The knowledge of metabolic traits important for the growth and competition of rhizobia within population is limited. It is known that local populations of rhizobia may differ significantly both on the genetic and physiological levels. Up to now studies concerning genetic and physiological diversity of rhizobial populations focused mainly on the strains colonizing the soil or particular legume plants species [2, 5, 7].

In this work the representative sample of local population of rhizobia that occupy the nodules of clover root systems growing in each other's vicinity in the soil was subjected to comprehensive analyses of their genetic and especially metabolic variability. We have described the possible interconnections between the metabolic-physiological properties of *R. leguminosarum* bv. *trifolii* and some genetic traits of their genomes.

Materials and methods

50 *Rhizobium leguminosarum* isolates were obtained from the nodules of red clover (*Trifolium pratense* L. cv. Dajana) growing in sandy loam. The nodules were surface-sterilized, crushed and their content was plated on 79CA medium [13]. Strains isolated from the nodules were purified by successive streaking of single colonies and pure cultures were used in further experiments. After analysis of PCR-RFLP and plasmid profiles, strains with the same profiles were discarded. 23 strains different in the PCR-RFLP pattern and plasmid profiles were considered as the representatives of the *Rlt* soil population and subjected to further physiological analyses.

The analyses of the plasmid content of the isolates were performed as it was described by Eckhardt (1978) [3]. The estimation of plasmid size was performed using BIO-PROFIL BioGene Windows Application V11.01 (Vilber-Lourmat, France), using *R. leguminosarum* bv. *viciae* strain 3841 [15] as plasmid standard.

PCR assays of 16S-23S rDNA internal transcribed spacer (ITS) were carried out using genomic DNA of *Rlt* isolates as the templates and primers FGPS1490-5'-TGCGGCTGGATCACCTCCTT-3' and FGPL132-5'-CCGGGTTTCCC CATTCGG-3' [4]. PCR amplicons were digested with *Bsu*RI (*Hae*III) and *Taq*I restriction enzymes (FERMENTAS, Vilnius, Lithuania), and restriction fragments were separated by 3% agarose gel electrophoresis.

The utilization of different carbon and energy sources by *Rlt* isolates was assessed using BIOLOG GN2MicroPlate™ (Gram Negative Identification Test Panel) (BIOLOG, Hayward, USA) containing 95 carbon sources, including sugars, amino acids and organic acids as it had been described earlier [14].

For bacterial growth kinetics assays the isolates were grown overnight at 28 °C in 5 ml TY liquid medium. The cultures were then diluted to OD_{550} of 0.2, and the suspensions were used for inoculation (1:100 v/v) of 79CA, TY and M1 liquid media [12], the latter supplemented with vitamins (thiamine, 1 μg/ml, biotin, 0.5 μg/ml, panthotenate, 1 μg/ml). The cultures were grown in 79CA and TY media for 48 h and in M1 medium for 72 h at 28 °C. Optical density (OD_{550}) of cultures was measured at every 24 h. Each experiment was conducted in triplicate.

For cluster analysis and principal component analysis (PCA) the results of BIOLOG test were coded in the binary system. The cluster analysis was used to define similarity of rhizobia metabolic profiles which were calculated by a simple matching coefficient, following which the clustering was performed by the UPGMA method.

The principal component analysis (PCA) with varimax rotation [6] was used to analyze bacterial capability of utilization of particular substrates or groups of substrates. In this manner PCA method allowed us to transform the numerous variables

(utilization of individual substrates), possibly correlated as well into small groups of uncorrelated factors (utilization of groups of substrates) as well as to interpret the defined PCA factors named PC1, PC2 and PC3.

To compare the growth rates of bacteria belonging to different PCR-RFLP groups (groups 1-4) one-way ANOVA was used. All the described analyses were performed with STATISTICA software.

Results and discussion

50 isolates obtained from nodules of clover plants growing in each other's vicinity were characterized by PCR-RFLP analysis of 16S-23S rDNA ITS region and plasmid patterns. The analyses allow selecting of 23 strains different in these genetic traits. Four distinct PCR-RFLP genetic groups (Groups 1—4) were distinguished after RFLP analysis of PCR-amplified 16S-23S rDNA ITS region with *BsuRI* and *TaqI* restriction enzymes (Fig. 1, Tab. 1). The isolates of Group 1 and 2 may be considered as related to some extent due to the same *BsuRI* RFLP profile.

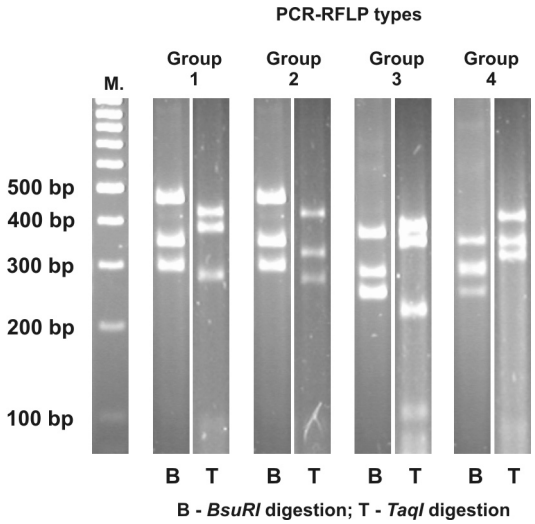

Fig. 1. PCR-RFLP groups identified in *Rlt* strains used in this study
M — 100 bp molecular weight marker (MassRuler™ DNA Ladder Mix, Fermentas).

The plasmid profiles obtained for all isolates showed the great variability, even within one PCR-RFLP group (Fig. 2). Each isolate contained from 2 to 5 plasmids ranging in size approximately from 170 kb to 1 Mb.

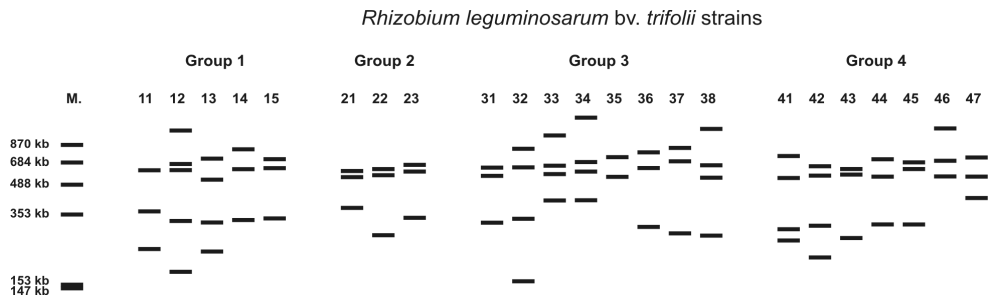

Fig. 2. Plasmid profiles of *Rlt* strains used in this study
M — molecular weight marker (*R. leguminosarum* bv. *viciae* 3841).

All 23 isolates belonging to four different PCR-RFLP genetic groups were examined with respect to their metabolic profiles using commercial Biolog GN2 MicroPlate™ test. This approach revealed that there were substantial differences between strains in number of utilized substrates, ranging from 37 (*Rlt 38*) to 59 (*Rlt 35*) substrates. On the other hand no differences were found between PCR-RFLP groups, where the number of utilized substrates was respectively 49 ± 4, 47 ± 6, 46 ± 8 and 49 ± 5 in Group 1, 2, 3 and 4.

Table 1

PCR-RFLP grouping of *Rlt* strains used in this study

PCR-RFLP group	Strain numbers
Group 1	11, 12, 13, 14, 15
Group 2	21, 22, 23
Group 3	31, 32, 33, 34, 35, 36, 37, 38
Group 4	41, 42, 43, 44, 45, 46, 47

Similarly when the Biolog test results were analyzed by the UPGMA method, no relationships were found between PCR-RFLP groups and clustering based on metabolic properties of strains (Fig. 3). Two main branches at 86% similarity level and two independent lineages can be distinguished, but the representatives of four PCR-FFLP groups was found in each of them (Fig. 3).

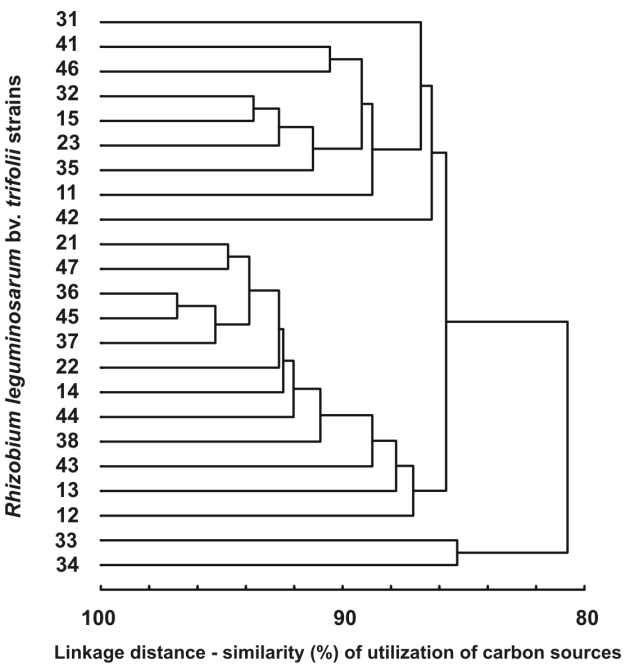

Fig. 3. UPGMA dendrogram of *R. leguminosarum* bv. *trifolii* strains constructed on the basis of the Biolog test results

Since cluster analysis did not allow identifying the qualitative metabolic differences between the clover nodule isolates, the results of Biolog test were subjected to principal component analysis (PCA). The total 95 carbon and energy sources used in the test were arbitrary divided into nine groups: monosaccharides [S], complex saccharides [cS], modified saccharides [mS], non-modified acids [A], modified acids [mA], sugar acids [sA], non-modified aminoacids [AA], modified aminoacids [mAA] and others (with amines predominating) [O]. The PCA analysis enabled us to group all mentioned carbon and energy sources into three factors explaining most of the total variance: principal component 1 (PC1) composed of A, mS, AA, O and sA; principal component 2 (PC2) composed of S, cS and mAA; and principal component 3 (PC3) including mA (detailed data not shown). Taking into account the chemical characteristics of the substrates, PC1 was interpreted as "utilization of non-sugar components", and PC2 as "utilization of sugar substrates". These two large groups of substrates were composed of 90 (35 "sugars" and 55 "non-sugars") of 95 tested carbon and energy sources. Substantial differences in capability to utilize of substrates belonging to these two groups by the tested rhizobia were found (Tab. 2). While individual strains used "sugars" more readily, the greater variance in substrate utilization by different strains was observed for "non-sugars" substrates. Therefore the differences in the number of utilized substrates by individual *Rlt* strains may be attributed rather to "non-sugars" than to "sugars" substrates (Tab. 2).

Table 2

Classes of metabolic substrates created by the principal component analysis (PCA) applied to *Rlt* strains

Substrates	Group of substrates		
	"sugars" (from PCA classification)	"non-sugars" (from PCA classification)	all tested Biolog's substrates
Total number of tested substrates	35	55	95
Minimum number of utilized substrates	24	7	37
Maximum number of utilized substrates	31	26	59
Average number of utilized substrates	28.2 ± 1.6	15.7 ± 5.0	47.7 ± 6.0
Variance (for number of utilized substrates)	2.4	25.3	36.5
% of utilized substrates	80.5 ± 4.5	27.7 ± 8.2	49.8 ± 5.8
Variance (for % of utilized substrates)	19.8	67.0	33.8

The relations in the utilization of "sugars" and "non-sugars" by the strains belonging to PCR-RFLP groups were examined (Tab. 3). Relatively high correlation coefficients (ranging from 0.700 to 0.996) between utilization of "sugars", "non-sugars" and "all substrates" were observed in Group 1 and 2. On the other hand correlation between the use of "sugars" vs. "non-sugars" and "all substrates" in Group 4 was not visible. This observation suggested that the utilization of "non-sugar" substrates reflected the differences of metabolic potential in this group of rhizobial strains (Tab. 3).

Table 3

Correlation coefficients for utilization of "sugar" and "non-sugar" substrates in four PCR-RFLP groups of *Rlt* strains

PCR-RFLP group	Groups of substrates		
		"non-sugars"	all substrates
Group 1	"sugars"	0.700	0.858
	"non-sugars"	-	0.965
Group 2	"sugars"	0.786	0.839
	"non-sugars"	-	0.996
Group 3	"sugars"	0.560	0.739
	"non-sugars"	-	0.972
Group 4	"sugars"	0.357	0.452
	"non-sugars"	-	0.995

The metabolic potential of the individual strains was also estimated by measurements of their growth rates in M1, 79CA and TY liquid media and substantial differences in their growth rates were observed (the details not shown). When ANOVA and post-hoc Tukey test were applied in relation to PCR-RFLP genetic classification (for groups composed of numerous strains), statistically significant difference ($p<0.05$) in the growth rates of isolates belonging to Group 4 vs. Group 1, 2 and 3 after 24 h of growth, as well between the isolates belonging to Group 4 vs. Group 3 after 48 h of growth was observed on TY medium (Tab. 4). The statistically significant differences were not observed in the case of strains grown on M1 and 79CA media.

In general a great diversity of studied local population with respect to PCR-RFLP profiles, plasmids content, metabolic profiles and growth rate was found. The degree of diversity observed within this population is comparable with that described for populations originating from the distinct soils [5]. It seems that in the context of population diversification studies, plasmid and metabolic profiles should be of special interest. A substantial part of genetic variability results from different plasmid content that constitutes an accessory rhizobial genome influencing bacterial metabolic potential and differentiation of strains even within a given genetic group.

Despite the lack of the straight correlation between PCR-RFLP genetic groups and plasmid content or PCR-RFLP and Biolog test results, some relationships could be found. In the case of the strains belonging to the Group 1 and 2 characterized by similar PCR-RFLP profile similarity in utilization of "sugars" and "non-sugars" were observed, while such correlation between the use of "sugars" vs. "non-sugars" and "all substrates" was not visible in the strains of genetically distinct Group 4. Moreover the differences in the growth rate in TY medium of the strains belonging to Group 4 suggest their variable metabolic potential. Though the genetic classification of the strains based only on the differentiation in 16-23S ITS rDNA is very useful, this type of study underestimates the biodiversity of especially complex populations such as rhizobial ones. The genetic characteristic supplemented by metabolic characterization provides more adequate assessment and demonstrates the correlation occurring between genetic and metabolic profiles of bacterial strains.

Table 4

The growth of *Rlt* strains belonging to four PCR-RFLP groups in TY, 79CA and M1 liquid media

PCR-RFLP group	OD_{550}			
	79CA medium		TY medium	
	24h	48h	24h	48h
Group 1	0.31 ± 0.07a	0.61 ± 0.14a	0.34 ± 0.05a	0.48 ± 0.05ab
Group 2	0.33 ± 0.07a	0.60 ± 0.09a	0.35 ± 0.06a	0.51 ± 0.07ab
Group 3	0.28 ± 0.05a	0.54 ± 0.11a	0.33 ± 0.05a	0.50 ± 0.04a
Group 4	0.26 ± 0.09a	0.56 ± 0.09a	0.28 ± 0.05b	0.46 ± 0.06b

	M1 medium		
	24h	48h	72h
Group 1	0.23 ± 0.07a	0.45 ± 0.19a	0.52 ± 0.21a
Group 2	0.29 ± 0.08a	0.56 ± 0.16a	0.67 ± 0.15a
Group 3	0.25 ± 0.07a	0.51 ± 0.17a	0.70 ± 0.22a
Group 4	0.27 ± 0.09a	0.57 ± 0.23a	0.69 ± 0.24a

[a, b] — the values for one medium at defined time of the growth marked with the same letter did not differ significantly at $P<0.05$

The strain numbers are on the left. The scale bar represents percent of metabolic diversity.

REFERENCES

1. *Brom S., Garcна-de los Santos A., Cervantes L., Palacios R., Romero D.* In *Rhizobium etli* symbiotic plasmid transfer, nodulation competitivity and cellular growth require interaction among different replicons // Plasmid. — 2000. — 44. — P. 34—43.
2. *Depret G., Laguerre. G.* Plant phenology and genetic variability in root and nodule development strongly influence genetic structuring of *Rhizobium leguminosarum* biovar *viciae* populations nodulating pea // New Phytol. — 2008. —179. — P. 224—235.
3. *Eckhardt T.* A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria // Plasmid. — 1978. — 1. — P. 584—588.
4. *Laguerre G., Mavingui P., Allard M. R., Charnay M. P., Louvrier P., Mazurier S. I., Rigottier-Gois L., Amarger N.* Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars // Appl. Environ. Microbiol. — 1996. — 62. — P. 2029—2036.
5. *Laguerre G., Louvrier P., Allard M.R., Amarger N.* Compatibility of rhizobial genotypes within natural populations of *Rhizobium leguminosarum* biovar *viciae* for nodulation of host legumes // Appl. Environ. Microbiol. — 2003. — 69. — P. 2276—2283.
6. *Morrison D.F.* Multivariate statistical methods. McGraw-Hill 3rd ed., New York, NY, USA, 1990.
7. *Mutch L.A., Young J.P.* Diversity and specificity of *Rhizobium leguminosarum* biovar *viciae* on wild and cultivated legumes // Mol. Ecol. — 2004. — 13. — P. 2435—2444.
8. *Oresnik I.J., Pacarynuk L.A.,. O'Brien S.H.P, Yost C., Hynes M.F.* Plasmid-encoded catabolic genes *in Rhizobium leguminosarum* bv. *trifolii*: evidence for a plant-inducible rhamnose locus involved in competition for nodulation // Mol. Plant Microbe Interact. — 1998. — 11. — P. 1175—1185.

9. Oresnik I.J., Liu S.L., Yost C.K., Hynes M. F. Megaplasmid pRme2011a of *Sinorhizobium meliloti* is not required for viability // J. Bacteriol. — 2000. — 182. — P. 3582–3586.

10. Palacios R., Newton W.E. Genomes and genomics of nitrogen-fixing organisms. Rafael Palacios and William E. Newton ed. — Dordrecht: Springer, 2005.

11. Perret X., Staehelin C., Broughton W.J. Molecular basis of symbiotic promiscuity // Microbiol. Mol. Biol. Rev. — 2000. — 64. — P. 180–201.

12. Sambrook J., Fritsch E.F., Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press 2nd ed., Cold Spring Harbor, NY, USA, 1989.

13. Vincent J.M. A manual for the practical study of root nodule bacteria. International biological program handbook no.15. Oxford: Blackwell Scientific Publications Ltd., 1970.

14. Wielbo J., Marek-Kozaczuk M., Kubik-Komar A., Skorupska A. Increased metabolic potential of *Rhizobium* spp. is associated with bacterial competitiveness // Can. J. Microbiol. — 2007. — 53. — P. 957–967.

15. Young J.P., Crossman L.C., Johnston A.W.,. Thomson N.R, Ghazoui Z.F., Hull K.H., Wexler M., Curson A.R., Todd J.D., Poole P.S., Mauchline T.H., East A.K., Quail M.A., Churcher C., Arrowsmith C., Cherevach I., Chillingworth T., Clarke K., Cronin A., Davis P., Fraser A., Hance Z., Hauser H., Jagels K., Moule S., Mungall K., Norbertczak H., Rabbinowitsch E., Sanders M., Simmonds M., Whitehead S., Parkhill J. The genome of *Rhizobium leguminosarum* has recognizable core and accessory components // Genome Biol. — 2006. — 7. — P. 34.

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РАЗЛИЧИЯ ЛОКАЛЬНЫХ ПОПУЛЯЦИЙ *RHIZOBIUM LEGUMINOSARUM* BV. *TRIFOLII*: СВЯЗЬ МЕЖДУ ГЕНЕТИЧЕСКИМИ И ФИЗИОЛОГИЧЕСКИМИ ПРИЗНАКАМИ

Реферат

Изучена генетическая и метаболическая вариабильность локальных популяций *Rhizobium leguminosarum* bv. *trifolii* в контексте возможных связей между метаболо-физиологическими свойствами ризобий. Были обнаружены значительные генетические различия в 16-23S рДНК регионах и в плазмидных профилях исследованных штаммов. Штаммы, разделённые на группы с помощью PCR-RFLP, значительно отличались. Метаболический профиль ризобий, исследованных с помощью Biolog теста, показал существенные различия даже в пределах одной генетической группы. PCA анализ метаболических свойств показал различия в утилизации двух больших групп субстратов (а) сахаров, и (б) органических кислот, аминокислот и модифицированных сахаров, названных в работе «не-сахара». Утилизация химически различных сахаров ризобиями наблюдалась более часто, чем «не-сахаров». Утилизация «сахаров» и «не-сахаров» положительно коррелировала с генетическими различиями штаммов, относящихся к PCR-RFLP группами 1, 2 и 3. «Сахара» утилизировались отлично от «не-сахаров» штаммами группы 4. Обнаружены значительные различия у этих PCR-RFLP групп штаммов в способности расти на разных средах. Сделан вывод о том, что локальные популяции

Rhizobium leguminosarum bv. *trifolii* не являются однородными. Они состоят из физиологически различных штаммов, разная метаболическая активность которых является отражением их генетической неоднородности.

Ключевые слова: *Rhizobium leguminosarum*, метаболические и генетические различия.

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ВІДМІННОСТІ ЛОКАЛЬНИХ ПОПУЛЯЦІЙ *RHIZOBIUM LEGUMINOSARUM* BV. *TRIFOLII*: ЗВ'ЯЗОК МІЖ ГЕНЕТИЧНИМИ І ФІЗІОЛОГІЧНИМИ ОЗНАКАМИ

Реферат

Досліджена генетична та метаболічна варіабільність *Rhizobium leguminosarum* bv. *trifolii* у контексті можливих зв'язків між метаболо-фізіологічними ознаками ризобій. Були встановлені значні генетичні відмінності у 16-23S рДНК регіонах та в плазмідних профілях досліджуваних штамів. Штами, розподілені на групи за допомогою PCR-RFLP, значно відрізнялися. Метаболічний профіль ризобій, досліджений з використанням Biolog тесту, показав суттєві відмінності навіть у межах однієї генетичної групи. PCA аналіз метаболічних властивостей виявив відмінності в утилізації двох великих груп субстратів: (а) цукрів і (б) органічних кислот, амінокислот та модифікованих цукрів, які у роботі названі «не-цукри». Утилізація хімічно різних цукрів ризобіями спостерігалася частіше, ніж «не-цукрів». Утилізація «цукрів» і «не-цукрів» позитивно корелювала з генетичними відмінностями штамів, що належали до PCR-RFLP груп 1, 2 і 3. Штами 4-ї групи по різному утилізували «цукри» і «не-цукри». Були виявлені значні відмінності у здатності цих PCR-RFLP груп штамів рости на різних середовищах. Зроблено висновок, що локальні популяції *Rhizobium leguminosarum* bv. *trifolii* не є однорідними. Вони складаються з фізіологічно відмінних штамів, різна метаболічна активність яких є відображенням їх генетичної неоднорідності.

Ключові слова: *Rhizobium leguminosarum*, метаболічні і генетичні відмінності.