ՀԱՅԱՍՏԱՆԻ ԳԻՏՈՒԹՅՈՒՆՆԵՐԻ ԱԶԳԱՅԻՆ ԱԿԱԴԵՄԻԱ
НАЦИОНАЛЬНАЯ АКАДЕМИЯ НАУК АРМЕНИИ
NATIONAL ACADEMY OF SCIENCES OF ARMENIA
ДОКЛАДЫ ԶԵԿՈՒՅՑՆԵՐ REPORTS

Հատոր Том Volume 119 2019 № 1

MICROBIOLOGY
BIOCHEMISTRY

УДК 595; 577

H. H. Panosyan,
corresponding member of NAS RA A. H. Trchounian

Polar Lipid Pattern and Fatty Acid Composition of Geobacilli and Their Temperature Induced Changes

(Submitted 9/II 2019)

Keywords: *geobacilli, polar lipids, straight, iso- and anteiso-ranched, fatty acids, chemotaxonomy.*

Membrane lipids have been used extensively for chemotaxonomic purposes [1]. The identification of bacteria is based on the large structural differences within membrane fatty acids (FA) including the variation in length (8 to 20 C-atoms), presence of saturated and monounsaturated bonds, occurrence of branched (*iso*- and *anteiso*-), cyclopropane (17:0c, 19:0c), hydroxy- (with an OH-group at position two or three of the molecule) FAs [1-3].

Bacteria are able to modify their membrane composition in response to environmental changes, such as in temperature, osmolarity, salinity and pH [4, 5]. The biophysical properties to control their membrane phospholipids allows bacteria to thrive in a wide range of physical environments [6, 7]. It was shown that bacteria precisely adjust their membrane lipid composition by modifying the types of FAs and altering the structures of pre-existing phospholipids [7].

In thermophilic bacterial species, polar lipids form a large proportion of the cellular membrane fractions and usually include phospholipids and glycolipids [8]. Comparative studies on the FAs composition of psychrophilic, mesophilic and thermophilic bacilli have shown that a certain preference for the synthesis of saturated, elongated and branched FAs existed at higher temperature [9-12].

The aim of this study is to determine polar lipid and FA composition of geobacilli strains isolated from Armenian geothermal springs. Temperature induced changes in membrane lipids and FAs profile were also analyzed. Chemotaxonomic implications based on FAs analysis were reported too.

Materials and methods. The objects of investigation were four thermophilic bacilli strains recently isolated from Arzakan (Armenian) geothermal spring and identified based on 16S rRNA gene sequence data as *Geobacillus caldoxylosilyticus* ArzA-3, *G. thermodenitrificans* ArzA-6, *G. toebii* ArzA-8 and *G. toebii* ArzA-33a. The sequences of strains have been deposited in GenBank under accession numbers JQ929017, JQ929020, JQ929022, and JQ929015 respectively [13].

Cultivation conditions. Batch cultivation of thermophilic bacilli was carried out using nutrient broth (Difco) at pH 7.2 and 65 °C (optimum temperature) under aerobic conditions with shaking at 240 rpm, for 24 h. In case of *G. thermodenitrificans* ArzA-6 incubation were carried out also at 45, 55, 60 and 70 °C. All strains were grown in 1 L Erlenmeyer flasks filled with 200 mL of medium. Cultures were grown to the late exponential phase (turbidity 0.6-0.7 OD at 600 nm) and 50 ml inocula were transferred into 1 L of fresh medium in 5 L flasks and incubated at the appropriate conditions up to late exponential phase. After incubation, the cells were harvested by centrifugation (10,000 rpm; 15 min) and lyophilized.

Lipid analysis. Total lipids were extracted by the method of Bligh and Dyer [14] usung by $CHCl_3/CH_3OH$ (1:1, v/v) mixture under shaken conditions overnight. Solvents were removed with a rotavapor and lipids were weighed for calculation of lipid yield. Lipids were fractionated by thin layer chromatography (TLC) on silica gel (0.25 mm, F_{254}, Merck KGA). The mixture of $CHCl_3/CH_3OH/H_2O$ (65:25:4, v/v/v) was used as a solvent system for the initial monodimensional TLC experiment. The phospholipids were identified also by using bidimensional TLC. The plate was developed in the first dimension in solvent system $CHCl_3/CH_3OH/H_2O$ (65:25:4, v/v/v) and in second dimension in solvent system $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (85:12:15:4,v/v/v/v) [15]. Cardiolipin disodium salt from bovine heart (CL), rac1,2-dipalmitoyl-glycero-3-phosphoethanolamine (PEA), 2,3-dipalmitol glycero-1-phosphocholine (PC_1), 1,2-dipalmitol-SN-glycero-3-phosphocholine monohidrate (PC_3), 1(3-SN-phosphatidyl)-rac-glycerol sodium salt (PG) and 3-SN-phosphatidyl-1-serine from bovine brain (PS) were used as standards. Lipids were detected by spraying the plates with Cs_2SO_4 in H_2SO_4 (w/v) followed by heating at 100 °C for 5 min. Staining tests for complex lipids were performed by using specific reagents: molybdenum blue (for detection of phospholipids); 3.4% N-(1-naphthyl)-ethylenediamine in 97:3 MeOH–H_2SO_4 (for detection of glycolipids), ninhydrin (for visualization of aminolipids) [16-18].

FA analysis. FA methyl esters (FAMEs) were obtained from complex lipids by acid methanolysis (CH_3OH/HCl anhydrous 9:1 at 80 °C overnight). Samples were treated repeatedly with the mixture of CH_3OH and $CHCl_3$ and dried under nitrogen until disappearance of HCl smell. The purity of samples was assessed by TLC analysis. Samples were eluted with N-hexane/CH_3CH_2OH (96:4 v/v), detected by exposure to J_2 vapors and compared with standards. FAME analyses were performed by gas liquid chromatography (GLC, Carlo Erba HRGC 5300 Instrument, FID, OV 1.25m x 0.32mm, 0.25μm). Iden-

tification was accomplished by comparison with a standard mixture of FAMEs. In addition to GLC, identification of each sample component was confirmed by mass spectroscopy analyses, which were performed with an GC-MS HP5890 series II TRIO 2000 VG analytical Instrument, using flame-ionization detector (FID) under following conditions: HP-5 column, temperature programme of 120°C (1 min), from 120 to 230 °C at 2 °C /min, from 230 °C to 250 °C at 10 °C/min, injection volume 1µl. The identification of the compounds was performed by parallel runs of pure standards (Sigma), and by interpretation of mass spectra [15].

Results and discussion. The total lipid content of studied thermophilic strains ranged between 2.0-2.5 % of the cells biomass at the optimal growth temperature. Phospholipids formed a large proportion of the cellular membrane fractions for all studied strains (Fig. 1). A major glycolipid (phosphoglycolipid) was detected only for *G. thermodenitrificans* ArzA-6 (Fig. 1) in case of incubation at higher temperatures (60, 65 and 70 °C). Other glycolipids were not visualized in chromatograms probably due to their absence in studied strains. It was recently reported also that relative proportion of the main glycoloipids increase with growth temperature [19]. Presumably, sugar-containing lipids increase the hydrogen-bonding capacity of the lipid bilayer surface, thus stabilizing the membrane at high temperatures.

Analogy in aminolipid content between all studied bacilli was observed. Major aminolipids and minor aminolipids and amniophospholipids and or their traces were also detected in strains. A major aminolipid and aminophospholipid was detected for *G. thermodenitrificans* ArzA-6 at lower than the optimal growth temperatures.

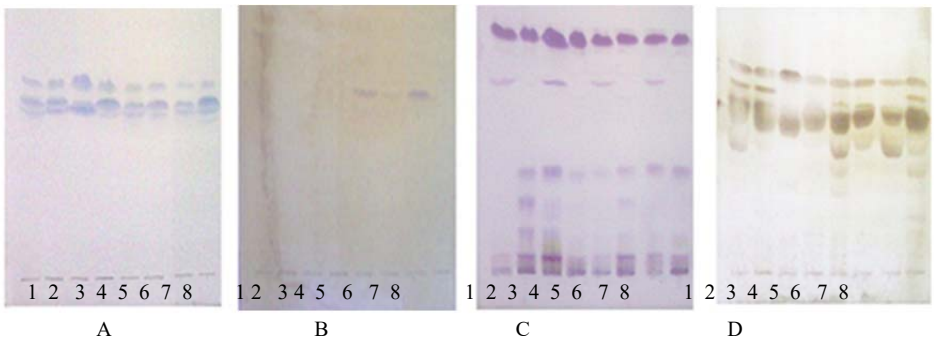

Figure 1. TLC of polar lipids. A, Phospholipids; B, Glycolipids; C, Aminolipids; D, Total lipids. The solvents systems and staining reactions were given under Materials and methods. 1-3 – *Geobacillus caldoxylosilyticus* ArzA-3, *G. toebii* ArzA-8, *G. toebii* ArzA-33a, respectively, at 65 °C; 4-8 – *G. thermodenitrificans* ArzA-6 at 45, 55, 60, 65 and 70 °C, respectively.

Significant differences in the lipid pattern of *G. thermodenitrificans* ArzA-6 were observed at lower, optimal and higher growth temperatures. The relative proportions of different polar lipids in *G. thermodenitrificans* ArzA-6 was

obviously affected by temperature. As the growth temperature was increased, the phosphoglycolipids content increased in contrast to aminophospholipids, amount of which was decreased. Moreover, phosphoglycolipids was absent at the lowest temperature of growth (45 °C). Obtained results are in good agreement with data reported earlier confirming that lipid headgroup compositional changes are part of the mechanism regulating membrane fluidity [9, 15, 20].

Different strains of Geobacillus were investigated and different polar lipids, mainly phospholipid species such as phosphatidylethanolamines, phosphatidylglycerols, cardiolipins (glucopyranosylcardiolipin, alanylcardiolipin or lysylcardiolipin, bisphosphatidylglycerol, acylglycosylcardiolipins) were isolated [12]. Analysis of bidimensional TLC of lipid extracts from studied geobacilli also revealed components that could be identified as PEA (Fig. 2). Other tested phospholipids (CL, PC_1, PC_3, PG and PS) were absented or probably due to their small amount were not identified.

The results for determination of FA composition of geobacilli strains are shown in Table 1. All strains were characterized by the predominance of branched chain *iso*- and *anteiso*- FAs (mainly *i*C15:0, *ai*C15:0, *i*C17:0, *ai*C17:0, often *i*C16:0, rarely *ai*C16:0, *ai*+*i*C14:0). The other major component was *n*C16:0. FAs of *n*C15:0 and *n*C17:0 were presented in low amount, while FAs of *n*C14:0 were absent or in trace amounts. The presence of *n*C18:0 as a minor component was detected only for *G. toebii* ArzA-33a. In general, at the optimal growth temperature branched FAs were predominant, ranging from 80 % to more than 89 % of all FAs measured, while straight-chained FAs were minor components (6-8 %).

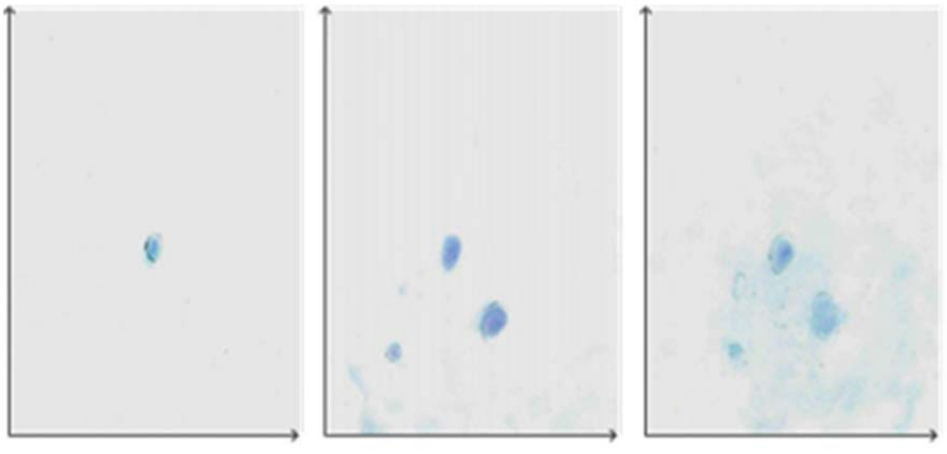

Figure 2. Bidimensional TLC of phospholipids of *G. thermodenitrificans* ArzA-6 compared with PEA using as standard. 1 – PEA; 2 – lipid solution extract of *G. thermodenitrificans* ArzA-6; 3 – mixture of PEA and lipid solution extract of *G. thermodenitrificans* ArzA-6.

Table 1

FA composition (%) of geobacilli strains*

Strains	T (°C)	Lipids % cell dry wt	Total %			*i*C15/*ai*C15	*i*C15/*i*C17	Individual FAs (%)											
			i-branched	*ai*-branched	Normal			*n*C14:0	*i*+*ai*C14:0	*i*C15:0	*n*C15:0	*ai*C15:0	*i*C16:0	*n*C16:0	*ai*C16:0	*i*C17:0	*n*C17:0	*ai*C17:0	*n*C18:0
G. caldoxylo-silyticus ArzA-3	65	2.6	83	6	10	24.6	1.01	0.5	1.1	39.4	1.7	1.6	6.3	7.4	0.3	37.2	tr	3.8	-
G. toebii ArzA-8	65	2.4	81	6	12	23.9	0.94	-	0.5	35.9	1.8	1.5	7.1	8.9	tr	37.9	1.1	4.6	tr
G. toebii ArzA-33a	65	2.7	80	6	13	21.9	0.93	-	Tr	35.1	1.8	1.6	6.9	8.2	tr	37.8	1.1	4.7	2.1
G. thermodenitri-ficans ArzA-6	70	2.0	82	7	10		0.99	Tr	0.5	36.6	1.6	1.2	7.8	6.7	tr	36.9	1.4	6.4	tr
	65	2.6	83	8	9	37.4	1.17	0.2	Tr	41.1	1.4	1.1	7.0	6.8	tr	34.9	1.0	6.4	-
	60	2.3	82	10	7		1.23	0.6	Tr	41.0	1.3	1.9	6.9	6.6	1.1	33.2	tr	6.5	-
	55	1.4	80	10	9		1.35	1.0	-	42.2	1.1	3.1	7.0	6.1	1.0	31.2	tr	6.3	-
	45	1.1	79	11	9		1.49	1.2	-	42.8	0.8	3.8	7.2	5.8	1.3	28.7	tr	6.0	-
[a]*G. caldoxylosily-ticus* DSM 12041^T	65	-	90	4	4	51.5	2.1	-	-	56.7	1.7	1.1	6.8	2.2	-	26.7	-	2.5	-
[b]*G. toebii* SK-1^T	60	-	86	-	-	-	0.97	-	-	34.03	-	-	17.46	-	-	34.86	-	-	-
[c]*G. thermodenitri-ficans* DSM 465^T	65	-	78	8	13	27.8	1.04	0.4	0.38	35.64	1.79	1.28	8.07	6.77	-	34.23	1.61	6.4	0.55

* Values are the means of the least two determinations.

tr: Trace amounts; -: Not detected.

Abbreviation for FAME: *i*, *iso*-branched; *ai*, *anteiso*-branched; *n*, *normal*-elongated unbranched; C14:0-C18:0, saturated straight chains.

[a]Data from Fortina et al. (2001); [b]Data from Sung et al. (2002); [c]Data from Manachini et al. (2000) and Nazina et al. (2001).

The branched-*iso*-family was the most abundant component of the FAs mixture for all tested strains and greatly predominated over *anteiso*-branched FAs. The presence of branched FAs is considered to be a means of maintaining membrane fluidity; *iso*-branched FAs generally have higher melting points, while *anteiso*-branched FAs typically have lower melting points. The studied bacterial strains contained less of the lower melting point pair (*ai*C15:0, *ai*C17:0) and more of the higher melting point pair (*i*C15:0, *i*C17:0).

Unsaturated FAs were not detected. It is comprehensible: unsaturated FAs do not have appropriate properties suitable for maintaining membrane fluidity at higher temperature and they are not widespread in thermophilic microbes. Furthermore, in cold-adapted species the levels of unsaturated FAs or polyunsaturated FAs are known to be relatively high [10, 21].

For *G. thermodenitrificans* ArzA-6 the change in FAs composition at different growth temperature were determined. At different growth temperatures the ratios of branched to straight and of *iso*- to *anteiso*-FAs did not change significantly. The branched-chain FAs were major constituents at all temperature tested. The highest growth temperatures of the strain induced the synthesis of longer chains, while at the minimum temperature shorter chains were biosynthesized. Study of FA profiles in relation to the different temperatures tested showed that *i*C17:0 were preferred at the higher growth temperatures and *i*C15:0 at the lower temperatures, accordingly the ratio of *i*C15:0/*i*C17:0 was decreased along with the temperature rise. With increasing temperature, a decrease of *n*C14 FAs and an increase of *n*C15-*n*C17 FAs were observed. Despite of trace amounts, *n*C18:0 were found only at the highest growth temperature.

The strong presence of elongated, saturated and branched *iso*-FAs with high melting points and, on the other side, the absence of unsaturated acids with low melting points were detected for all tested thermophilic strains. The melting point of the major membrane constituents can be regarded as a factor influencing the flexibility and stability of the membrane. Obtained results are in agreement with previously published data, confirming significance of the membrane lipids structure and composition in thermophility formation [8, 10, 15, 21]. It was shown bacteria have elaborate mechanisms by which they regulate the FAs composition at temperatures just above the phase transition temperature [9].

The chemotaxonomic importance of ratio of the quantitatively predominant FAs *i*15C:0 and *ai*15C:0 for bacilli has been reviewed by Kaneda and later by Kampfer [2, 22]. It was shown for obligatory thermophilic bacilli at the optimal growth temperatures the *i*15C:0 was the predominant FA and *i*15C:0/*ai*15C:0>2. FA profiles of studied strains were compared with those of the following thermophilic reference strains: *G. caldoxylosilyticus* DSM 12041^T [23], *G. thermodenitrifcans* DSM 465^T [24, 25] and *G. toebii* SK-1^T [26]. The ratio of *i*15C:0/*ai*15C:0 of studied thermophilic strains varied from 21.9 to 37.4 (Table 1). This evidence and also high amounts of *i*C17:0 in all strains confirming their affiliation to genus *Geobacillus* including only obligatory thermophiles. Thus, FAs analysis was used as chemotaxonomic approach for complete identification of isolates.

This research was carried out at the Institute of Biomolecular Chemistry (C.N.R.) Pozzuoli (Naples, Italy).

Acknowledgements. The work was supported by the FEBS Short Fellowship-2009 H.P. The invaluable assistance of Prof. B. Nicolaus, Dr. A. Poli and technical support of Mr. E. Pagnotta is to be highly emphasized.

Yerevan State University
hpanosyan@ysu.am

H. H. Panosyan, corresponding member of NAS RA A. H. Trchounian

Polar Lipid Pattern and Fatty Acid Composition of Geobacilli and Their Temperature Induced Changes

Polar lipid patterns and fatty acid (FA) compositions of membrane of four geobacilli strains were analyzed. Phospholipids were found as the main polar lipids. The branched chain of *iso*- and *anteiso*- FAs were predominant, ranging from 80 % to 89 % of total FAs. The branched-*iso*-family FAs (*i*C15:0, *i*C17:0) were the most abundant components (73-76.6 %). Unsaturated FAs were not detected. Temperature induced changes in polar lipids and FA composition were analyzed. With increasing temperature, a decrease of aminophospholipids and short straight (*n*C14) chains of FAs and an increase of phosphoglycolipids and long straight (*n*C15-*n*C17) chains of FAs were observed. The ratio of *i*C15:0/*i*C17:0 was decreased along with the growth temperature rise. FAs analysis was used as chemotaxonomic approach for complete identification of isolates.

Հ. Հ. Փանոսյան, ՀՀ ԳԱԱ թղթակից անդամ Ա. Հ. Թռչունյան

Գեոբացիլների բևեռային լիպիդների և ճարպաթթուների կազմը և դրանց ջերմաստիճանով մակածված փոփոխությունները

Ուսումնասիրվել է չորս գեոբացիլների մեմբրանային բևեռային լիպիդների և ճարպաթթուների բևեռային լիպիդների և ճարպաթթուների (ՃԹ) կազմը: Հաստատվել է, որ բևեռային լիպիդներից գերակշռել են ֆոսֆոլիպիդները: *Իզո*- և *անթեիզո*-ճյուղավորված ՃԹ-ները կազմել են գումարային ՃԹ-ների 80 – 90%-ը: Քանակապես գերակշռել են *իզո*-ճյուղավորված (*ի*C15:0, *ի*C17:0) ՃԹ-ները՝ կազմելով ճյուղավորված ՉԹ-ների 73 – 76.6%-ը: Չհագեցած ՃԹ-ներ չեն հայտնաբերվել: Ցույց է տրվել, որ բևեռային լիպիդների համամասնությունը և ՃԹ-ների կազմը կախված են ջերմաստիճանից: Ջերմաստիճանի բարձրացմանը զուգընթաց դիտվել է ամինալիպիդների և կարճ չճյուղավորված ՃԹ-ների (*ն*C14) քանակի նվազում ու ֆոսֆոգլիկոլիպիդների և երկար չճյուղավորված ՃԹ-ների (*ն*C15-*ն*C17) քանակի ավելացում: Ջերմաստիճանի բարձրացմանը զուգընթաց *ի*C15:0/*ի*C17:0 հարաբերությունը նվազել է: Ճարպաթթուների վերլուծությունը կիրառվել է որպես քեմոտաքսոնոմիական մոտեցում ամբողջականացնելու շտամների նույնականացումը:

О. А. Паносян, член-корреспондент НАН РА. А.А. Трчунян

Состав полярных липидов и жирных кислот геобацилл и их температурные изменения

Изучен состав полярных липидов и жирных кислот (ЖК) мембран четырех штаммов геобацилл. Установлено, что фосфолипиды являются основными полярными липидами изученных штаммов. Показано доминирование длинноцепочечных *изо*- и *антеизо*-разветвленных ЖК, составляющих 80-89% суммарных ЖК. Длинноцепочечные *изо*-разветвленные ЖК (*i*C15: 0, *i*C17: 0) оказались наиболее распространенными компонентами (73-76.6%). Ненасыщенные ЖК не обнаружены. Были обнаружены также изменения пропорции полярных липидов и состава ЖК, вызванные температурой. С повышением температуры наблюдалось уменьшение количества аминофосфолипидов и коротких неразветвленных (*n*C14) ЖК и увеличение количества фосфогликолипидов и длинных неразветвленных цепей (*n*C15-*n*C17) ЖК. Соотношение *i*C15:0/*i*C17:0 уменьшалось с повышением температуры роста. Анализ ЖК использован как хемотаксономический подход для полной идентификации штаммов.

References

1. *Sharmili A.S., Ramasamy P.* – European Journal of Experimental Biology. 2016. V. 6. Issue 5. P. 1-7.
2. *Kaneda T.* – Microbiol. Rev. 1991. V. 55. P. 288-302.
3. *Vandamme P., Pot B., Gillis M., De Vos P., Kersters K., Swings J.* – Microbiol. Rev. 1996. V. 60. P. 407-438.
4. *Diomandé S.E., Nguyen-The C., Guinebretière M.H., Broussolle V., Brillar J.* – Front. Microbiol. 6:813. doi:10.3389/fmicb.2015.00813, 2015.
5. *Parsons J.B., Rock Ch.O.* – Prog Lipid Res. 2013. V. 52. № 3. P. 249-276.
6. *Al-Beloshei N.E., Al-Awadhi H., Al-Khalaf R.A., Afzal M.* – Can. J. Microbiol. 2015. V. 61. P. 48-59.
7. *Zhang Y.M., Rock Ch.O.* – Nature reviews: Microbiology. 2008. V. 6. P. 222-233.
8. *Dreissen A.J.M., Albers S.V.* In: Physiology and Biochemistry of Extremophiles, Eds. Charles Gerday Ch., Glansdorff N. ASM Press. 2007. P. 104-116.
9. *Koga Y.* Thermal Adaptation of the Archaeal and Bacterial Lipid Membranes. Hindawi Publishing Corporation. Archaea. ID 789652, doi:10.1155/2012/ 789652, 2012.
10. *Russell N.J., Fukunaga N.* – FEMS Microbiol. Rev. 1990. V. 75. P. 171-182.
11. *Shen P.Y., Coles E., Foote J.L., Stenesh J.* – J. Bacteriol. V. 103. № 2. P. 479-481.
12. *Siristova L., Melzoch K., Rezanka T.* – Extremophiles. 2009. V. 13. P. 101-109.
13. *Panosyan H., Birkeland N.K.* – J. Basic Microbiol. 2014. V. 54. P. 1240-1250.
14. *Bligh E.D, Dyer W.J.* – Can. J. Biochem. Biophysiol. 1959. V. 37. P. 911-917.
15. *Nicolaus B., Manca M.C., Lama L., Esposito E., Gambacorta A.* – System. Appl. Microbiol. 1995. V. 18. P. 32-36.
16. *Dittmer J.C., Lester, E.L.* – J. Lip. Res. 1964. V. 5. P. 126-127.
17. *Kates M*. Techniques of lipidology: isolation, analysis, and identification of lipids (3rd rev. ed). NewportSomerville, Ottawa, Canada, 2010.

18. *Kundu S.K.* In: Methods in Enzymology, Ed. Lowenstain J.M. N. Y. Academic Press Inc. 1981. P. 185-204.
19. *Yang Y.L., Yang F.L., Jao S,. Ch., Chen M.Y., Tsay S.S., Zou W., Wul S.H.* – J. Lipid Res. 2006. V. 47. P. 1823-1832.
20. *Panosyan H.H.* – Biolog. J. Armenia. 2010. V. 62. № 2. P. 100-107.
21. *Charlier D., Droogmans L.* – Cell Mol. Life Sci. 2005. V. 62. P. 2974-2984.
22. *Kampfer P.* – System. Appl. Microbiol. 1994. V. 17 P. 86-98.
23. *Fortina M.G., Mora D., Schumann P., Parini C., Manachini P. L., Stackebrandt E.* – Int. J. of Syst. Evol. Microbiol. 2001. V. 51. P. 2063–2071.
24. *Manachini P.L., Mora D., Nicastro G., Parini C., Stackebrandt E., Pukall R., Fortina M.G.* – Int. J. of Syst. Evol. Microbiol. 2000. V. 50. P. 1331–1337.
25. *Nazina T.N., Tourova T.P., Poltaraus A.B., Novikova E.V., Grigoryan A.A., Ivanova A.E., Lysenko A.M., Petrunyaka V.V., Osipov G.A., Belyaev S.S., Ivanov M.V.* – Int. J. of Syst. Evol. Microbiol. 2001. V. 51. P. 433–446.
26. *Sung M.H., Kim H., Bae J.W., Rhee S.K., Jeon C.O., Kim K., Kim J.J., Hong S.P., Lee S.G., Yoon J.H., Park Y.H.,. Baek D.H.* – Int. J. of Syst. Evol. Microbiol. 2002. V. 52. P. 2251–2255.