Հայաստանի Գիտությունների Ազգային Ակադեմիա
Национальная Академия Наук Армении
National Academy of Sciences of Armenia

Հայաստանի Կենսաբանական Հանդես
Биологический Журнал Армении
Biological Journal of Armenia

•Փորձարարական և տեսական հոդվածներ •Экспериментальные и теоретические статьи•
•Experimental and theoretical articles•

Biolog. Journal of Armenia, 2 (62), 2010

POLAR LIPID PATTERN AND FATTY ACID COMPOSITION OF THERMOPHILIC BACILLI ISOLATED FROM A SITE WITH GEOTHERMAL ANOMALY OF DILIJAN (ARMENIA)

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Polar lipid (phospho-, glyco- and aminolipids) pattern and fatty acid (elongated, saturated and unsaturated, iso- and anteiso-branched fatty acids) composition of cytoplasmic membrane of seven thermophilic bacilli at the optimal growth temperature were analyzed. Phospholipids and particularly phosphoglycolipids as the main polar lipids found in isolates were studied. The predominance of branched chain of iso- and anteiso- fatty acids ranging from 73 to 89% of total fatty acids was shown. The strong presence of elongated, saturated and branched chain fatty acids and absence of unsaturated acids confirmed the membrane lipids structure and composition significance for thermophility formation of all tested isolates. Chemotaxonomic implications on the basis of branched chain fatty acid analysis were reported.

Polar lipids - fatty acids – thermophiles - Bacillus and related genera

Ուսումնասիրվել է յոթ ջերմասեր բացիլների (թերմոֆիլ) պլազմային մեմբրանի բևեռային լիպիդների (ֆոսֆոլիպիդներ, գլիկոլիպիդներ և ամինոլիպիդներ) և ճարպաթթուների (երկար շղթաներով, հագեցած և չհագեցած, իզո- և անթեիզոճյուղավորված ճարպաթթուներ) կազմը աճի օպտիմալ ջերմաստիճանում: Հաստատվել է, որ ֆոսֆոլիպիդները, մասնավորապես ֆոսֆոգլիկոլիպիդները, հանդիսանում են մեկուսացված բացիլների հիմնական բևեռային լիպիդները: Ցույց է տրվել երկար շղթաներով իզո- և անթեիզո-ճյուղավորված ճարպաթթուների գերակշռումը, որոնք կազմում են գումարային ճարպա-թթուների 73-ից մինչև 89%: Երկար շղթաներով հագեցած, ճյուղավորված ճարպաթթուների գերակշռումը և չհագեցած ճարպաթթուների բացակայությունը հաստատում են թաղանթային լիպիդների կառուցվածքի և կազմի նշանա-կությունը ուսումնասիրված ջերմասեր բացիլների ջերմադիմացկունության համար: Ըստ ճյուղավորված շղթաններով ճարպա-թթուների վերլուծության մեկուսացված բացիլները խմբավորվել են քեմոտաքսոնոմիայի նպատակով:

Բևեռային լիպիդներ – ճարպաթթուներ – թերմոֆիլներ – ցեղ Bacillus և հարակից տեսակներ

Изучен состав полярных липидов (фосфо-, глико- и аминолипидов) и жирных кислот (длинноцепочечные, насыщенные и ненасыщенные, изо- и антеизо-разветвленные жирные кислоты) цитоплазматической мембраны штаммов термофильных бацилл, при оптимальной для роста температуре. Установлено, что фосфолипиды, в частности, фосфогликолипиды, являются основными полярными липидами изолятов. Показано доминирование длинноцепочечных изо- и антеизо-разветвленных жирных кислот, состав-

ляющих 73-89 % суммарных жирных кислот. Наличие длинноцепочечных насыщенных и изо-разветвленных жирных кислот и отсутствие ненасыщенных жирных кислот подтверждает значение структуры и состава мембранных липидов для формирования термофилии изученных изолятов. Для хемотаксономических целей изоляты сгруппированы на основании анализа разветвленных жирных кислот.

Полярные липиды - жирные кислоты – термофилы – род Bacillus и родственные виды

The ability of microbes to thrive in high-temperature environments has recently prompted researchers to study these microorganisms to understand better their interesting physiological traits [3]. The phenomenon of thermostability of microorganisms represents an intricate problem involving many factors, which could be responsible for the stability of entire cells and cellular components [1,5]. Cell membranes are an obvious target when subjected to severe treatments. In thermophilic species polar lipids form a large proportion of the cellular membrane fractions and usually include phospholipids and glycolipids [7, 16]. Comparative studies on the fatty acid (FA) 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, 14, 15]. This is determined by the changes occurring as a result of temperature alterations. In fact, microorganisms synthesize their lipids rapidly, and therefore, they must respond to a changing environment with equal rapidity, altering their membrane lipid composition. It is one of the adaptation mechanisms to allow the survival of thermophiles in extreme conditions [3, 5].

The analysis of the membrane lipids is also a useful chemotaxonomic approach for classification and identification of some bacteria. The significance of the branched-chain FA for classification of representatives of the genus Bacillus and related genera is impressive [8, 9, 17].

The aim of this study is to determine polar lipid and FA composition of seven thermophilic strains of bacilli isolated from soil sampled from the site with geothermal anomaly (Dilijan, Armenia) to ascertain lipids role in thermophility formation mechanism. Chemotaxonomic implications on the basis of fatty acid analysis were reported too.

Material and Methods. The objects of investigation were seven selected thermophilic bacilli designated as T-2, T-3, T-4, T-6, T-7, T-9 and T-10 isolated from the soil sampled from site with geothermal anomaly (40o44'27,7" N, 44o52'01,52" E) near the town of Dilijan [2].

Cultivation conditions. All cells were cultivated in a medium containing (g/l) NaCl, 5; $CaCO_3$, 3; yeast extract, 5; peptone, 5; glucose, 10; pH 7.2. Solid medium was prepared by the addition of 2% agar [2]. After incubation of slants at optimal temperature (for T-4 and T-7 at 50°, for T-2, T-3 and T-6 at 55°, and for T-9 and T-10 at 65°) for 24 hr cells were inoculated into flasks containing 5 ml of liquid medium and cultured in corresponding conditions on shaker. Up to 1 liter of liquid culture was obtained for each isolate by using gradual inoculation method. Cell growth was monitored by a spectrophotometer. Cultures were grown up to the late exponential phase (turbidity 0,5 OD at 540 nm) [13]. Cells were harvested by a centrifugation (10000 g, 15 min).

Lipid analysis. Total lipids were extracted from freshly harvested cells by CHCl3/CH3OH (1:1, v/v) mixture under shaken conditions overnight [4]. 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, F254, Merck KGA) [4]. 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 [4]. The phospholipids were identified by using also 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) [13]. Lipid solutions extracted from the strains Thermus sp. and Bacillus (Geobacillus)

thermoleovorans from the Collection of Extremophilic Bacilli at the Institute of Biomolecular Chemistry (C.N.R., Naples, Italy), as well as cardiolipin disodium salt from bovine heart (CL), rac1,2-dipalmitoyl-glycero-3-phosphoethanolamine (PEA), 2,3-dipalmitol glycero-1-phosphocholine (PC1), 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 100^0 for 5 min. Staining tests for complex lipids were performed by using specific reagents for phospho-, glyco- and aminolipids [6, 10, 11].

Fatty acid analysis. FA methyl esters (FAME) were obtained from complex lipids by acid methanolysis (CH_3OH/HCl anhydrous 9:1 at 80^0 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 J2 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). Identification 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° (1 min), from 120 to 230° at 2°/min, from 230° to 250° at 10°/min, injection volume 1μl. The identification of the compounds was performed by parallel runs of pure standards (Sigma), and by interpretation of mass spectra [13].

Results and discussion. The total lipid content of studied seven thermophilic isolates ranged between 1.8-2.64% of the cells biomass at the optimal growth temperature. Phospho-, glyco- and aminolipids of thermophilic isolates were extracted and fractionated by monodimensional TLC [4]. Phospho- and glicolipids formed a large proportion of the cellular membrane fractions for all seven isolates examined. The phospholipids represented the main polar lipids found in all isolates studied. The glico- and phospholipids content of all studied bacilli compared with lipids extracted from *Thermus sp.* and *Bacillus (Geobacillus) thermoleovorans* is shown in figures 1 and 2. A major and some minor phosphoglycolipids were detected in each isolate. A major and a minor glycolipid, with the exception of minor phosphoglycolipids, were detected only for the isolate T-9 (Fig. 1). Other glycolipids were not visualized in chromatograms probably due to their absence in most of studied isolates. It was shown earlier that glycolipids were widely distributed within species of the *Bacillus* and related genus and their absence was reported only for few bacilli species [12, 13]. Recently It was recently reported also that relative proportion of the main glycoloipids increase with growth temperature [18]. Presumably sugar-containing lipids increase the hydrogen-bonding capacity of the lipid bilayer surface, thus stabilizing the membrane at high temperatures. Consequently the high proportion of phosphoglycolipids in all isolates and additional glycolipids in strain of T-9 could contribute to the ability of the bacteria to grow at high temperature.

Analogy in aminolipid content between all studied bacilli was observed. Major aminolipids and aminoglicolipids and minor phospholipids, phosphoglycolipids, aminolipids, aminophospholipids and or their traces were also detected in isolates. As shown in Figures 1 and 2, in general, by phospho- and glycophospholipids content isolates T-9 and T-10 were similar to *Bacillus (Geobacillus) thermoleovorans* that indicated the belonging of theses isolates to obligatory thermophilic ones of *Bacillus* or related genera. Analogy in polar lipids content of studied bacilli and Thermus sp. were not observed.

Analysis of mono- and bidimensional TLC of lipid extract from some of studied bacilli revealed components that could be identified as PEA (Fig. 3). For the isolates of

T-9 and T-10 were detected also PG and CL. Other tested phospholipids, probably due to their small amount were not identified.

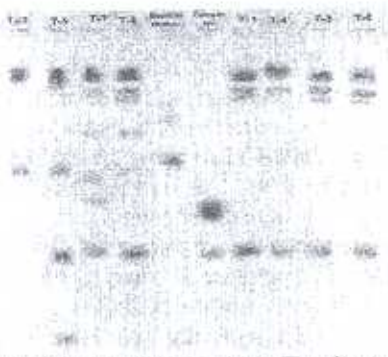

Figure 1. TLC of sugar containing lipids. The plates were treated with α-naphtol for detection glycolipids.

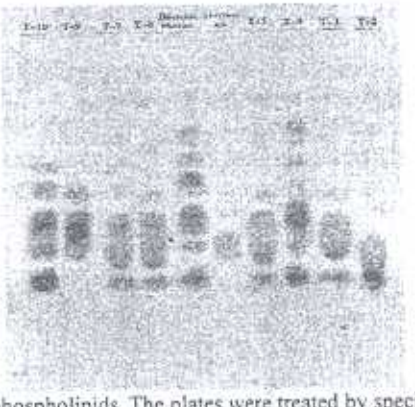

Figure 2. TLC of phospholipids. The plates were treated by specific reagent as reported Dittmer&Lester [6] for detection phospholipids.

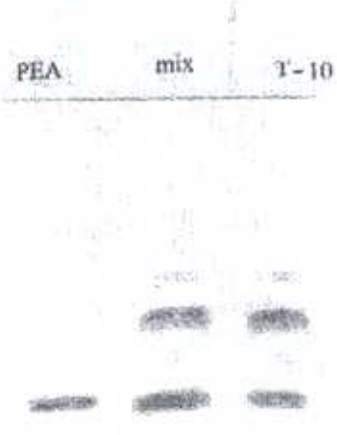

Figure 3. TLC of phospholipids of isolate T-10 compared with PEA using as standard. Mix – mixture of PEA and lipid solution extract of T-10.

The results for determination of FA composition of thermophilic isolates at the optimal temperature of growth are shown in Table 1. All isolates 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, *ai*C16:0, rarely *i*C14:0, *i*C18:0 *i*C19:0). The other major components were *n*C16:0 (11-27%) and *n*C18:0 (2-15%). FAs *n*C14:0, *n*C15:0, *n*C17:0 and *n*C19:0 were absent or in trace amounts. The presence of *i*C18:0, *i*C19:0 and *ai*C19:0 as a minor component was detected only for the isolate T-2. The ratio of normal and branched (both *iso*- and *anteiso*-) FAs are shown in figure 4. In general, branched FAs were predominant, ranging from 73% to more than 89 % of of all FAs measured, while straight chained fatty acids were minor components.

The branched-*iso*-family was the most abundant component of the FA mixture for major part of tested isolates. 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. *Iso*-branched FAs of isolates T-2, T-3, T-6, T-9 and T-10 constituted 42-65% of total FAs and greatly predominated over anteiso-branched FAs, which constituted 17-35%. By contrast, anteiso-branched FAs of isolates T-4 and T-7 slightly predominated over the iso-branched. This can be explained by optical growth temperature, which is the lowest among all tested strains (50° for both isolates).

Unsaturated acids 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 [3, 14].

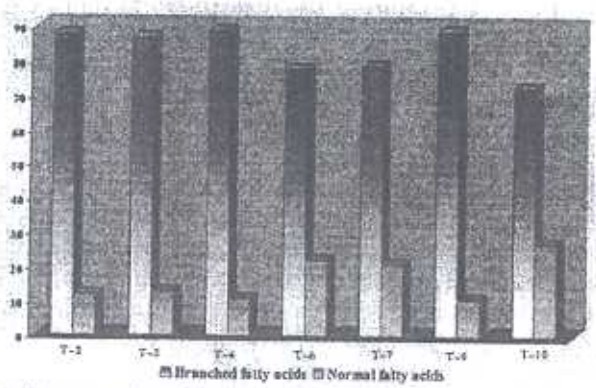

Fig. 4. The comparison of normal and branched (iso- and anteiso-) FAs contents (weight per cent of fatty acids) of tested isolates.

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 isolates. The melting point of the major membrane constituents can be regarded as a factor influencing the flexibility and stability of the membrane. In general the results of the present study are in agreement with previously published data [5, 7, 13-16], confirming that the membrane lipids structure and composition are fundamental for thermophility formation of bacilli isolates.

The importance of branched chain FA analysis for the chemotaxonomy of the genus Bacillus has been reviewed by T. Kaneda [9]. According to Kaneda it is possible to create fingerprints of the genus Bacillus by using FAs analysis. Later P. Kampfer [8] showed that the genus could be divided into major and minor groups based on the ratio of the quantitatively predominant FAs i15C:0 and *ai*15C:0. The *ai*15C:0 FA (12-methyltetradecanoic acid) was the predominant FA in the major group (*i*15C:0/*ai*15C:0<2), and the *i*15C:0 FA (13-methyltetradecanoic acid) was the predominant FA in the minor group (*i*15C:0/*ai*15C:0>2) including mainly obligatory thermopiles.

The ratio of *i*15C:0/*ai*15C:0 of studied thermophilic isolates varied between 0.3-10.5 (Table 1). Isolates T-9 and T-10 were characterized by a ratio of *i*15C:0/*ai*15C:0>2 and could be grouped into minor group, while all others, exhibited a ratio of *i*15C:0/*ai*15C:0<2, could be grouped into major group. Although isolates T-9 and T-10 could group in the same cluster, FA composition was different. The determination of FAs composition of isolate T-9 has shown high amounts of *i*C17:0 suggesting a possible relationship with geobacilli which are known to be obligatory thermophiles. The isolates T-4 and T-7 possessed low amounts of *i*17C:0 and the same value of *i*15C:0/*ai*15C:0 ratio, indicating possible relationship between those isolates. The ratio of *i*15C:0/*ai*15C:0 and relatively high amount of *i*17C:0 content was similar for isolates T-2, T-3 and T-6. Moreover, the differences at the level of *i*15C:0/*ai*15C:0 ratios and *ai*17C:0 content indicate that isolates might be belonging to the different subgroups. Final identification can be proved by studies of other phenotypic and genotypic properties.

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

Acknowledgements

The research was supported by the FEBS Short Fellowship-2005. The invaluable assistance of Prof. B. Nicolaus, Dr. A. Poli and technical support of Mr. E. Pagnotta is to be highly emphasized.

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Received 02.03.2010

Table 1. Fatty acid composition of thermophilic isolates at the optimal temperature of growth

Isolates	Lipid, % cell biomass	*i*C15/*ai*C15	Individual fatty acids (%)													
			i+*n*C14:0	*n*C14:0	*i*C15:0	*ai*C15:0	*n*C15:0	*i*+*n*C16:0	*n*C16:0	*i*C17:0	*ai*C17:0	*n*C17:0	*i*C18:0	*n*C18:0	*i*C19:0	*ai*C19:0
T-2	1.8	1.3	0.54	0.32	22.80	15.77	-	9.20	7.09	23.23	14.66	-	0.75	4.97	0.75	0.66
T-3	2.2	1.4	-	-	24.50	16.50	-	9.11	5.88	23.30	13.43	-	-	7.20	-	-
T-4	2.6	0.3	1.17	0.85	13.40	39.01	-	4.09	7.14	14.78	16.76	-	-	2.84	-	-
T-6	2.9	1.5	-	-	22.63	14.24	-	8.40	6.50	21.01	12.01	-	-	15.20	-	-
T-7	2.4	0.3	-	-	11.07	35.75	-	8.32	8.06	14.85	19.15	-	-	2.80	-	-
T-9	2.6	10.5	-	-	21.55	2.05	1.54	22.84	5.90	32.53	9.70	1.80	-	2.10	-	-
T-10	2.1	2.7	-	1.68	16.90	6.05	-	10.62	18.76	14.90	26.80	-	-	4.17	-	-

-: Not detected.

Abbreviation for FA: *i*, *iso*-branched FA; *ai*, *anteiso*-branched FA; *n*, *normal*-elongated unbranched FA; C14:0-C19:0, saturated straight chains.