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FATTY ACIDS COMPOSITION OF ACTINOBACTERIA ISOLATED FROM MUSSELS *MYTILUS GALLOPROVINCIALIS* OF THE BLACK SEA ODESA BAY

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Analysis of the fatty acid composition of total cellular lipids is important chemotaxonomic characteristic that is used to identify microorganisms, in particular actinobacteria, with the help of fatty acid spectra libraries. Actinobacteria are well known as producers of secondary metabolites, which are of pharmacological and commercial interest. A significant number of actinomycetes is associated with various marine benthic communities. The aim of this study was determination of the fatty acid composition and preliminary identification of actinobacteria isolated from mussels Mytilus galloprovincialis of the Black Sea Odesa Bay. Actinobacteria of 12 isolated strains were grown in a liquid medium at 28° C for 72 h. Methyl esters of fatty acids were determined on a gas chromatograph Agilent. The MIDI Sherlock microorganisms identification system was used to identify the studied strains. It was found that the fatty acids methyl esters profiles of studied actinobacteria strains were characterized by the predominance of 12-17 saturated branched-chain fatty acids with high content of 12-methyltridecanoic, 12-methyltetradecanoic, 14-methylpentadecanoic and 14-methylhexadecanoic fatty acids. All 12 actinobacteria strains isolated from mussels (Mytilus galloprovincialis) of Odesa Bay were identified as members of the Streptomyces genus.

K e y w o r d s: Mytilus galloprovincialis, fatty acid compositions, marine actinobacteria, Streptomyces, Black Sea.

Marine actinobacteria exist in a unique environment characterized by extreme conditions (high pressure, high salinity, temperature changes, nutrients limited availability), and this promotes their synthesis of new biologically active metabolites by forming unusual, for land, metabolic pathways [1, 2].

Marine actinobacteria synthesize a variety of metabolites with antibiotic and antitumor activity, and also they are capable of inhibiting the biofilms formation by antibiotic-resistant human pathogens. To date, it is well known that a significant number of actinomycetes is associated with various marine benthic communities members, such as sponges, corals, ascidians, sea anemones, sea cucumbers, jellyfish, fish, sea urchins, and algae. Thus, the isolation and study of actinobacteria from marine aquatic organisms as potential producers of new antibiotics are promising. Most marine actinobacteria usually are members of the genus *Streptomyces* [1, 3, 4].

The fatty acid composition analysis of total cellular lipids is an important chemotaxonomic characteristic that correlates with the results of molecular genetic identification [5, 6], and is used to identify microorganisms with the help of fatty acid spectra libraries [7]. Differences in fatty acid composition influenced membrane functions, particularly membrane permeability, which consequently could favor the synthesis of antimicrobial compounds [8].

Actinobacteria are divided into two types according to the fatty acids composition. The first type includes species with cells dominated by fatty acids with a branched chain, and species of the second type show a significant percentage of saturated or monounsaturated fatty acids with a straight chain [9, 10].

The study aimed to determine the fatty acids composition of actinobacteria isolated from mussels *Mytilus galloprovincialis* of the Black Sea Odesa Bay, and their preliminary identification.

Materials and Methods

The study was carried out on 12 actinobacteria strains isolated in 2020 from mussels *Mytilus galloprovincialis* that were collected in the Mechnikov Odesa National University Hydrobiological Station sea area from a depth of 3-5 meters of the Black Sea Odesa Bay [11].

Fatty acids methyl esters (FAME) determination of the studied strains was performed according to the MIS Operating Manual [9]. To do this, actinobacteria were grown in 20 ml of Tryptic Soy Broth (Biolife, Italia) at 28 ± 1°C and 150 rpm shaking for 72 h. The resulting liquid culture was passed through 0.45 μm pore size filters, and about 40 mg of separated cells biomass was transferred into glass vials with Teflon-coated lids for fatty acids extraction. Isolation and chromatographic separation of fatty acids was performed according to a standard protocol. Cell lysis and saponification of microorganism's cell lipids were performed by adding 1 ml of a 1.125 M NaOH solution in a methanol mixture at 95–100°C for 30 min. Subsequent fatty acid methylation was performed by adding an acidic solution (2 ml of 6.0 N HCl in methanol) at 80°C for 10 min. Extracted fatty acid methyl esters were neutralized with 0.3 M NaOH solution [7].

Determination of the bacteria fatty acids composition was performed by gas chromatography using an automatic system for the identification of the microorganisms MIDI Sherlock based on gas chromatograph Agilent 7890 (Agilent Technologies, USA), capillary column ULTRA 2 (25 m×0.2 mm×0.33 μm), flame ionization detector. The sample (2 μl) was injected in *split* mode with a coefficient of 40:1, the evaporator temperature 250°C. The separation was performed in the mode of programming temperature – the initial temperature of 170°C with a subsequent gradient of 5°C/min up to 270°C. The fatty acid content was expressed as a percentage of the peak area's total amount. The Sherlock Microbial Identification System library (MIDI Sherlock version 6.2, MIDI Library ACTIN 3.80) was used to identify the studied strains. Statistical analysis of FAME profiles was done with MIDI Library Generation System software version 6.2, which also includes a built-in quantitative multivariate statistical approach [12].

The dendrogram was built based on the fatty acids composition determination results using the *ape* package *as.phylo* function in the R 4.1.1 environment software in the R programming language. The ape package in R 4.1.1 is widely used for any analysis related to phylogenetics and comparative evolutionary studies of organisms. In order to be able to create an object belonging to "phylo" it is necessary first to cluster the data using the dist and hclust methods, which are used in hierarchical cluster analysis. In this case, the function as.phylo.hclust (or as.phylo) transforms the clustering tree, computed with hclust, into a phylogenetic tree, i.e., into a "phylo" class object. Additional analysis was performed using the pvclust package in R 4.1.1, which provides an estimate of the uncertainty in hierarchical cluster analysis.

Results and Discussion

We have previously studied the morphological, cultural, physiochemical properties of actinobacteria isolated from mussels *Mytilus galloprovincialis* in the Odesa Bay of the Black Sea [11]. The importance of chemotaxonomic characteristics, one of which is cellular fatty acids, for the taxonomy of bacteria is well-proven [5, 13]. Bacterial fatty acid profiles can vary significantly with culture age and culture conditions. But provided a standard and reproducible approach, as well as a data library with profiles of reference typical strains, the method can be used to identify bacteria [5-7]. The similarity indices (Sim Index) of the studied strains were established (from 0.01 to 0.302) with the indices of the reference strains from the database of the ACTIN 3.80 version 6.2 library. The Sim Index is a numerical indicator of the FAME profile similarity of the studied strain to the FAME profile of the strains that were used to create the library reference element. The numerical indicator lower than 0.300 suggests that these species are not in the database. Therefore, we can only talk about the preliminary identification of the species, which will be confirmed by 16S sequencing in future works [11]. Thus, 12 actinobacteria strains isolated from mussels (*Mytilus galloprovincialis)* were preliminarily identified as members of the genus *Streptomyces* with different similarity indices [7, 12, 14].

In this chromatographic analysis of the FAME profile all of the studied actinobacteria strains, fatty acids with a carbon chain length ranging from C_{11} to C_{17} were detected, including unsaturated 14-methylhexadecenoic acid ($C_{16:1\ iso\ H}$), 9-cis-hexadecenoic acid ($C_{16:1\ cis\ 9}$), 3-methylheptadecenoic acid ($C_{17:1\ anteiso\ C}$), as well as saturated 12-methyltridecanoic acid ($C_{14:0iso}$), 12-methyltetradecanoic acid ($C_{15:0\ anteiso}$),

T a b l e. Cellular fatty acids composition of Streptomyces strains, (%)

Fatty acid	The strain of *Streptomyces sp.*											
	myt1	myt2	myt3a	myt3b	myt4	myt5	myt6	myt7	myt8b	myt10	myt11	myt12a
$C_{11:0\ anteiso}$	–	–	0.09	0.06	0.13	0.05	0.13	0.10	–	0.05	–	–
$C_{12:0\ iso}$	–	–	0.11	0.12	0.11	0.05	0.13	0.18	–	0.07	0.08	–
$C_{13:0\ iso}$	0.24	0.36	–	0.40	0.58	0.46	0.41	0.43	–	0.14	0.31	0.45
$C_{13:0\ anteiso}$	0.42	0.46	0.34	0.58	0.59	0.31	0.57	0.45	–	0.41	0.46	0.25
$C_{14:0\ iso}$	4.13	2.86	3,24	3.79	3.51	2.05	3.24	1.87	8.03	2.89	3.57	1.43
$C_{14:0}$	0.73	0.58	0.53	0.50	0.52	1.28	0.45	1.09	–	0.26	0,56	0.46
$C_{15:1\ anteiso\ A}$	0.17	–	0,11	–	0.02	–	–	–	–	–	–	–
$C_{15:0\ iso}$	6.64	12.81	12.27	12.64	14.24	13.81	13.37	14.89	–	7.51	9,87	22.18
$C_{15:0\ anteiso}$	36.05	34.32	31.98	33.10	43.30	30.29	44.20	33.67	12.41	48.90	33.53	29.67
$C_{15:0}$	2.46	2.41	–	2.55	1.54	1.94	1.31	2.03	34.84	1,67	2.13	1.21
$C_{15:1\ B}$	–	0.21	0.27	0.32	0.16	0.11	0.15	0.16	–	0.17	0.22	–
$C_{16:1\ iso\ H}$	2.59	2.51	3.09	3.60	1.02	0.80	1.54	0.99	5.38	0.97	2.97	0.64
$C_{16:0\ iso}$	15.05	11.45	13.18	14.10	7.36	6,08	7.42	7.26	15.76	10.82	12.75	6.83
$C_{16:1\ cis\ 9}$	6.79	5.53	4.77	4.92	4.62	6.69	4.03	5.93	6.41	1.61	4.89	2.14
$C_{16:0}$	6.44	6.52	6.25	5.23	5.78	11.23	5.26	10.24	3.53	3.32	5.81	5.96
$C_{16:0\ 9\ methyl}$	1.44	3.12	3.04	3.06	2.43	2.92	2.54	3.21	2.41	1.26	2.07	5.30
$C_{17:1\ anteiso\ C}$	3.03	3.39	3.37	3.45	2.05	2.49	2.74	2.81	4.82	2.58	2.94	2.88
$C_{17:0\ iso}$	1.59	2.70	2.49	2.37	3.16	3.05	3.23	3.43	–	1.52	1.96	8.14
$C_{17:0\ anteiso}$	10.41	8.74	8.08	7.69	6.98	7.59	7.70	9.46	6.42	9.49	7.75	11.39
$C_{17:1\ cis\ 9}$	0.64	0.81	0.74	0.75	0.42	0.24	0.31	0.32	–	0.39	0.70	–
$C_{17:0\ cyclo}$	–	0.52	0.43	0.28	0.46	0.29	0.64	0.34	–	1.12	0.44	–
$C_{17:0}$	0.58	0.45	0.70	0.40	0.32	0.62	0.27	0.58	–	0.27	0.41	0.59
Sim Index	0.288	0.32	0.098	0.302	0.136	0.01	0.113	0.203	0.091	0.075	0.03	0.216

14-methylpentadecanoic acid ($C_{16:0\ iso}$), hexadecanoic acid ($C_{16:0}$), 16-methylhexadecanoic acid ($C_{16:0\ 9\ methyl}$), 14-methylhexadecanoic acid ($C_{17:0\ anteiso}$) (Table).

It was found that the *Streptomyces* genus actinobacteria's FAME profile was dominated by 12-methyltetradecanoic acid ($C_{15:0\ anteiso}$) from 12.41% to 48.9%, 13-methyltetradecanoic acid ($C_{15:0\ iso}$) – 6.64-22.18%, 14-methylpentadecanoic acid ($C_{16:0\ iso}$) – 6.83-15.05%, 14-methylhexadecanoic acid ($C_{17:0\ anteiso}$) – 6.98-11.39%, hexadecanoic acid ($C_{16:0}$) – 3.32-11.23%. The *Streptomyces sp.* myt8b differed from other strains: in its fatty acids composition pentadecanoic acid ($C_{15:0}$) – 34.84% and 12-methyltridecanoic acid ($C_{14:0\ iso}$) – 8.03% were dominated.

The FAME profiles of actinobacteria strains isolated from mussels *Mytilus galloprovincialis* of the Black Sea Odesa Bay were characterized by the predominance of 12-17 saturated branched-chain fatty acids with iso and anteiso positions of the methyl group. Actinomycetes markers are methyl-branched fatty acids, which is consistent with many studies [15-17]. Also, a suitable marker for the *Streptomyces* genus identification is the fact that almost all the strains contained a $C_{17:0\ cyclo}$ small percentage [12]. Unsaturated acids are proposed to have a minor role in microbes containing branched-chain acids, although unsaturation is often regarded as one of the most important regulators of membrane flexibility

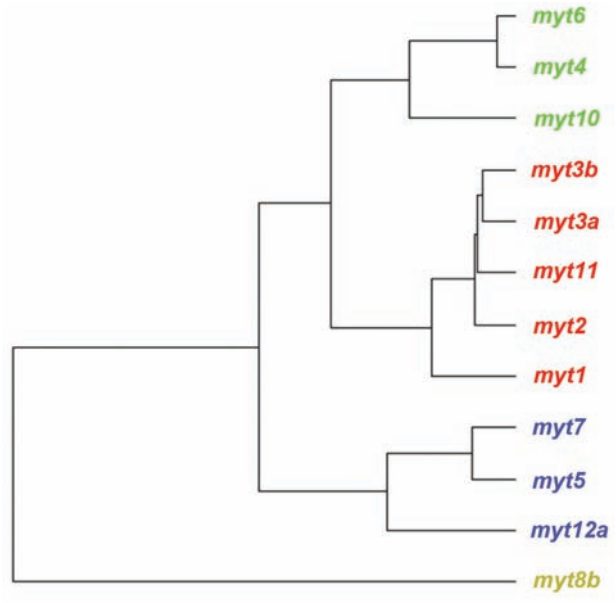

Fig. Dendrogram of results of clustering by the fatty acid composition of streptomycetes isolated from mussels Mytilus galloprovincialis

and stability. The FAME profiles of the streptomycetes contained up to 10% of unsaturated fatty acids, most of which were iso or anteiso acids.

According to the obtained results, most strains were assigned to one cluster that was divided into three subclusters. It can be assumed that, according to the cell wall fatty acid composition results, *Streptomyces sp.* myt3b, myt3a, myt2 showed the greatest affinity for each other. The subcluster also includes *Streptomyces* sp. myt1 and myt11. The other two subclusters include *Streptomyces sp.* myt7, myt5, myt12a and *Streptomyces sp.* myt4, myt6, myt10, respectively. According to the cell wall fatty acids composition, strain myt8b showed the greatest difference from all the other strains that are reflected in the separate cluster forming.

Therefore, this type of clustering may also indicate that not all isolated strains can be attributed to representatives of the genus *Streptomyces*. That is, the affiliation of some of them, such as myt8b, to the genus *Streptomyces* may be incomplete and requires further research.

Conclusions. Fatty acids profiles of actinobacteria strains isolated from mussels *Mytilus galloprovincialis* of the Black Sea Odesa Bay were characterized by the branched saturated fatty acids isomers presence. Thus, by fatty acids chromatographic analysis the 12 studied actinobacteria strains were identified as members of the *Streptomyces* genus. However, the clustering showed the necessity for further specification of classification obtained.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

ЖИРНОКИСЛОТНИЙ СКЛАД АКТИНОБАКТЕРІЙ, ВИДІЛЕНИХ ІЗ МІДІЙ *Mytilus galloprovincialis* ОДЕСЬКОЇ ЗАТОКИ ЧОРНОГО МОРЯ

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Аналіз жирнокислотного складу загальних ліпідів клітини є важливою хемотаксономічною характеристикою, яка використовується для ідентифікації мікроорганізмів, зокрема актинобактерій, за допомогою бібліотек спектрів жирних кислот. Актинобактерії добре відомі як продуценти вторинних метаболітів, які становлять фармакологічний і комерційний інтерес. Значна кількість актиноміцетів пов'язана з різноманітними морськими бентосними угрупованнями. Метою даної роботи було визначення жирнокислотного складу та попередня ідентифікація актинобактерій, виділених із мідій *Mytilus galloprovincialis* Одеської затоки Чорного моря. Актинобактерії 12 виділених штамів вирощували в рідкому середовищі при 28°С протягом 72 год. Метилові ефіри жирних кислот визначали на газовому хроматографі Agilent. Для ідентифікації досліджуваних штамів використовували систему ідентифікації мікроорганізмів MIDI Sherlock. Встановлено, що профілі метилових ефірів жирних кислот досліджуваних штамів актинобактерій характеризуються переважанням 12-17 насичених розгалужених жирних кислот із високим вмістом 12-метилтридеканової, 12-метилтетрадеканової, 14-метилпентадеканової та 14-метилгексадеканової жирних кислот. Усі 12 штамів актинобактерій, виділених із мідій *Mytilus galloprovincialis* Одеської затоки, були ідентифіковані як представники роду *Streptomyces.*

Ключові слова: мідії *Mytilus galloprovincialis*, жирнокислотний склад актинобактерій, *Streptomyces*, Чорне море.

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