УДК 579.68.8

E.A. Kiprianova, V.V. Klochko, L.B. Zelena, L.N. Churkina, L.V. Avdeeva

Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, 154 Acad.Zabolotny St., Kyiv, MSP, 03680, Ukraine

PSEUDOMONAS BATUMICI SP. NOV., THE ANTIBIOTIC-PRODUCING BACTERIA ISOLATED FROM SOIL OF THE CAUCASUS BLACK SEA COAST

Four novel strains of saprophytic bacteria were isolated from the soil samples collected in the moist subtropics region (the Black Sea coast of the Caucasus) and studied using methods of polyphasic taxonomic analysis. Microorganisms were Gram-negative, oxidase positive, aerobic, rod-shaped motile bacteria that produced antibiotic named batumin with high and selective activity against staphylococci; its total formula was $C_{30}H_{48}N_2O_7$. *Phylogenetic analysis of 16S rRNA gene sequences (1376 bp, accession number in Genbank – JF306642) indicated that the isolates belonged to the γ-Proteobacteria, formed a separate branch within the genus Pseudomonas and had 98% 16S rRNA gene sequence similarity with Pseudomonas gingeri. The latter essentially differed from the studied strains in its phenotypic characteristics.*

The predominant cellular fatty acids of isolates were similar and included C16: 0, C16:1, C18:1, and up to 22.9 % of ΔC17:0; their DNA G+C content was 64.0 mol%. An analysis of taxonomic data indicated that the studied isolates represented a novel species, for which the name Pseudomonas batumici sp. nov. is proposed with the type strain UCM B-321 (Ukrainian Collection of Microorganisms).

K e y w o r d s *: polyphasic taxonomic analysis, new species Pseudomonas batumici*

To date the authentic genus *Pseudomonas* consists of more than 120 validly described species which are assigned to several large intrageneric clusters [9]. The list of species isolated from various sources is permanently enlarged which is caused with remarkable adaptability of pseudomonads to different conditions of the environment.

During investigation of diversity and antibiotic activity of pseudomonads from the different natural habitats we isolated four heterotrophic, oxidase positive, Gram-negative rods, motile by few polar flagellae from the soil samples collected in the Black Sea coast of the Caucasus (moist subtropics region). It has been shown that the isolates belonged to the *Pseudomonas* genus and produced a new antibiotic with high and selective activity against staphylococci. According to the region of Batumi where the strains have been isolated the antibiotic was named batumin and the antibiotic-producing isolates were designated as *Pseudomonas batumici*. During many years of intensive study attention was fixed upon antibiotic, its biosynthesis, chemical structure, perspectives of medical use [1, 3, 4] while the taxonomic status of batumin-producing strain remained unclear. This study was aimed at clarifying the taxonomic affiliation of *Pseudomonas batumici*.

Materials and Methods

Strains of *Pseudomonas batumici* UCM B-303, B-311, B-321 and B-362 were isolated by direct plating of soil suspensions on peptone-meat-infusion agar. Stock cultures were preserved lyophilized at 4 °C. The strains were routinely grown at peptone-meat-infusion agar at 26 °C. Cell morphology was examined by using a light microscope and a transmission electron microscope (Hitachi H-800). Growth was tested at various temperatures in the range of 5-40 °C. The ability of bacteria to produce pigments was tested on King A and B medium. Examined physiological and biochemical properties of the isolated bacteria included their glucose metabolism, oxidase activity, ability to denitrify and liquefy gelatin, arginine dihydrolase, lysine and ornithine decarboxylase activities, the ability to secrete extracellular hydrolytic enzymes (proteases, lecithinases, lipases, amylases) and to produce levan from sucrose. Assimilation of 106 carbon compounds was determined in Koser agar medium containing 0.1% of appropriate C-source. All physiological and biochemical tests were carried out according to the methods described by Stanier et al. [12]. Susceptibility to antibiotics was tested by the routine diffusion plate method, employing peptone-meat-infusion agar and disks impregnated

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with penicillin, streptomycin, oleandomycin, lincomycin, erythromycin, tetracycline, cefazolin, rifampicin, chloramphenicol, ciprofloxacin, imipenem.

Cell morphology analyses were carried out by mounting cells onto 400 mesh copper grids, counterstaining with 1% uranyl acetate and examining in transmission electron microscopy (TEM) JEM-1400 (Jeol ltd., Japan) at 80 kV [14].

In order to study the fatty acid profiles bacteria were cultivated during 24 hours in peptone-meat-infusion agar at 26 °C; cellular fatty acids were extracted and methylated by standard procedure.

Fatty acid methyl esters (FAMES) were extracted from aliquots of cell suspensions by acid methanolysis and quantified by GC/MS with an Agilent 6890 gas chromatograph and 6890N Mass Selective Detector (Palo Alto, CA, USA). Helium was used as carrier gas; the gas-chromatography capillary column was a HP-5MS (J&W Scientific, USA). Mass spectra were recorded in electron impact ionization at 70 eV in SCAN mode. Data were processed with Workstation software (Agilent Technologies) and compounds were identified by relative retention time with standards of bacterial FAMEs (Supelco, № 4708-U, USA) and from the comparison of mass spectra in NIST02 MS library [2].

DNA isolation, amplification and 16S rRNA sequencing. Genomic DNA from bacterial cultures was extracted using DNA isolation kit (“AmpliSens”, Russia). The G+C content of the DNA was determined by the thermal denaturation method of Marmur and Doty [8].

The universal primers 27f and 1492r targeting bacterial 16S rRNA genes were used to obtain a PCR product of approximately 1.5 kb by using the protocol [6]. The purified PCR product was sequenced by the dideoxynucleotide chain termination method using a model ABI 310 (Applied Biosystems). The primers used for sequencing were the same as those used for PCR amplification.

Analysis of sequence data. The resulting 16S rRNA gene sequence was compared with sequences obtained from public databases to find the most closely related species. All sequences were identified using the NCBI BLASTN tool (http://www.ncbi.nlm.nih.gov/blast). The sequence of 16S rRNA gene of new isolate has been deposited in Genbank and assigned accession number JF306642. Phylogenetic analysis was performed using the software packages MEGA 4 [13] after multiple alignments of sequence data by CLUSTAL W. The evolutional instances (corrected by Kimura’s 2-parameter model) were calculated and clustering was performed with the neighbor-joining method. Bootstrap analysis was performed using 1000 replications.

Results and Discussion

The isolates exhibited the phenotypic characteristics of *Pseudomonas* sensu stricto; their characteristics are shown in the species description and Table 1. With few not essential exceptions all of them were identical in their properties.

They were Gram-negative rods (2.2×1.0 µm) motile with few (more than 2) polar flagellae. The length of flagella was 6-8 µm*,* with 4-6 waves (Fig.1).

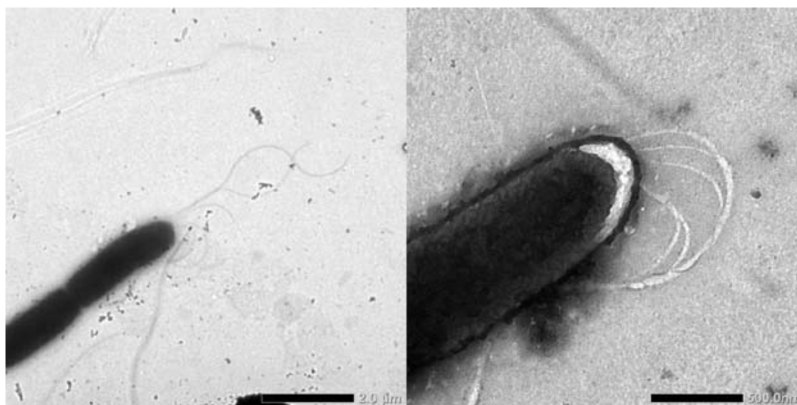

Fig.1. Electron micrograph of cells of strain *P. batumici* UCM B-321.

The isolates did not accumulate poly-β-hydroxybutyrate, did not form spores or microcysts. They grew well in common media, did not require any growth factors. Mineral nitrogen forms (ammonia, nitrates) were utilized.

The temperature optimal for their growth was 27 °C, growth was poor at 37 °C, bacteria did not grow at all at 40 °C.

Oxidase, arginine dihydrolase, levan sucrase and lecitinase were positive, ornitine and lysine decarboxylase were negative. They could not denitrify, did not hydrolyse gelatin, starch and aesculin, did not oxidize gluconate, did not produce the yellow-green fluorescent pigment typical of many species of *Pseudomonas*.

In some glucose-containing media bacteria secreted into the medium the phenazine-1-carboxylic acid – antibiotically active phenazine derivate produced by few *Pseudomonas* species (*P.fluorescens, P.chlororaphis* etc). Simultaneously the complex of chemically similar colourless antibiotics among which batumin was the most abundant and active have been produced. This new antibiotic had extremely high and selective activity against staphylococci. The following studies have shown that batumin formula proved to be $C_{30}H_{48}N_2O_7$ and that it was (2E, 10Z, 12E)-20-(3-aminocarboxy-2 -methyl-1-oxobutyl) amino-7-methylen-17-oxo-19-oxy-3, 5, 15-trimethyl-eicosa-2,10,12-trienic acid [3, 4]. Batumin is the stereo isomer of kalimantacins isolated in 1995 from *Alcaligenes sp*. [5]. In 2010 antibiotic of similar structure named Kalbat (kalimantacin-batumin) was isolated from the strain of *Pseudomonas fluorescens* [7], but properties of antibiotic-producing strain and the evidence of its identification have not been presented.

Batumin-producing strains utilized more than 40 substances (on the whole carbohydrates, organic acids, amino acids) as single sources of carbon and energy (see the species description).

In their sensitivity to antibiotics the studied bacteria were similar to most of *Pseudomonas* species: all of them were sensitive to streptomycin, chloramphenicol, ciprofloxacin, tetracycline, imipenem; weakly sensitive to cefazoline, resistant to penicillin, erythromycin, lincomycin, oleandomycin, furadonin.

The fatty-acid profiles of isolates were as a whole typical of *Pseudomonas* genus representatives with predominance of C 16:0 and C 16:1 acids (Table 1). Yet they contained less of C 18:1 than other *Pseudomonas* species and unusually large amounts of cyclopropanic Δ17 acid (the only exception was strain B-362).

According to data of 16S rRNA gene sequence analysis (1376 bp) the new isolate constituted an independent cluster from the other Pseudomonas species on the phylogenetic tree shown in Fig.2. Its nearest phylogenetic neighbour *Pseudomonas gingeri* has shown 98.0% of 16S rRNA gene sequence similarity. It is suggested that bacterial strains with less than 98.7 % of 16S rRNA sequence identity are members of different species [11]. The sequence similarity between strain UCM B-321 and strains of other *Pseudomonas* species (*P.fluorescens, P.chlororaphis* etc) was 97 % and lower.

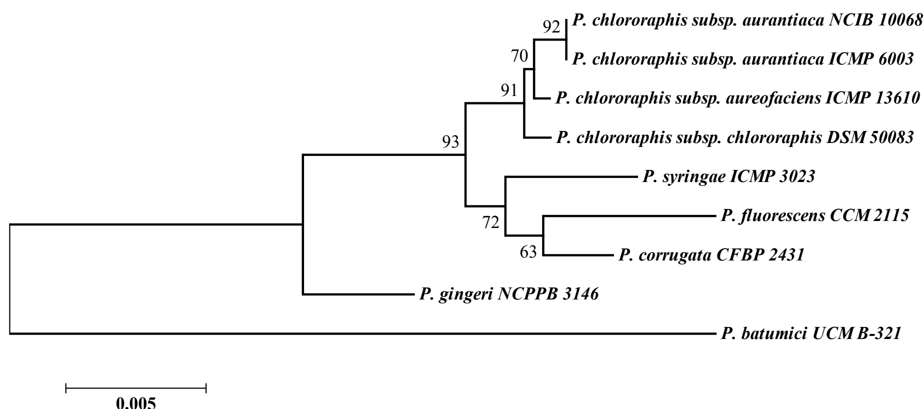

Fig. 2. Phylogenetic position of *Pseudomonas batumici* UCM B-321 within the genus *Pseudomonas* based on 16S rRNA gene sequences. The tree constructed using the neighbor-joining method. Bootstrap analyses were performed with 1000 repetitions. Bar, 0.005 substitutions per nucleotide position.

Table 1

Cellular fatty acid composition of novel strains

Fatty acids	Content of fatty acids, % in strains			
	B-311	B-362	B-303	B-321
C12:0	0.15	0.25	0	0.25
2OH C12:0	0.58	0.58	0	0.96
C14:0	0.50	0.31	1.2	0.69
C16:0	49.63	59.69	40.00	46.10
C16:1	18.00	30.30	24.00	27.50
C17:0	0.25	0.12	0.10	0.19
ΔC17:0 (cis9,10 C17:0)	22.59	3.52	22.90	14.90
C18:0	4.30	3.05	1.40	4.69
TransC18:1	4.02	2.28	9.50	4.91

P.gingeri is the causative agent of cultivated mushrooms disease called ginger blotch disease. Its description was published in 1982 [15], but the information about its properties was rather brief. Some data (which we use in this paper) were added later during study of other *Pseudomonas* species causing the mushrooms diseases [10]. The smooth forms of *P.gingeri* are mucoid and non-fluorescent; they produce toxin and are pathogenic to mushrooms, whereas rough forms are fluorescent and non-pathogenic. In their phenotypic properties, particularly pigments, antibiotics, toxin production and nutritional spectra *P.gingeri* strains essentially differ from Batumi isolates (see Table 2).

Table 2

Characteristics that differentiate *Pseudomonas batumici* strains from *P. gingeri*

Characteristic	*Pseudomonas batumici*	*Pseudomonas gingeri*
Green fluorescent pigment	-	+ (in smooth forms)
Yellow pigment phenazine-1-carboxylic acid	+	-
Batumin production	+	-
Assimilation of		
d-xylose	-	+
l-arabinose	+	-
Propionate	-	+
Valeriate	-	+
Acetate	-	+
Tartrate	-	+
Sorbitol	-	+
o-hydroxybenzoate	-	+
Sarcosine	+	-
l-serine	-	+
l-leucine	-	+
l-lysine	-	+

In summary, all of the presented results, based on phylogenetic, phenotypic and genotypic data illustrate that the *P. batumici* strains represent a new species within the genus *Pseudomonas*.

Description of *Pseudomonas batumici* sp. nov.

Pseudomonas batumici (ba.'tu.mi.ci ; refers to Batumi, the town in the Black Sea coast of the Caucasus, where bacteria have been isolated).

Cells are Gram-negative straight rods (2.2×1.0 μm) motile with few (most often 2) polar flagellae. The length of flagella 6-8 μm, with 4-6 waves. Oxidase positive, strict aerobes. They do not accumulate poly-β-hydroxybutyrate, do not form spores or microcysts, do not produce the yellow-green fluorescent pigment typical of many species of *Pseudomonas*.

They grow well in common media, do not require any growth factors. Mineral nitrogen forms (ammonia, nitrates) are utilized. The temperature optimal for their growth is 27 °C, growth is poor at 37 °C, they do not grow at 40 °C.

Arginine dihydrolase, levan sucrase and lecithinase are positive, ornitine and lysine decarboxylase are negative. They cannot denitrify, do not hydrolyse gelatin, starch and aesculin, do not oxidize gluconate.

Bacteria produce the phenazine-1-carboxylic acid – antibiotically active phenazine derivate produced by few other *Pseudomonas* species. Simultaneously antibiotic batumin with extremely high and selective activity against staphylococci is produced.

Batumin producing strains utilize a wide spectrum of organic compounds as single sources of carbon and energy: d-glucose, saccharose, tregalose, d-fructose, l-arabinose, d-galactose, acetate, succinate, fumarate, glutarate, lactate, citrate, pyruvate, α-ketoglutarate, itaconate, mannitol, inositol, glycerol, chinate, α-alanine, β-alanine, isoleucine, l-aspartate, l-glutamate, arginine, ornitine, citrulline, γ-aminobutyrate, l-hystidine, l-proline, l-tyrosine, l-phenylalanine, betaine, sarcosine.

Xylose, d-arabinose, d-fucose, l-rhamnose, maltose, cellobiose, lactose, starch, inulin, salicin, propionate, butyrate, valeriate, caproate, malate, tartrate, hydroxybutyrate, glycollate, dulcitol, sorbitol, adonitol, ethylene- and butylene glycol, methanol, ethanol, propanol, n-butanol, mandelic, benzoic, o-, m- and p-hydroxybenzoic acid, phtalate, phenol, naphthalene, glycine, serine, threonine, lysine, tryptophane, anthranilate, creatine, hippurate, acetamide, nicotinic acid are not utilized.

The cellular fatty-acid profiles are typical of *Pseudomonas* genus representatives with predominance of C16:0 and C16:1 acids. Yet bacteria contain less of C18:1 than other *Pseudomonas* species and unusually large amounts (up to 22.9 %) of cyclopropanic Δ17 acid .

The DNA G+C content of strain UCM B-321 is 64.0 mol%.

Isolated from soil samples collected in the moist subtropics region (the Black Sea coast of the Caucasus).

The type strain is *Pseudomonas batumici* UCM B-321 (Ukrainian Collection of Microorganisms, Kyiv, Ukraine).

О.А. Кіпріанова, В.В. Клочко, Л.Б. Зелена, Л.М. Чуркіна, Л.В. Авдєєва

Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України
вул. Академіка Заболотного 154, Київ МСП 03680, Україна

PSEUDOMONAS BATUMICI SP. NOV., УТВОРЮЮЧІ АНТИБІОТИК БАКТЕРІЇ, ІЗОЛЬОВАНІ З ГРУНТУ ЧОРНОМОРСЬКОГО УЗБЕРЕЖЖЯ КАВКАЗУ

Р е з ю м е

Чотири нових штами сапрофітних бактерій були ізольовані з проб ґрунту, відібраних в зоні вологих субтропіків (Чорноморське узбережжя Кавказу) і досліджені методами поліфазного таксономічного аналізу.

Мікроорганізми були грамнегативними оксидазопозитивними рухомими паличками, які утворювали антибіотик, названий батуміном (брутто-формула $C_{30}H_{48}N_2O_7$), з високою і селективною активністю щодо стафілококів. Філогенетичний аналіз нуклеотидної послідовності гену 16S рРНК (1376 п.н., номер у Genbank – JF306642) свідчив, що ізоляти належали до γ-протеобактерій, формували окрему гілку в групі представників роду *Pseudomonas* і мали 98 % подібності нуклеотидної послідовності з *"Pseudomonas gingeri"*. Останній істотно відрізнявся від досліджуваних штамів за своїми фенотиповими властивостями.

Основні клітинні жирні кислоти ізолятів були подібними і включали C16:0, C16 :1, C18:1 та до 22,9 % ΔC17:0; їх Г+Ц вміст ДНК був 64,0 мол%. Аналіз таксономічних даних показав, що досліджувані бактерії представляють новий вид, для якого пропонується назва *Pseudomonas batumici* sp. nov. з типовим штамом УКМ В-321 (Українська Колекція Мікроорганізмів).

К л ю ч о в і с л о в а : поліфазний таксономічний аналіз, новий вид *Pseudomonas batumici*.

Е.А. Киприанова, В.В. Клочко, Л.Б. Зеленая, Л.Н. Чуркина, Л.В. Авдеева

Институт микробиологии и вирусологии им. Д.К. Заболотного НАН Украины
ул. Академика Заболотного 154, Киев МСП 03680, Украина

PSEUDOMONAS BATUMICI SP. NOV., ОБРАЗУЮЩИЕ АНТИБИОТИК БАКТЕРИИ, ИЗОЛИРОВАННЫЕ ИЗ ПОЧВЫ ЧЕРНОМОРСКОГО ПОБЕРЕЖЬЯ КАВКАЗА

Р е з ю м е

Четыре новых штамма сапрофитных бактерий были изолированы из почвенных образцов, отобранных в зоне влажных субтропиков (Черноморское побережье Кавказа) и исследованы методами полифаз-

ного таксономического анализа. Микроорганизмы представляли собой грамотрицательные, оксидазоположительные аэробные подвижные палочки, образующие антибиотик, названный батумином (брутто-формула $C_{30}H_{48}N_2O_7$), с высокой и селективной активностью в отношении стафилококков. Филогенетический анализ последовательности гена 16S рРНК (1376 п.н., номер в Genbank – JF306642) показал, что изоляты принадлежали к γ-протеобактериям, формировали отдельную ветвь в группе представителей рода *Pseudomonas* и имели 98 % сходства нуклеотидной последовательности с *«Pseudomonas gingeri»*. Последний существенно отличался от исследуемых бактерий по своим фенотипическим свойствам.

Важнейшие клеточные жирные кислоты изолятов включали С16:0, С16:1, С18:1 и до 22,9 % ΔС17:0; их Г+Ц содержание ДНК составляло 64,0 мол%. Анализ таксономических данных свидетельствовал о том, что изучаемые изоляты относятся к новому виду, для которого предложено название *Pseudomonas batumici* sp. nov. с типовым штаммом В-321 (Украинская Коллекция Микроорганизмов).

К л ю ч е в ы е с л о в а: полифазный таксономический анализ, новый вид *Pseudomonas batumici*.

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Отримано 15.11.2010