UDC 579
Orginal scientific paper

THE CELLULAR FATTY ACID COMPOSITION OF *Bradyrhizobum japonicum* AND *Sinorhizobium meliloti* STRAINS

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Gojgić-Cvijović Gordana, Dragoslava Radin, Bogić Miličić and Dejan Djoković (2006): The cellular fatty acid composition of *Bradyrhizobum japonicum* and *Sinorhizobium meliloti strains*. Mikrobiologija, Vol. 43. No. 2. 93-102, Beograd.

Microbial fatty acid analysis represents a simple and rapid method for identification of strains mainly for taxonomic purpose. Fatty acid composition in the whole cells and lipopolysaccharides in *Rhizobiaceae* were studied to point at biochemical events of the nitrogen-fixing symbiosis too.

In this study the fatty acid profiles of six strains *Bradyrhizobium japonicum* and three strains *Sinorhizobium meliloti* were analysed. The strains were isolated from soils of Serbia and obtained from collection of Institute of Soil Science. Methyl esters of total fatty acids were prepared from lyophilised biomass using toluene-methanol-sulphuric acid mixture and analysed using GC-MS method.

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In *Sinorhizobium meliloti* strains the main components are 16:0, Σ18:1, *cy*19:0 and in lesser proportion are *cy* 17:0, 17:0, 18:0, 16:1, 3-OH 14:0, 15:0 and 14:0 fatty acids. The results obtained are in accordance with previosly reported data. The absence of 20:3ω 6,9,12*cis* and 3-OH 16:0 fatty acids in all chromatograms might be explained by differences in derivation method or growth medium used. The ineffective strain (Nod^+Fix^-) contains remarkable less *cy*17:0 acid.

Dominant components in *Bradyrhizobium japonicum* strains are Σ18:1 and 16:0 fatty acids. Also *cy*19:0, 18:0 and 16:1 components were detected and certain shifts in the amount of these minor components observed. However, on the basis of our results there is no correlation between the relative content of particular fatty acid and the fixing effectiveness of tested strain.

Keywords: fatty acid analysis, *Bradyrhizobium japonicum*, *Sinorhizobium meliloti*

INTRODUCTION

The most important nitrogen fixing system is the symbiosis between legumes and rhizobia. During this interaction the rhizobia induce nodules on the roots of their leguminous host plant in which they convert gaseous nitrogen to ammonium obviating the presence of other nitrogen sources for the plant growth. In return, the host plant supplies the bacteria with carbon compounds and other nutrients necessary for the support of bacterial growth and nitrogen fixation within the nodule.

The atttachment of bacteria to the root hair and recognition of the correct partner on the part of both plant and bacterium involves a signal exchange such as Nod factors secreted by bacteria and lectins and flavonoides secreted by the root cells. A successful infection process (and later nodule formation) reqiures correct rhizobial cell-surface components. Defects in these components may cause the absence or early abortion of infection thread development (MICHIELS *et.al.* 1994).

Phylogenetic data place the rhizobia and agrobacteria in the α-subdivision of the subclass *Proteobacteria* and divide them into seven genera: nitrogen fixing *Rhizobium*, *Sinorhizobium*, *Mezorhizobium*, *Azorhizobium*, *Allorhizobium* and *Bradyrhizobium* and plant pathogen *Agrobacterium*. The cellular proliferation rate of genus *Bradyrhizobium* is slower than other rhizobia and these bacteria are genetically different from other rhizobia.

The groups were distinguished from each other by nodulation abilities, DNA relatedness and 16 S rRNA gen sequence data. It was demonstrated that the application of cellular fatty acid analysis for establishing a phylogenetical position of rhizobia gave results compatabile with genetical data (TIGHE *et al.*, 2000).

In microbiology fatty acid analysis is used for the taxonomic purpose as well as comparison of some biochemical characteristics like productivity of some metabolities. In rhizobia fatty acids as components of the outer layer of the cell wall and Nod factors may represent a useful indicator for the selection of strains.

The aim of this work is to analyse fatty acids profiles of *Sinorhizobium meliloti* and *Bradyrhizobium japonicum* strains and establish an eventual connection of this composition and nitrogen fixing effectiveness.

MATERIAL AND METHODS

Microorganisms Six strains *Bradyrhizobium japonicum* and three strains *Sinorhizobium meliloti* are used in this study. The strains are obtained from Collection of Institute of Soil Science. Two strains (258 and 507) are isolated from the soil samples collected in Serbia. The origin of all strains is summarized in Tables 1 and 3.

The symbiotic effectiveness of *S. meliloti* was tested in separate trials on the alfalfa K-22 cultivar host (RADIN 1993). For *Bradyrhizobium japonicum* testing was carried out with soybean (*Glycine max*) according to the standard method (VINCENT 1970),

Biomass preparation The *Sinorhizobium meliloti* strains were cultivated in the yeast mannitol broth in aerobic conditions at 28°C for 2 days. The *Bradyrhizobium japonicum* strains were cultivated in a fermenter in a soybean extract-glucose medium for 5-6 days at 28°C. Microbial biomass was separated by centifugation at 12000 rev/min and lyophilised.

Fatty acid analysis 100 mg of biomass was boiled under reflux on water bath in toluene-methanol-sulphuric acid mixture 5 : 5 : 0,2 (V/VV) 3 (MINNIKIN *et la.*, 1975). After cooling 10 mL of saturated sodium chloride solution was added and methyl esters were extracted two times with chloroform : hexane 1 : 4 (V/V) mixture. Extracts were washed with water, dried with anhydrous sodium sulfate and evaporated to dryness in nitrogen steam. The methyl esters of the fatty acids were determined using GC and GC/MS.

Gas chromatography Varian 3400 GC equipped with Split/Splitless injector (1:20) operates at 266°C. Column was JW DB-5 30 m, 0.25 mm ID, 0.25 μm film. Carrier gas was hydrogen, 1 mL/min measured at 210 °C. Column temperature was linearly programmed from 60 to 285 °C at 4.3 °C/min. Detector was FID at 300°C.

GC/MS Ion Trap Detector ITD-705 Finnigan was used for this observation with Varian 3400 GC equipped with Split/Splitless injector (1:20) operates at 266°C. Column Supelco PTE-5 30 m, 0.325 mm ID, 0.25 μm film inserted directly in Ion Trap via transfer line at 240°C. Carrier gas was hydrogen, 1mL/min measured at 210°C. Ion manifold and exit nozzle temperatures of 240°C were used. Column temperature was linearly programmed from 60 to 285°C at 4.3°C/min. Scan range was 39-333 daltons,1 scan/second. Version 3 of ITDS

software was used. CG-MS data were analysed using AMDIS program version 2 (STEIN 1999) and our compilation library.

Identification of the detected compounds was done by comparing the retention times with standard Bacterial Acid Methyl Esters CP Mix , Supelco (Fig.1).

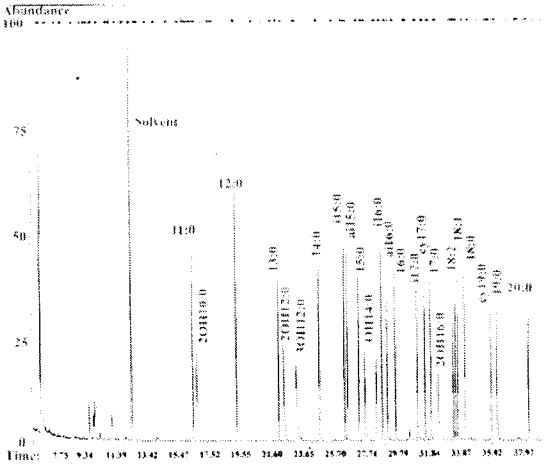

Fig.1. Chromatogram of standard mixture of fatty acid methyl esters

All experiments were done in duplicate and results in Tables 2 and 4 are mean value.

For designation of fatty acids usual abbrevation was used : the first number refers to the number of C atoms, the second number after (:) is number of double bonds, *cy* is cyclopropane supstitued and OH is hydroxylated fatty acid. The double bond position is indicated by ω.

RESULTS AND DISCUSSION

Sinorhizobium meliloti

In Table 1. are shown characteristics of *Sinorhizobium meliloti* strains.

Table 1. Origin and nitrogen fixing effectiveness of Sinorhizobium meliloti strains

	Origin	% Nitrogen in plant	Total nitrogen content (mg/plant)	Fixed nitrogen (mg/plant)
203(Nod$^+$Fix$^-$)	Rothamsted, England	1.58	0.16	0.11
236	Beltsville, USA	3.37	0.84	0.79
258	Chernozem, Ljukovo	3.01	0.77	0.72
Control 1**		0.84	0.05	/
Control 2		2.73	1.42	/

*ineffective strain; **control 1 - noninoculated without N in medium, control 2 - noninoculated with KNO_3 in medium

The strains 236 and 258 are effective nitrogen fixing strains, while the strain 203 forms nodule in *Medicago sativa* but without nitrogen fixing.

Table 2 lists fatty acids identified in *Sinorhizobium meliloti* strains and Figure 2 shows fatty acid profile for strain 236.

Table 2.-Fatty acid composition of Sinorhizobium meliloti strains
Values are the percentage of the total amount of fatty acid components

Fatty acid	Strain 203	Strain 236	Strain 258
cy 19:0	21.73	22.1	25.33
18:0	4.45	2.78	2.54
Σ18:1*	48.27	41.41	40.63
17:0	0.44	Nd**	0.86
cy 17:0	0.97	5.29	5.43
16:0	15.1	16.8	11.6
16:1	1.03	3.29	3.37
3-OH 14:0	5.82	5.52	5.14
15:0	0.59	1.0	1.37
14:0	1.67	1.8	2.2
13:0	Nd	Nd	1.52

*in chromatograms appeared as broad peak corresponding summed feature 18:1 ω7*cis* /ω9 *trans*/ω12 *trans*, 18:1 ω7*cis* /ω9 *cis* /ω12 *trans*, ** Nd. Not detected

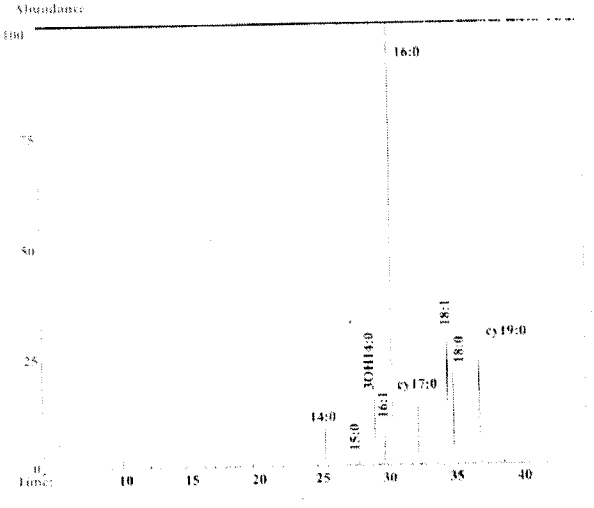

Fig.2. Chromatogram of fatty acids of *Sinorhizobium meliloti* strain 236

As could be seen from Table 2 in *S. meliloti* strains the main components are *cy*19:0, Σ18:1, 16:0 and in lesser proportion are 18:0, 17:0, *cy*17:0, 16:1, 3-OH 14:0, 15:0 and 14:0 fatty acids. The presence and percentage of particular

components in total fatty acid spectrum are in accordance with the reported data (TIGHE *et al.*, 2000; JARVIS *et al.*, 1996). The only exception is the absence of 20:3 ω 6,9,12*cis* and 3-OH 16:0 fatty acids in all chromatograms. These are minor components (average values are 0.5 %) and beside this, the other hydroxylated fatty acids (3-OH 14:0 in samples and 2-OH 16:0 in Supelco standard) are already detected by our GC/MS equipment. We supposed that the absence of these components is due to differences in growth medium or in method of methyl esters preparation used.

Comparison of fatty acid profiles of effective and ineffective strains indicated that strain 203 contains remarkably less *cy*17:0 and 16:1 components. Because of a small number of strains used this observation need to be tested by another methods for example in lipopolysaccharides (LPS) or phospholipids (PL).

In the attachment of the rhizobia cell to the host plant root polysaccharides have the most important role. All types exo, capsular, lipo (EPS, CPS, LPS, respectively) polysaccharides possess the ability to bind the plant lectin. For example, alfalfa nodule invasion by *S. meliloti* (PELLOCK *et al.*, 2000) can be mediated with different efficiency by three polysaccharides: succinoglycan, EPS II or K antigen.

Chemical characterization of effective and ineffective strains of *Rhizobium leguminosarum* bv. *viciae* (IZMAILOV *et al.*, 1999) indicate that the main difference is in the content of hydroxylated mainly 3-OH fatty acids of LPS. The degree of the affinity of the host lectin to LPS of ineffective strain was half that to LPS of effective strain. Very significant is the ratio of unsaturated to saturated fatty acids of a peribacteroid membrane formed by the strains, too.

Bradyrhizobium japonicum

The origin of *Bradyrhizobium japonicum* strains used in this study as well as their nitrogen fixing effectiveness are given in Table 3.

Table 3.-Origin and nitrogen fixing effectiveness of Bradyrhizobium japonicum strains

Strain	Origin	Nitrogen in grain, %	Fixed nitrogen, mg/pot
507	Chernozem, Stig	5.33	59.35
518	Beltsville,USA	5.43	55.15
523	Porto Allegre, Brazil	5.31	56.92
526	Beltsville,USA	6.12	68.55
532*	Beltsville,USA	5.43	61.47
542	Milwackie,USA	6.2	67.30
Control 1**		3.15	/
Control 2		5.47	/

*mutant of 518 resistant to 200 μg /mL streptomycin

**control 1 - noninoculated without N in medium, control 2 - noninoculated with KNO_3 in medium

The nitrogen fixing effectiveness was tested with soybean *Glycine max*. The most effective strains are 526 and 542.

The fatty acid profiles of tested strains are shown in Table 4 and corresponding chromatogram for the strain 523 in Figure 3.

Table 4.-Fatty acid composition of Bradyrhizobium japonicum strains
Values are the percentage of the total amount of fatty acid components

Fatty acid	507	518	523	526	532	542
cy19:0	10.85	3.77	4.08	Nd*	3.09	8.22
18:0	0.94	9.43	4.08	7.79	6.79	Nd
Σ18:1**	64.62	67.92	73.47	73.12	69.14	68.49
16:0	18.87	13.21	14.97	16.88	12.34	18.49
16:1	4.72	5.67	3.4	1.95	8.64	4.79

*Nd. Not detected

**in chromatogams appeared as broad peak corresponding summed feature 18:1 ω7*cis* /ω9 *trans*/ω12 *trans*, 18:1 ω7*cis* /ω9 *cis* /ω12 *trans*,

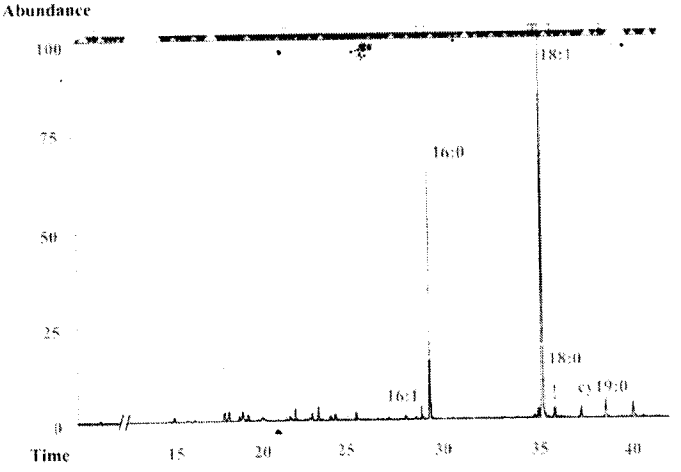

Figure 3 Chromatogram of fatty acids of *Bradyrhizobium japonicum* strain 523

Comparisons of the bradyrhizobia fatty acid profiles with those of other members of the genera indicate that they possess a unique ratio of fatty acids. Unlike other rhizobia, bradyrhizobia are composed primarily of 16:0 and Σ18:1 that make up 90% of the total fatty acids. Additional fatty acids detected in the tested strains are *cy*19:0, 18:0 and 16:1. Our results are in accordance with the data of other researchers (TIGHE *et. al.*, 2000; RATLEDGE *et al.*, 1988). Tighe *et al.* detected in *B. japonicum* strains also 17:0 fatty acid in small concentration.

As Table 4 shows certain shifts in the amount of minor components were observed particularly in the *cy*19:0 and 18:0 content. The maximum degree of similarity was expressed by strains 518 and his mutant 532 without regard to nitrogen fixing effectiveness. It should be noted that the tested strains have different growth rates and probably such differences in fatty acid content resulted from that. On the basis of our results there is no correlation between the relative content of particular fatty acid and nitrogen fixing ability of the tested strains.

These data could be supported by the results of other authors (BOUMAHDI *et al.*, 2001). During the course of growth of *B. japonicum*, an interchange of *cis*-vaccenic (18:1 *cis* ω11) with lactobacillic acid (*cy* 19:0) and a slight increase in palmitic acid were observed while other fatty acids remained constant. The degree of unsaturation was significantly higher in the exponential phase of growth.

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Received September 22, 2003
Accepted December 1, 2006

ĆELIJSKE MASNE KISELINE SOJEVA *Bradyrhizobum japonicum* i *Sinorhizobium meliloti*

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Izvod

Analiza masnih kiselina mikroorganizama, jednostavna i brza metoda za identifikaciju sojeva, često se koristi u taksonomiji. Masne kiseline celih ćelija i lipopolisaharida kod *Rhizobiaceae* su od posebnog značaja za proučavanje simbiotske azotofiksacije.

U ovom radu su analizirani profili masnih kiselina šest sojeva *Bradyrhizobium japonicum* i tri soja *Sinorhizobium meliloti*. Sojevi su ili izolovani iz uzoraka zemlje iz Srbije ili potiču iz kolekcije Instituta za zemljište. Metil estri ukupnih masnih kiselina su pripremani digestijom liofilizovane biomase u smesi toluol-metanol-sumporna kiselina i analizirani GC-MS metodom.

Kod sojeva *Sinorhizobium meliloti* glavne komponente su 16:0, Σ18;1, *cy*19:0 i u manjem procentu *cy* 17:0, 17:0, 18:0, 16:1, 3-OH 14:0, 15:0 i 14:0 masne kiseline. Dobijeni rezultati su u saglasnosti sa literaturnim podacima, a odsustvo 20:3ω 6,9,12*cis* i 3-OH 16:0 masnih kiselina u svim hromatogramima se može objasniti razlikama u sastavu podloge korišćene za gajenje i načinu pripreme metil estara. Neefektivni soj (Nod^+Fix^-) sadrži znatno manje *cy*17:0 kiseline.

Dominantne komponente kod sojeva *Bradyrhizobium japonicum* su Σ18:1 i 16:0 masne kiseline. Pored toga detektovane su i *cy*19:0, 18:0 and 16:1 masne kiseline. Uočene razlike u koncentraciji ovih minornih komponenata su verovatno posledica različite brzine rasta, pa na osnovu dobijenih rezultata nema korelacije izmedju sadržaja masnih kiselina i efektivnosti azotofiksacije kod ispitivanih sojeva.

Primljeno 22. septembra 2003.
Odobreno 1. decembra 2006.