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V.V. Klochko

D.K. Zabolotny Institute of Microbiology and Virology,
National Academy of Sciences of Ukraine,
154 Zabolotny St., Kyiv 03143, Ukraine

ECOLOGY, SYSTEMATICS AND ANTIBIOTIC ACTIVITY OF *PSEUDOMONAS BATUMICI* AND *ALTEROMONAS MACLEODII* IN CONNECTION WITH ANALYSIS OF THEIR GENOME STRUCTURE

New data about ecology, systematics and synthesis of biologically active substances of the type strain Pseudomonas batumici UCM-321 producing the antibiotic batumin, highly effective against staphylococci, and of Alteromonas macleodii strains, as representatives of marine species widely inhabiting the world ocean, was obtained based on a complex analysis of their biological properties and genomic structure.

Analysis of taxonomic data indicated that P. batumici is a novel species. Illumina Hi-Seq sequencing of the chromosomal DNA enabled to obtain the complete genome sequence of P. batumici UCM B-321. Its DNA contained 127 contigs of the total length of 6608172 bp. Batumin biosynthesis operon was identified as 77 kbp operon containing in total 28 protein coding genes. This operon sequence was significantly less GC-rich and the program SeqWord Genomic Island Sniffer predicted a horizontal acquisition of this region. The closes relatives of UCM-321 were P. gingeri and P. protegens; both have no batumin operon in their genomes. HLPC-analysis of the culture broth has shown the presence of batumin in P. batumici broth and no any similar substances in P. gingeri culture medium.

The phenotypic, chemotaxonomic and genetic peculiarities of 5 deep-water strains of A. macleodii (isolated from a depth of 1000–3500 m) and 5 strains of the same species isolated from the surface layer have been studied. Electron microscopy has shown that the deep strains' cells were, on average, two times longer (2.1±0.2×0.7±0.1 μm) than the surface strains (1.1±0.1×0.6±0.1 μm). Using fatty acid analysis the deep and surface isolates were clearly separated into two clusters. The distinctions between them were also found in different lectin binding capacity, which was probably determined by the structure of their extracellular polysaccharide matrix. Analysis of the PCR results with the primers to repeated nucleotide sequences revealed a higher level of genetic polymorphism in surface strains in comparison to the deep-water isolates. The described peculiarities probably reflect the specific conditions in which A. macleodii strains live on the surface or in the depth of the World Ocean.

K e y w o r d s: Pseudomonas batumici, antibiotic batumin, Alteromonas macleodii, systematics, genomic structure.

Analysis of bacterial genome is one of the important and necessary elements of research in all areas of modern fundamental and applied microbiology. To a full extent it is relevant for the *Pseudomonas* genus which covers one of the ecologically most significant groups of bacteria with a remarkable degree of genomic diversity and ecological adaptability [1]. To the present time the complete genome sequence was established for 14 *Pseudomonas* species (data present in Genbank). A considerable progress was also achieved in the study of marine proteobacteria.

The two named groups of microorganisms were studied at the antibiotics department of Zabolotny Institute of Microbiology and Virology (National

Academy of Sciences of Ukraine) during the last decade. Isolation, polyphasic taxonomic analysis and description of new species of pseudomonads and *Alteromonas*-like marine bacteria and their antibiotic activity were the main results of this work. The genome analysis was included in the arsenal of methods used, which allowed to establish the connection between some genetic and biological features of the studied microorganisms, their ecology and antibiotic producing activity.

In this article we consider the new data obtained in this area using two objects as examples – *Pseudomonas batumici* strain producing the polyketyde antibiotic batumin highly effective against staphylococci and *Alteromonas macleodii* strains, representatives of marine species widely inhabiting the world ocean.

Strains of *Pseudomonas batumici* were isolated during an investigation of diversity and antibiotic activity of pseudomonads from different natural habitats. Four heterotrophic, oxidase positive, Gram-negative rods, motile by means of few polar flagellae, were isolated from soil samples collected on the Black Sea coast of Caucasus (moist subtropical region) [9].

The results of 16S rRNA gene sequence analysis have shown that *Pseudomonas batumici* UCM B-321 strain formes a separate branch within the genus *Pseudomonas* and has 98 % of 16S rRNA gene sequence similarity with evolutionally most related species *P. gingeri* and *P. baetica* (Fig. 1) [11].

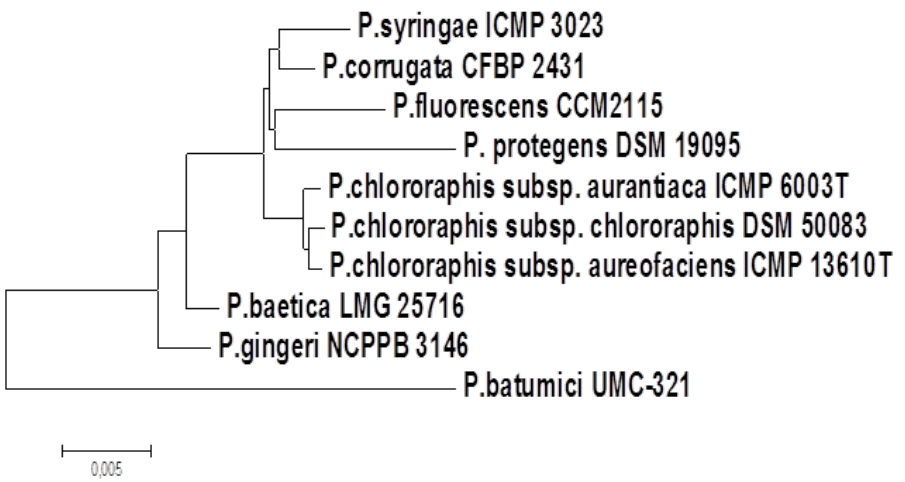

Fig. 1. Phylogenetic position of *Pseudomonas batumici* UCM B-321 within the genus *Pseudomonas* based on 16S rRNA gene sequences

The differences between the mentioned species were determined by polyphasic taxonomic analysis along with identification of some enzymes, pigments production ability, spectra of assimilated carbon sources, the fatty acid profiles, antagonistic activity and synthesis of antistaphylococcal antibiotic batumin. Analysis of taxonomic data indicated that *P. batumici* represents a novel species [9].

Illumina Hi-Seq sequencing of the chromosomal DNA enabled to obtain the complete genome sequence of *P. batumici* UCM B-321^T (Fig. 2). These and supplementary data were deposited at the National Center for Biotechnology

Information (NCBI) under the accession no. JXDG00000000, BioProject PRJNA270768 and BioSample SAMN03273282. The DNA contained 127 contigs of the total length of 6608172 bp [13].

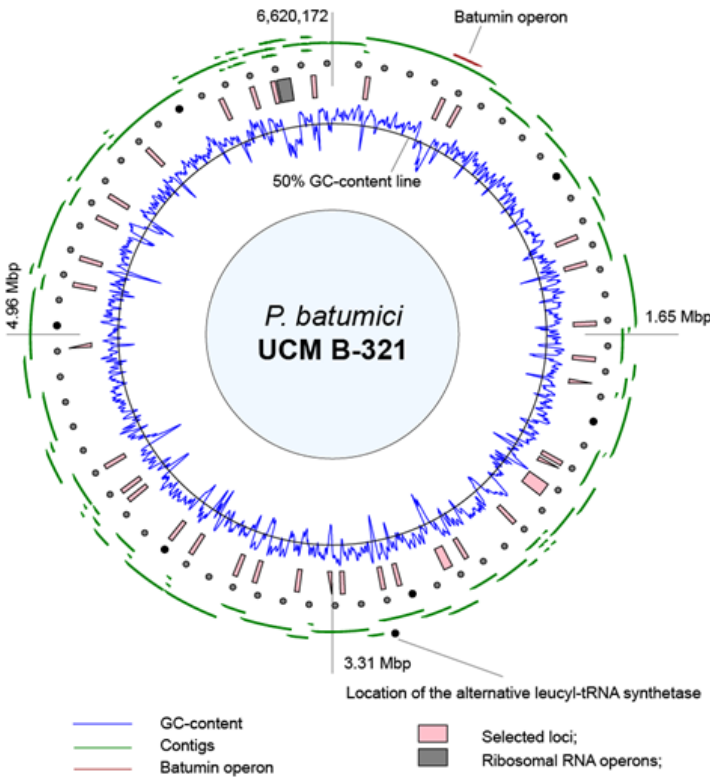

Fig. 2. Atlas of the concatenated contigs of *Pseudomonas batumici* UCM B-321 shows the locations of predicted genomic islands

The average GC content of *P. batumici* UCM B-321 genome constitutes 61.78 %. In the locus covering the batumin operon, the GC content is <50 %. The batumin biosynthesis operon was identified in the center of the large contig 3. Surprisingly the identified 77 kbp long operon contains in total 28 protein coding genes. This operon sequence is significantly less GC-rich (50 % against 64 % in average) than the whole genome sequence and the program SeqWord Genomic Island Sniffer predicted horizontal acquisition of this region [6].

The availability of the whole genome sequence of UCM-321 allowed precise identification of its phylogenetic position among other sequenced *Pseudomonas*. The closes relatives of UCM-321 were identified as *P. gingeri* NCPPB 3146 and *P. protegens* CHA0; both have no batumin operon in their genomes. HLPC-analysis of cultural broth showed the prepense of batumin and its minor derivate descarbamoyl batumin in *P. batumici* broth and no any similar substances in *P. gingeri* cultural medium (Fig. 3).

The highest similarity to batumin operon was observed for gene operons encoding bacillaen biosynthesis in *Bacillus* [6, 7]. During many years of intensive study the attention was fixed on antibiotic batumin, its high and selective activity against staphylococci, its biosynthesis, chemical structure, perspectives of medical use [2, 12, 16]. Batumin synthesis in fermenter occurs in the process of culture growth and its maximal value is achieved after 50–55 hours. Antibiotic yield comprises 175–180 mg/l and depends on intensity of aeration [10].

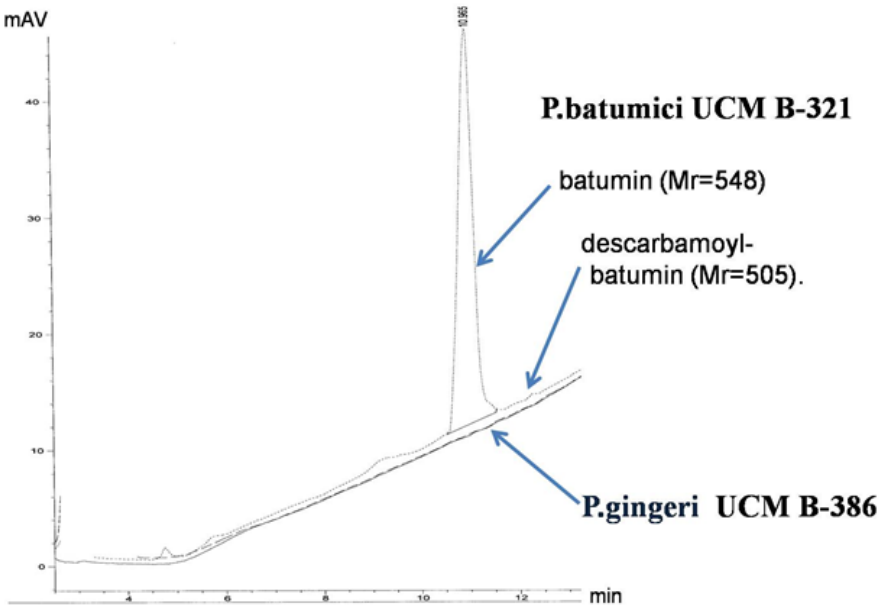

Fig. 3. Determination and identification of culture medium components by LC/MC

Batumin (Fig. 4) was highly active against all studied strains belonging to 10 species of *Staphylococcus* genus (minimal inhibitory concentration – 0.25–0.5 μg/ml); it showed a moderate activity against enterobacteria of *Salmonella*, *Bordetella*, *Escherichia*, *Klebsiella* genera (MIC 8–64 μg/ml) and practically did not inhibit any strains of micrococci, streptococci, sporeforming bacteria including *Clostridium sporogenes* (MIC 256 μg/ml or higher). Strains of yeasts and microscopic fungi *Candida tropicalis, C. utilis, C. albicans, Saccharomyces cerevisiae and Aspergillus niger* were resistant to batumin.

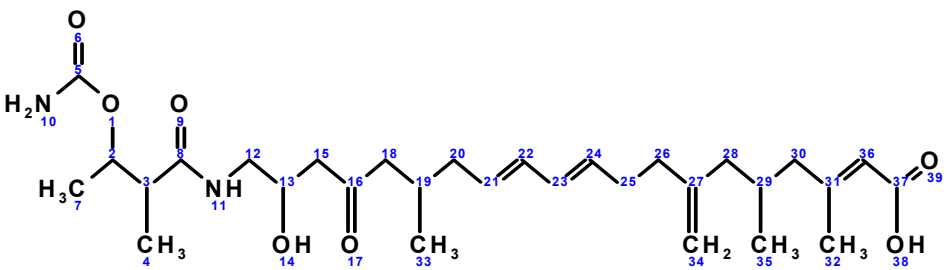

Fig. 4. Chemical structure of antibiotic batumin

It was shown that the unique batumin antimicrobial effect depends on the presence of double bonds, $CONH_2$–, COOH– and OH-groups. Replacement or modification of each of these groups reduced the activity against *Staphylococcus* and qualitatively changed the antimicrobial spectrum of the antibiotic [8].

At the same time, batumin may target a broader range of aminoacyl tRNA synthetases, including leucyl-tRNA synthetase. Paralogues of diverse leucine-tRNA synthetases in the genome of *P. batumici* indicat that this protein might be the prime target of batumin. It appears plausible that the leucyl-tRNA

synthetase and the batumin operon were acquired by horizontal gene transfer from the same unknown Gram-positive bacterium [13].

The simultaneous *P. batumici* growth and antibiotic biosynthesis and this molecule's capacity to optical isomerisation allow us to suppose that batumin plays a certain role in metabolism of the producing strain. So it is interesting to study not only the ecological role of batumin (which is probably the instrument of competition with other rhizosphere microorganisms), but its other functions in metabolism and survival of this species.

Studies of *Alteromonas macleodii* – the next object of our experiments – concern one of the most widespread heterotrophic marine bacteria found in the North Sea as well as marine waters in tropical and moderate latitudes, inhabiting surface and deep waters [3]. *A. macleodii* was also detected in the Black Sea during isolation of *Alteromonas*-like proteobacteria from the Black Sea water [15]. This species was widely studied by scientists of different countries within the framework of the "Global Ocean Sampling project" devoted to fundamental study of genetic biodiversity of marine microbial groups and finding out their role in the natural ecological processes.

Data on the biogeography of *A. macleodii* indicated that there were two different ecotypes from the surface and deep sea waters. These ecotypes were differentiated according to the results of molecular-genetic analysis of *A. macleodii* strains. It was observed that the size of internal transcribing spacer (ITS) between genes of 16S and 23S rRNA [15] and the nucleotide sequences of 16S rRNA, *gyrB*, *rpoB* genes and several other genes were different for the two ecotypes [4, 15]. The complete genome sequence of the deep-sea strain *A. macleodii* DSM 17117 [5] revealed the connection between the genome loci and the biochemical peculiarities and ecological features of these bacteria.

We studied the *A. macleodii* strains isolated from different geographical locations: five strains from sea-surface water samples (D7, D12 were from the Andaman Sea; MED64 was from the Aegean Sea; 621 was from the Atlantic Ocean near the British shore; 29–06 was from the Black Sea) and other five strains from deep water samples: U4, U8, UM4b from the Ionian Sea (3,500 m); Adriatic1, Adriatic2 from the Adriatic Sea (1,000 m). The strains were kindly supplied by Prof. Francisco Rodriguez-Valera (Universidad Miguel Hernandez, Spain); strain 29–06 was isolated from surface water of the Black Sea by O. Onyshchenko (Zabolotny Institute of Microbiology and Virology NASU, Ukraine).

We studied new phenotypic and genetic peculiarities of the named *A. macleodii* strains and attempted to connect the marked differences with ecology of these bacteria [14]. The ability of *A. macleodii* strains to grow at different temperatures (18, 26, 37 and 42 °C) was studied under decompressed conditions to compare their growth rates and morphological features. No strains were able to grow at 42 °C. The surface strains, in contrast to the deep ones, were able to grow at 37 °C. The optimal growth rate was marked at 26 °C for all strains. We discovered that the cell size of *A. macleodii* strains depended on the depth of their isolation only: the deep strain cells were two times longer than the surface ones.

A finer dissection of the *A. macleodii* genome shows an extremely efficient recruitment of four GI genes present in the island that were all annotated as metal efflux pumps. Metal detoxification seems to be the common motive

of the gene clusters in GI2, including putative efflux pumps for cobalt, zinc, cadmium, silver or copper. The heavy metal resistance of the 23 strains of *A.macleodii* present in the laboratory of Universidad Miguel Hernandez [15] was assessed by determining the minimal inhibitory concentration for zinc, mercury and lead. It was shown that most of the deep-water isolates were more resistant.

We have studied other heavy metals ions and did not find any significant distinction between deep and surface isolates sensitivity to those. *A. macleodii* strains survived at lead ions concentration of 200–400 μg/ml and copper ions 20–50 μg/ml. Sensitivity to cadmium ions substantially varied from strain to strain (10–200 μg/ml).

With the help of fatty acid analysis the deep and surface isolates were clearly separated into two clusters (Fig. 5).

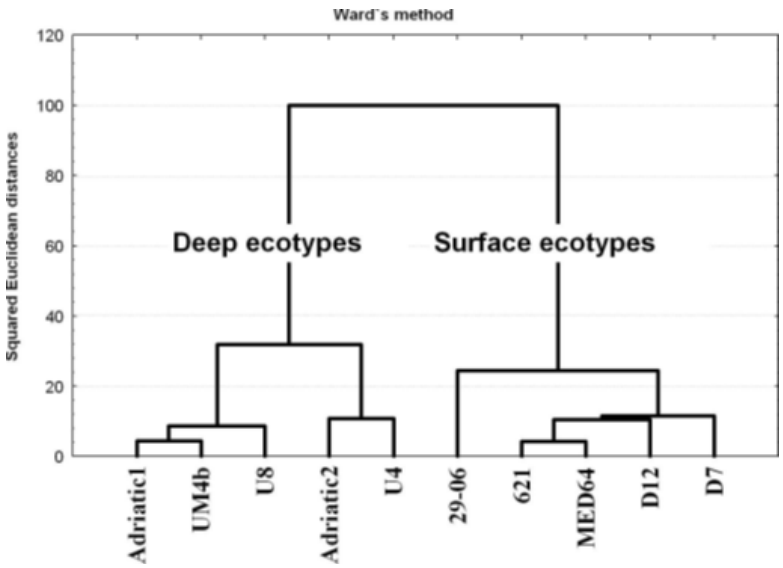

Fig. 5. Dendrogram of *A. macleodii* strains according to their fatty acid profiles

The strains isolated from the deep sea waters contained a 10–15 % larger portion of monounsaturated (C16:1 and C18:1) fatty acids in comparison with the strains isolated from the surface layers of water. This observation may show a relation to the specific conditions of the habitat of the deep sea: low temperature and high pressure.

Restriction analysis of 16S rRNA seemed to reveal insufficient genomic variability among *A. macleodii* strains isolated from different econiches: we were able to distinguish one among 10 *A. macleodii* strains. Our results are consistent with data from other studies that determined heterogeneity of the fragments of this gene among marine organisms, including *A. macleodii* [5]. We used DNA-markers that are highly polymorphic and allow an evaluation of genome variability to study intraspecies genomic heterogeneity of *A. macleodii*. Patterns of PCR products obtained by using M13 primer and 3 primers to short nucleotide repeats were scored, explored and compared between the strains (Fig. 6).

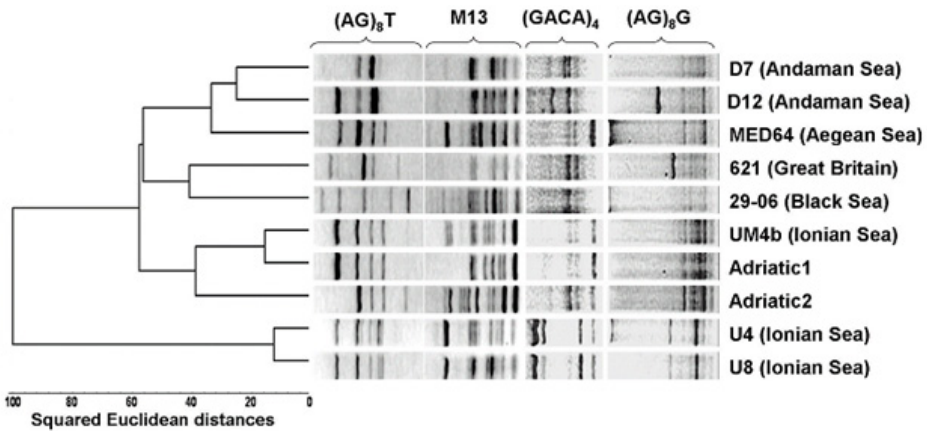

Fig 6. Dendrogram of *A. macleodii* strains according to their amplicon patterns

The level of polymorphic bands was high and amounted to up to 79 %. The group of surface strains possessed a higher level of polymorphism in comparison with the group of deep ones (72 % and 62 % respectively) [14].

We observed that different *A. macleodii* strains possessed varied affinity to the tested lectins. Cluster analysis of polysaccharide profiles resulted in separation of the strains into two major groups. One group was composed of the deep water strains (from the 3,500 m); the second was divided into two subclusters: the first subcluster was composed of the strains isolated from the 1,000 m deep water, the second subcluster included strains isolated solely from surface waters.

In our research, we conducted a comprehensive comparative analysis of two *A. macleodii* ecotypes and showed the differences in morphology and fatty acid content between the strains inhabiting deep and surface waters. The most significant features were marked, first of all, in the cell size (deep strains were two times longer than strains from surface water), and, second, in the fatty acid composition (strains were readily divided into two separate clusters). Strains were also distinct in their affinity to various lectins, which can be connected to the structural peculiarities of their polysaccharide matrices.

The data obtained in the current research allow concluding that the marked peculiarities mainly reflect specific environmental conditions of depth and surface waters from which the strains were isolated. To a lesser degree, they are related to the geographical area of the bacterial habitat. On the other hand, such considerable differences in ecotypes morphology and physiology allow to assume that probably they are representatives of two subspecies or even two different species of *Alteromonas*.

The presented data illustrate how analysis of genome structure allows to find areas, which determine certain (sometimes new and unknown) properties of bacteria: the metabolites biosynthesis, the enzymes activity, resistance to chemical agents etc. The presence of such characteristics must be experimentally proved. Only then we may answer the question: what is the metabolic, evolutionary or ecological role of the analyzed properties? This allows to obtain an integral picture of the organization and functioning of a microbial cell. In this article we tried to present one of the approaches to answer these questions.

В.В. Клочко

Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України, вул. Академіка Заболотного, 154, Київ, 03143, Україна

ЕКОЛОГІЯ, СИСТЕМАТИКА ТА АНТИБІОТИЧНА АКТИВНІСТЬ *PSEUDOMONAS BATUMICI* І *ALTEROMONAS MACLEODII* ТА ЇХ ЗВ'ЯЗОК ЗІ СТРУКТУРОЮ ГЕНОМІВ

Резюме

На основі комплексних досліджень структури геному і ряду біологічних ознак отримано нові дані з екології, систематики та синтезу біологічно активних сполук типовим штамом-продуцентом високоефективного антистафілококового антибіотика батуміну *Pseudomonas batumici* УКМ В-321 і широко розповсюджених у Світовому океані штамів *Alteromonas macleodii*.

Таксономічним аналізом показано, що *P. batumici* являє собою новий вид бактерій. Повним сіквенсом геному типового штаму *P. batumici* УКМ В-321 методом Illumina Hi-Seq встановлено, що ДНК штаму містить 127 контигів загальною довжиною 6608172 п.н. Ідентифіковано оперон біосинтезу батуміну, який складається з 77000 п.н., містить 28 кодуючих генів і має більший Г+Ц вміст ДНК; комп'ютерною програмою SeqWord Genomic Island Sniffer встановлено горизонтальний перенос оперону батуміну. Найбільш філогенетично близькими до *P. batumici* УКМ В-321 видами виявилися *P. gingeri* і *P. protegens*, однак у них був відсутній оперон біосинтезу батуміну. HPLC-аналіз культуральної рідини *P. batumici* УКМ В-321 показав наявність у ній батуміну; в культуральній рідині *P. gingeri* батумін або його аналоги були відсутніми.

Вивчено фенотипові, хемотаксономічні і генетичні властивості 5 глибоководних морських штамів *A. macleodii,* ізольованих з глибини 1000–3500 м та 5 штамів, виділених з поверхневих шарів води. Методом електронної мікроскопії встановлено, що глибоководні штами виявилися вдвічі довшими (2,1±0,2×0,7±0,1 мкм), аніж поверхневі (1,1±0,1×0,6±0,1 мкм). Аналіз жирнокислотних профілів глибоководних і поверхневих штамів дозволив розділити їх на два кластери. Знайдена різниця у лектинзв'язуючій активності штамів, виділених з різних глибин, свідчила про їх різні за структурою екзополісахаридні матрикси. Аналіз результатів ПЛР з ДНК-маркерами показав більш високий рівень поліморфізму у поверхневих штамів у порівнянні з глибоководними. Знайдені особливості штамів *A. macleodii,* очевидно, є відображенням специфічних умов існування, в яких вони знаходяться на поверхні або в глибинах Світового океану.

Ключові слова: Pseudomonas batumici, антибіотик батумін, *Alteromonas macleodii*, систематика, структура геномів.

В.В. Клочко

Институт микробиологии и вирусологии им. Д.К. Заболотного НАН Украины, ул. Академика Заболотного, 154, Киев, 03143, Украина

ЭКОЛОГИЯ, СИСТЕМАТИКА И АНТИБИОТИЧЕСКАЯ АКТИВНОСТЬ *PSEUDOMONAS BATUMICI* И *ALTEROMONAS MACLEODII* И ИХ СВЯЗЬ СО СТРУКТУРОЙ ГЕНОМОВ

Резюме

На основе комплексных исследований структуры генома и ряда биологических свойств получены новые данные по экологии, систематике и синтезу биологически

активных соединений типовым штаммом-продуцентом высокоэффективного антистафилококкового антибиотика батумина *Pseudomonas batumici* УКМ В-321 и широко распространенных в Мировом океане штаммов *Alteromonas macleodii*.

Таксономическим анализом показано, что *P. batumici* представляет собой новый вид бактерий. Полным сиквенсом генома типового штамма *P. batumici* УКМ В-321 методом Illumina Hi-Seq установлено, что ДНК штамма содержит 127 контигов общей длиной 6608172 п.н. Идентифицировано оперон биосинтеза батумина, который состоит из 77000 п.н., содержит 28 кодирующих генов и имеет более высокое Г+Ц содержание ДНК; компьютерной программой SeqWord Genomic Island Sniffer установлен горизонтальный перенос оперона батумина. Наиболее филогенетически близкими к *P. batumici* УКМ В-321 видами являются *P. gingeri* и *P. protegens*, однако в них отсутствовал оперон биосинтеза батумина. HPLC-анализ культуральной жидкости *P. batumici* УКМ В-321 показал наличие в ней батумина; в культуральной жидкости *P. gingeri* батумин или его аналоги отсутствовали.

Изучены фенотипические, хемотаксономические и генетические свойства 5 глубоководных морских штаммов *A. macleodii*, изолированных из глубины 1000–3500 м и 5 штаммов, выделенных с поверхностных слоев воды. Методом электронной микроскопии установлено, что глубоководные штаммы оказались вдвое длиннее (2,1±0,2×0,7±0,1 мкм), чем поверхностные (1,1±0,1×0,6±0,1 мкм). Анализ жирнокислотных профилей глубоководных и поверхностных штаммов позволил разделить их на два кластера. Выявленные отличия в лектинсвязывающей активности штаммов, выделенных из различных глубин, свидетельствовали о различной структуре их экзополисахаридного матрикса. Анализ результатов ПЦР с ДНК-маркерами показал более высокий уровень полиморфизма у поверхностных штаммов по сравнению с глубоководными. Найденные особенности штаммов *A. macleodii,* очевидно, являются отражением специфических условий существования, в которых они находятся на поверхности или в глубинах Мирового океана.

Ключевые слова: Pseudomonas batumici, антибиотик батумин, *Alteromonas macleodii*, систематика, структура геномов.

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