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T.S. Todosiichuk[1], V.V. Klochko[2], L.B. Zelena[2]

[1] National Technical University of Ukraine «KPI», 37 Peremogy Pr., Kyiv, 03056, Ukraine
[2]Zabolotny Institute of Microbiology and Virology, National Academy of Science of Ukraine
154 Acad. Zabolotny St., Kyiv, MSP, 03680, Ukraine

TAXONOMIC ANALYSIS OF THE *STREPTOMYCES* SP. 2435 STRAIN, A PRODUCER OF ANTIMICROBIAL SUBSTANCES

The study of the taxonomic status of the antimicrobial substances producer strain Streptomyces sp. 2435 was conducted. The nucleotide sequence of 16S rRNA gene of the strain was determined and deposited in the Genbank (№ JN129837) database. Results of morphological, biochemical and cell wall fatty acids content analyses, evaluation of biosynthesis features of Streptomyces sp. 2435, together with the phylogenetic analysis have provided the basis to identify this strain as Streptomyces albus.

Key words: taxonomic analysis, systematics, Streptomyces albus, antagonistic activity

Actinomycetes of the genus *Streptomyces* are one of the most used in the production of biologically active substances. Modern scientific literature is not only the result of numerous studies on screening of new strains of streptomycetes, selection, biosynthesis conditions, genome analysis, but also analyzes, linking the practical applications of new and known producers with questions of their morphology, taxonomy and systematics [1, 6, 9].

N.A. Krasil'nikov and S. Waksman became one of the most renowned scientists and researchers of streptomycetes in the 20th century, whose fundamental works allowed establishing the taxonomic status of these organisms and their main characteristics. Contemporaries and followers have developed the doctrine of streptomycetes and proposed various approaches to taxonomy and the study of their properties [7, 8]. However, a large variety of actinomycetes species, and streptomycetes in particular, along with the high variability of cultures have caused difficulties in the classification and identification of these microorganisms. Thus, as a result of the international project on the Streptomyces species diversity revision (ISP, 1966 [7]) only 459 of the previously described and referred 1,000 species have been left in the list. For the comparison, the number of streptomycetes species stated in various collections in the 80's is: ATCC - 673, DSM - 473, JCM - 600. At the same time, researchers estimate that at the end of the 90's the total amount of streptomycetes species referred to in the various sources was approximately 3000 [7].

Modern researchers of streptomycetes recognize the existence of problems related to their species identification and taxonomy as a whole, calling the latter «contradictory», and the existing classification schemes fairly «subjective» [1, 7, 9]. All this poses challenges for scientists studying culture optimization techniques, using modern techniques and facilities. It should be noted that when using and patenting various streptomycetes strains as producers of a wide range of biologically active substances, taxonomic identification is of particular importance.

One of the cultures of streptomycetes, isolated in the 70's as a producer of the bacteriolytic enzyme complex, was identified as *Actinomyces* (now *Streptomyces) recifensis* var. *lyticus* 2435 [4]. The strain is the subject of research in a number of scientific laboratories, studying various fundamental aspects of the biosynthesis and the practical applications of this producer strain [2, 4]. The authors of this work developed the technology for production of the enzyme preparation Cytorecifen-M using selected strain *S. recifensis* var. *lyticus* 2435/M (Ukrainian Collection of Microorganisms Ac-5001). Research is now being conducted to optimize the process and the creation of various formulations of the drug [3].

Interest in this particular culture is due to peculiarity of the synthesized product – a complex of proteinase enzymes, peptidases, glucosaminidase, muramidase, the joint action of which causes lysis of the cell wall of bacterial cultures of a wide spectrum, such as *Staphylococcus aureus*, *Bacil-*

lus cereus, *Corynebacterium gravis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus rettgeri*, *Streptococcus thermophilus*, *Schigella sonnei*, *Salmonella typhi*, *Klebsiella pneumonia* can be destroyed, when treatment with higher doses is used, although these bacteria are more resistant. We previously demonstrated the possibility of using the enzyme complex synthesized by streptomycetes in medical and veterinary antimicrobial drugs, in detergents with an antiseptic effect, analytical studies of microbial cells, etc. [2, 3]. Despite the broad perspectives of a given culture, its reporting as bacteriolysin producer was only found in the work of the Ukrainian scientists and in Russia, where it has been deposited with the original strain *S. recifensis* var. *lyticus* 2435 (Russian National Collection of Industrial Microorganisms № AC 668).

Analysis of current research of streptomycetes and problems in their taxonomy, requirements for industrial producers as well as the fact that *S. recifensis* var. *lyticus* 2435 strain was identified only on the basis of a number of phenotypic traits over 30 years ago, set the task for the authors to re-identify the producer strain using modern methods of taxonomic analysis.

Materials and Methods. *Streptomyces* sp. 2435 from the collection of the Department of Industrial Biotechnology National Technical University of Ukraine "KPI" was used in our research.

Morphological and biochemical features of the given culture were studied on typical streptomycetes agar media: Czapek agar, oatmeal agar, Gause mineral agar, Gause organic agar, glucose-asparagine agar [1]. Ability to utilize carbon sources by cultures of the strains and reference cultures was studied on ISR medium 19 [1].

To study the antagonistic activity the following reference strains of test organisms from Ukrainian Collection of Microorganisms (Institute of Microbiology and Virology, National Academy of Sciences of Ukraine) were used: gram-positive – *Bacillus subtilis* ATCC 6633, *B. cereus* ATCC 11778, *Staphylococcus aureus* ATCC 6538, *Kocuria* (*Micrococcus*) *varians* ATCC 9341, gram-negative – *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 6896, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enterica* NTCT 9027; yeasts – *Candida albicans* ATCC 10231, *Candida utilis* ATCC LIA-01.

Antagonist activity was determined by radial grooves, using agar Gause medium. Petri dishes were inoculated with the culture of *Streptomyces* sp. 2435, and grown for 5 days at 28 °C. Then test microorganism strains were sown by radial strokes (concentration 1×10^5 cells / ml), Petri dishes were placed into the incubator for 24 hours, (t = 37 °C). Areas of growth retardation of test strains were measured.

Cell morphology analyses: counterstaining with 1% uranyl acetate and examining by transmission electron microscopy (TEM) JEM-1400 (Jeol ltd., Japan) at 80 kV.

Fatty acid methyl esters (FAMES) were quantified by GC/MS with an Agilent 6890 gas chromatograph and 6890N Mass Selective Detector (Palo Alto, CA, USA). Data was 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.

Genomic DNA from cell suspension was isolated using Proba-CTAB DNA isolation kit (Russia) according to the manufacturer's instructions. 16S rRNA gene was amplified using primers 27f and 1492r; procedure of direct sequencing of the PCR product and analysis of the data are described in Safronova et al. [12].

Results and Discussion. Analysis 16S rRNA sequence of *Streptomyces* sp. 2435 against the GenBank database using BLASTN search revealed 99% matches with the following species of streptomycetes: *S. albus*, *S. saprophyticus*, *S. sampsonii*, *S. albidoflavus*, *S. coelicolor*, *S. limosus* and *S. flavofungini*.

Shown in Fig. 1 results indicate, that *Streptomyces* sp. 2435 strain formed the cluster with two other streptomycetes species – *S. albus* and *S. saprophyticus*. Thus, we can speak of a high probability of the studied culture belonging to these species, but not to the species *S. recifensis*, as stated earlier based on the studies of morphological, biochemical and cultural characteristics of the strain.

The sequence of *Streptomyces* sp. 2435 16S rRNA gene has been deposited in Genbank and assigned with an accession number JN129837**.**

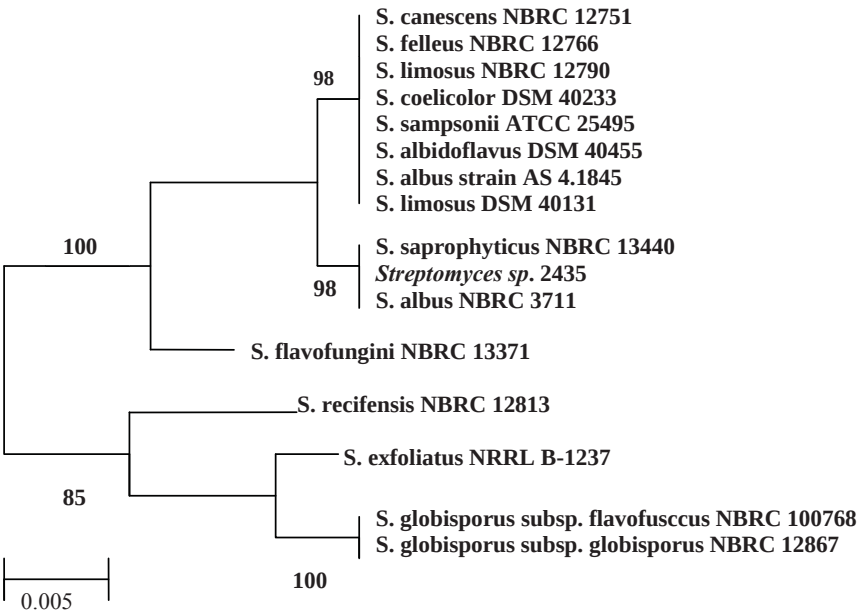

Fig. 1. Phylogenetic position of *Streptomyces sp.* 2435 within the genus *Streptomyces* based on 16S rRNA gene sequences. The tree was constructed using Neighbor-Joining method.

The complex of physiological, biochemical and morphological studies of *Streptomyces* sp. 2435 was carried out on the next phase of the work, and data on the current taxonomic position of *S. saprophyticus* and *S. albus* was analyzed.

The determined pattern of studied culture growth on a variety of typical streptomycetes agar media showed almost complete identity with the same characteristics of the typical strains of *S. saprophyticus*, *S. albus* (Table 1). The greatest differences were observed when the growth parameters of *S. saprophyticus* and *Streptomyces* sp. 2435 were compared on oat and glucose-asparagine agar. In these environments, aerial mycelium of *S. saprophyticus* has a yellow tone, while the test strain has a white to yellowish grey.

Table 1

Growth test of *Streptomyces* sp. 2435 and reference strains [1, 11, 13]

Medium	mycelium: air (A), substrate (S)	*S. saprophyticus* ATCC 3351	*S. albus* ATCC 25426	*Streptomyces* sp. 2435
Czapek agar	A	white	*white*	***colorless***
	S	colorless	colorless	colorless
Oat agar	A	***yellow***	white	*white*
	S	colorless	colorless	colorless
Gause mineral agar	A	white	white	white
	S	colorless	colorless	colorless
Gause organic agar	A	white to cream	white to cream	white to cream
	S	colorless	colorless	colorless
Glucose-asparagine agar	A	***yellow***	grey and yellow	***grey and yellow***
	S	colorless	colorless	colorless

These findings of the ability to utilize different carbon sources by the studied and reference strains (Table 2) also confirm the identity of the given cultures by biochemical characteristics.

Thus, only while utilizing arabinose a tiny difference in growth intensity was observed between *Streptomyces* sp. 2435 and streptomycetes species *S. saprophyticus* and *S. albus*. None of the compared cultures formed melanoid pigments when grown on media containing carbon sources. Data common to all was the formation of oval spores with a smooth surface. The last characteristic of the study of culture was determined by electron microscopy (Fig. 2). The photos showed a smooth surface of the spores of the studied culture, typical for the species *S. saprophyticus, S. albus* and *S. recifensis* [1].

Table 2

Morphological and biochemical characteristics of the studied *Streptomyces* sp. 2435 and reference strains [1, 11, 13]

Feature	*S. saprophyticus* ATCC 3351	*S. albus* ATCC 25426	*Streptomyces* sp. 2435
Utilization:			
glucose	+	+	+
fructose	+	+	+
sucrose	±	±	±
arabinose	±	±	+
xylose	+	+	+
rhamnose	±	±	±
raffinose	±	±	±
inositol	±	±	±
mannitol	+	+	+
Melanoid pigment formation	does not form	does not form	does not form
Spore form, method of formation	oval, smooth, fragmentation	oval, smooth, fragmentation	* oval, smooth , fragmentation
Sporophores form	wavy or spiral	wavy or spiral	wavy

Note: + normal growth, ± weak growth or variable, * indicated in Fig. 2

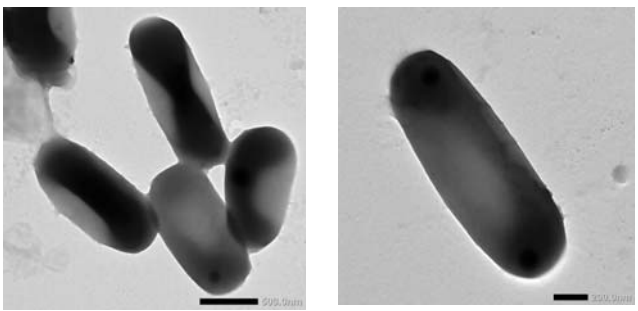

Fig. 2. Electron micrographs of *Streptomyces* sp. 2435 spores surface.

Fatty acids spectrum of *Streptomyces* sp. 2435 obtained in this study and their content in the cell wall is a general characteristic of streptomycetes. The spectrum was dominated by the saturated branched fatty acids and no acids with carbon number less than 12 and more than 19 were defined.

The main component of the fatty acid composition of the strain was straight-chain saturated octadecanoic (C18: 0) acid, the content of which was 72.55%. Contents of other saturated fatty acids with straight carbon chain (C16: 0) was 17, 79%. Thus, some other branched saturated acids (iC14: 0, iC15: 0, cis-9 ,10-C17: 0) were practically absent.

It must be noted that no fatty acids with double bonds were found in the studied strain, including monoenic acids, which are sometimes the components of streptomycetes lipids. However, according to the literature, the identification of streptomycetes lipid composition of the cell wall may only be considered as an additional feature, since it is largely dependent on the development stage of culture [1].

Experimental and literature data presented in the paper indicate a high degree of compliance with the studied culture and *S. saprophyticus* and *S. albus* species, which forces to turn to their modern systematic position and taxonomic status.

Species *S. albus* is claimed to be typical for the genus *Streptomyces* by many sources. It is the species that has been described and studied by Waksman and Pridham as one of the first in streptomycetes and is often used to compare the new species [1, 11]. The current literature mentions tens strains of this species, many of which are producers of antibiotics, enzymes and other biologically active substances, including those used in the industry.

Unlike *S. albus*, the reporting of *S. saprophyticus* is extremely limited not only in experimental studies, but also in the collections and individual systematics. Only individual information about this

species, as a producer of any biologically active substances occurs. In fundamental and integrated studies on the streptomycetes systematics the reporting of *S. saprophyticus* is found only in the collective work [14], where this species is placed into subscluster 1A of cluster 1 and marked in inverted commas (as well as a number of other species which taxonomic status is the matter of discussions). In a more recent survey on this subject conclusively established certain types of streptomycetes, which include *S. albus*, but not *S. saprophyticus* are listed [7]. The paper also provides evidence that the names of *S. albus* and *S. saprophyticus* were once used in relation to the same culture [11]. This fact explains the close taxonomic properties of the species listed in this paper.

On the basis of the information provided, there is a question about the current status of the *S. saprophyticus* species. Obviously, it is still ambiguous and leaves room for discussion among specialist taxonomists, who recommend streptomycetes species identification to be based on physiological (biochemical) features of cultures as well.

According to the literature, namely *S. albus* strains, in contrast to *S. saprophyticus*, are known as producers of a number of lytic enzymes and antibiotics [5, 10]. As indicated above, the analyzed strain *Streptomyces* sp. 2435 is characterized by the biosynthesis of the bacteriolytic enzyme complex that is active against several bacterial species (*Staphylococcus aureus*, *Bacillus cereus*, *Corynebacterium gravis*, *Pseudomonas aeruginosa* and others) [2, 3]. In our research *Streptomyces* sp. 2435 antagonistic properties assay showed that the strain is active against *Candida albicans* – growth retardation zone 20 mm (high activity), *Kocuria varians* – 10 mm (middle activity) and *Candida utilis* – 6 mm (low activity). Thus, *Streptomyces* sp. 2435 possesses both antibacterial and antifungal activities.

In previous work on the culture it was shown that culture liquid concentrate containing the lytic enzyme complex does not exhibit antibiotic activity, including one against the *Candida* species. Therefore, presumably active antifungal antibiotic can have endogenous localization.

Thus, on the basis of 16S rRNA gene sequence of *Streptomyces* sp. 2435, comparative studies of the morphology, biochemistry and physiology of the strain, as well as analysis of the current literature on the systematics and practical use of streptomycetes species we have established the identity of the strain *Streptomyces* sp. 2435 to the *S. albus* species.

Т.С. Тодосійчук[1], В.В. Клочко[2], Л.Б. Зелена[2]

[1]Національний технічний університет України «КПІ»
просп. Перемоги 37, Київ 03056, Україна
[2]Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України
вул. Академіка Заболотного 154, Київ МСП 03680, Україна

ТАКСОНОМІЧНИЙ АНАЛІЗ STREPTOMYCES SP. 2435 – ПРОДУЦЕНТА АНТИМІКРОБНИХ СУБСТАНЦІЙ

Р е з ю м е

Проведено вивчення таксономічного статусу штаму-продуцента антимікробних субстанцій *Streptomyces* sp. 2435. Визначена нуклеотидна послідовність гена 16S рРНК даного штаму, яка задепонована в базі даних Genbank за номером № JN129837. Результати морфологічного, біохімічного аналізів, жирнокислотного складу клітинних ліпідів, біосинтетичних ознак *Streptomyces* sp. 2435 разом з філогенетичним аналізом дали підстави віднести даний штам до виду *Streptomyces albus*.

К л ю ч о в і с л о в а: таксономічний аналіз, систематика, *Streptomyces albus*, антагоністична активність.

Т.С. Тодосийчук[1], В.В. Клочко[2], Л.Б. Зеленая[2]

[1]Национальный технический университет Украины «КПИ»,
просп. Победы 37, Киев 03056, Украина
[2]Институт микробиологии и вирусологии им. Д.К. Заболотного НАН Украины
ул. Академика Заболотного 154, Киев МСП 03680, Украина

ТАКСОНОМИЧЕСКИЙ АНАЛИЗ STREPTOMYCES SP. 2435 – ПРОДУЦЕНТА АНТИМИКРОБНЫХ СУБСТАНЦИЙ

Р е з ю м е

Проведено изучение таксономического статуса штамма-продуцента антимикробных субстанций *Streptomyces* sp. 2435. Определена нуклеотидная последовательность гена 16S рРНК данного штамма и депонирована в базе данных Genbank под номером № JN129837. Результаты морфологического, биохимического анализов, жирнокислотного состава клеточных липидов, биосинтетических особенностей у *Streptomyces* sp. 2435 наряду с филогенетическим анализом дали основание отнести данный штамм к виду *Streptomyces albus*.

Ключевые слова: таксономический анализ, систематика, *Streptomyces albus*, антагонистическая активность.

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