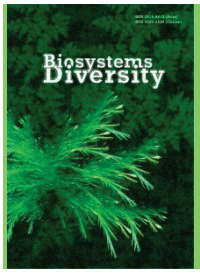

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Characteristics of marine strain *Streptomyces* sp. with antimicrobial and cytotoxic activity

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The Black Sea is a unique water basin consisting of a thin superficial oxygenic layer with moderate salinity, and a deep anoxic water mass. The microbiota of the Black Sea remains relatively understudied, which makes it interesting first of all from the most practical point of view of the search for producers of new biologically active compounds. A strain of actinobacteria *Streptomyces* sp. ONU 561 was isolated from the surface of mussel shells collected in the coastal zone of Odesa. It demonstrated a wide range of antagonistic activity, inhibiting the growth of a set of opportunistic pathogens, including representatives of *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, and *Klebsiella pneumoniae*. In addition, bacteria of this strain were able to inhibit the growth of all tested strains of mycelial fungi, including representatives of *Aspergillus niger*, *A. flavus* and *Fusarium oxysporum* species, and *Candida albicans* yeast. A significant cytotoxic effect was revealed in the cell cultures of human malignant cells – human rhabdomyosarcoma (RD) and human laryngeal adenocarcinoma (Hep-2). Analysis of the exometabolome of the strain did not explain these effects.The strain was comprehensively characterized, including physiological, biochemical, and morphological traits. The complete genome of the strain was sequenced using Illumina HiSeq 4000 (2x150) and ONT and annotated using NCBI PGAP. Its genome has a size of 8 359 197 bp. GC content – 71.59%. Using antiSMASH 7.0, 35 biosynthetic clusters were revealed. The indices of digital DNA-DNA hybridization and orthoANI for all of the type strains with *Streptomyces* sp. ONU 561 are much lower than threshold values for the species separation. The obtained results, including a comparative analysis of the genome, indicate the possible affiliation of the strain *Streptomyces* sp. ONU 561 to a new species and the potential ability of these actinobacteria to synthesize previously unknown antibiotic compounds.

Keywords: marine actinobacteria; *Streptomyces*; genome; exometabolite; cytotoxic activity; antimicrobial activity.

Introduction

Oncological and infectious diseases are important causes of death worldwide. Therefore, the search for new antimicrobial and antitumor compounds is an urgent problem. The gradual depletion of terrestrial biotopes as sources of new potential products, considering the tough selection that all potential clinically significant compounds undergo, creates requirements for a comprehensive search for new compounds in the marine environment (Papon et al., 2022). The marine microbiota is much older than terrestrial microbiota, and much less studied in all aspects (Landwehr et al., 2016; García-Davis et al., 2021). The study of the biological activity of low-molecular-weight substances of marine origin, in particular with accent on antimicrobial and antitumor activity, is one of the main trends in applied biological research in recent years (Malve, 2016). Various compounds with antimicrobial and cytotoxic activity have recently been found in representatives of a wide range of taxonomic groups of marine prokaryotes, primarily Actinomycetota, Bacillota, and Cyanobacteriota, which indicates that the genetic potential of the marine microbial community is understudied and underused (Ruiz-Torres et al., 2017; Potapenko et al., 2021; Ngamcharungchit et al., 2023). Many new compounds and chemical scaffolds of marine microbial origin are already undergoing clinical trials, e.g. salinosporamide A, and bryostatin-1 (Gulder & Moore, 2010; Tian et al., 2023).

Mycelia forming representatives of the phylum Actinomycetota are the main resource for the search for new natural products due to their ability to produce various secondary metabolites. In particular, actinobacteria produce almost 40% of bioactive secondary metabolites used in medicine. Of these, 80% are synthesized by representatives of the *Streptomyces* genus (Mu et al., 2021). *Streptomyces* natural products have a wide range of biological activities, including antiproliferative, antimicrobial, herbicidal, insecticidal, and more (Kim et al., 2020; Chen et al., 2021; Karim et al., 2021; Amelia-Yap et al., 2022). For some of them, no biological activity was revealed (Suzuki et al., 2021).

Considering the lack of research on the microbiota of the Black Sea and the specificity of its physical geography, it is possible to assume a high biosynthetic potential of its representatives. Actinobacteria of the Black Sea were detected mainly by metagenomic methods in samples of the Black Sea origin and remain poorly studied today (Jaiani et al., 2020).

During the previous studies, the biological diversity of the microbiota of the Odesa Bay of the Black Sea was studied and a large collection of actinobacteria isolated from biological fouling in the marine environment was collected (Vasylieva et al., 2018; Korotaeva et al., 2021).

This article presents the results of studying the biological characteristics of a strain of actinobacteria ONU 561 which was isolated from the Odesa Bay of the Black Sea and showed a wide range of antibiotic activity.

Materials and methods

Isolation. The studied strain of actinobacteria was isolated from shells of mussels collected in June 2020 in the Odesa Bay of the Black Sea at a point with coordinates 46°27′01′′ N 30°46′14′′ E at a depth of 5 m (Fig. 1). The collected mussels were put into flasks with sterile seawater and shaken for 30 min in a rotary shaker at 150 rpm and a temperature of 28 °C. The obtained suspension was heated at a temperature of 50 °C for 15 min, and then inoculated on agar medium Gauze 2 (pH 6.8–7.2),

prepared on seawater with the addition of nalidixic acid (10 mg/L), followed by cultivation at a temperature of 28 °C for 21 days. From the obtained isolates, strain ONU 561 was selected because of its wide spectrum of antimicrobial activity. ONU 561 is kept in the collection of microorganisms of the Odesa I. I. Mechnykov National University at a temperature of –85 °C.

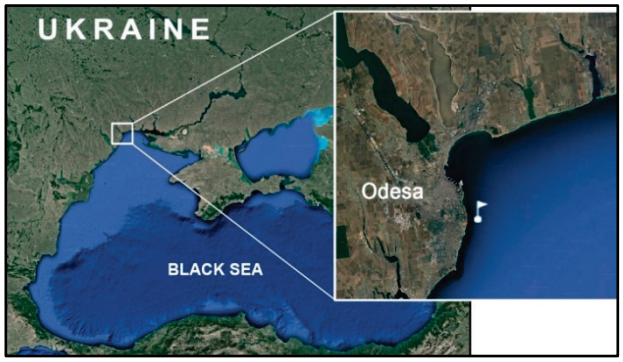

Fig. 1. Location of the sampling site in the northwestern part of the Black Sea in the Odesa city region

The morphological, cultural, physiological, and biochemical characteristics of the isolate were studied by the recommendations of the International *Streptomyces* Project (Shirling & Gottlieb, 1968).

The morphology and character of growth were studied on media: Nutrient Agar (Biolife) (NA), Gauze 1 (Soluble starch 20.0 g/L, KNO_3 1.0 g/L, NaCl 0.5 g/L, $MgSO_4$ x $7H_2O$ 0.5 g/L, K_2HPO_4 0.5 g/L, $FeSO_4$ x $7H_2O$ 10.0 mg/L, Agar 15.0 g/L), Gauze 2 (Peptone 5.0 g/L, NaCl 5.0 g/L, Glucose 10.0 g/L, Hottinger broth 30.0 mL/L, Agar 20 g/L), Oatmeal agar (OA) (Oatmeal 20.0 g/L, Agar 18.0 g/L, 1 mL of trace salt solution: $FeSO_4$ x $7H_2O$ 0.1 g $MnCl_2$ x $4H_2O$ 0.1 g $ZnSO_4$ x $7H_2O$ 0.1 g, Distilled water 100.0 mL), Starch-casein agar (SCA) (Soluble starch 10.0 g/L, K_2HPO_4 2.0 g/L, Casein 0.3 g/L, $MgSO_4$ * $7H_2O$ 0.05 g/L, $CaCO_3$ 0.02 g/L, $FeSO_4$ * $7H_2O$ 0.01 g/L, Agar 15.0 g/L), and on ISP media (ISP 1-7) for 14–21 days at 28 °C (Li et al., 2016).

The morphology of aerial mycelium was described during cultivation on Gauze 1 medium. To do this, the culture in the sporulation stage was inoculated into the prepared channels in a sterile Gauze 1 medium, then covered with a sterile cover glass and incubated at 28 °C (Ravindragouda & Jeyasekaran, 2016). Microscopy of the mycelium grown on the cover glass was carried out using a Carl Zeiss AxioScope.A1 microscope at magnifications of ^{x}100 and ^{x}400 in light-field mode.

Utilization of carbon sources was investigated in the minimal medium of ISP 9. The carbon sources were glucose, fructose, galactose, glycerol, lactose, xylose, rhamnose, mannitol, sorbitol, mannose, arabinose, and sucrose, which were added separately to the final concentration in a medium of 1%. Incubation was carried out for 14 days at 28 °C. The ability to utilize these carbon sources was recorded visually.

Catalase and oxidase activities (Bactident Oxidase MERKC) were determined as proposed for *Streptomyces*-like bacteria (Goodfellow, 2012; Espinoza et al., 2013). The ability to synthesize the enzymes amylase, protease, gelatinase, lipase, urease, cellulase, nitrate reductase, and to produce hydrogen sulfide was determined using the API 20E kit (bioMérieux, Inc., Durham, NC).

The fatty acids profile was determined using the Sherlock Microbial Identification System (MIDI Sherlock version 6.2, MIDI Library ACTIN 3.80) after culturing the isolate in Tryptic Soy Broth medium (Biolife) at 28 °C and 150 rpm for 3 days. Isolation and determination of fatty acids was carried out according to the MIS Operating Manual (Sasser, 1990) using an Agilent 7890 gas chromatograph (Agilent Technologies, USA) and an ULTRA 2 capillary column (25 m x 0.2 mm x 0.33 μm) and flame ionization detector.

The antitumor activity of exometabolites was studied by the cytotoxicity index on the model of a monolayer of passaged cultures of malignant cells of human connective tissue - human rhabdomyosarcoma (RD) and tumor cells of the glandular epithelium of the human laryngeal adenocarcinoma (Hep-2) according to the previously described method (Abdelfattah et al., 2016). Extract of exometabolites of the studied strain was prepared as described in the section "Extraction of secondary metabolites". Stock solutions of extracted metabolites were prepared in dimethyl sulfoxide (DMSO) (Gaylord Chemical Corp., USA) at a concentration of 100 mg/mL. The work solution of exometabolites was prepared in the nutrient medium for cell cultures DMEM (BioWest, France) at a concentration of 1 mg/mL. Metabolite solutions were sterilized using membrane filters with a pore diameter of 0.22 μm (Millex®-GS Filter Unit with MF-Millipore MCE Membrane, Millipore Ireland Ltd) (Paulus et al., 2017).

Cultures of Hep-2 and RD cells were inoculated in 48 and 96-well plates (4×10^4 cells per well) with 100 μL of DMEM medium supplemented with fetal bovine serum (FBS Premium) (BioWest, France) in the amount of 10% of the total volume of cell suspension in the well. Cell cultures were incubated at 36 °C in a CCL-050T-8 CO_2 incubator (EscoMicroPte. Ltd (SINGAPORE) for 24 h.

In each well, extract of exometabolites of actinobacteria were added to concentrations of 2.5, 25.0, 50.0, 100.0, 250.0, and 500.0 μg/mL. As controls we used intact cell cultures in DMEM medium and cell cultures in DMEM medium with the addition of DMSO in concentrations of 0.50%, 0.25%, 0.10%, 0.05%, 0.025%, 0.0025%, which corresponds to DMSO concentration in respective wells with exometabolites' solution.

The cytotoxic effect of exometabolites of *Streptomyces* sp. ONU 561 was determined visually after 24 h by microscopic examination (magnification ^{x}100, microscope Zeiss AxioScope.A1) of the monolayer of cells by observing the morphological changes of individual cells and the degree of degeneration of the monolayer.

The degree of destruction of the monolayer was estimated by the number of viable cells in the monolayer by the optical density indicator, which was measured using a universal spectrophotometer for microplates (μQuant™ "Bio-Tek", USA) at 630 nm.

The degree of toxicity was assessed according to the following criteria:

– non-toxic – deviation of the optical density indicator from the control with DMSO <20%;

– average level of toxicity – deviation of the optical density indicator from the control with DMSO – from 21% to 50%;

– highly toxic – deviation of the optical density indicator from the control with DMSO >50%.

Antagonistic activity was determined by agar blocks method on test strains of bacteria: *Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* ATCC 4698, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* DSM 348, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 6896, *Salmonella enterica* NCTC 6017, *Klebsiella pneumoniae* ATCC 10031, *Pseudomonas aeruginosa* ATCC 27853, *P. putida* KT 2440) and yeast *Candida albicans* ATCC 18804 on LB broth (MILLER) for Microbiology (Merck) medium, supplemented with 0.7% agar-agar and collection strains of mycelial fungi: *Alternaria alternata* UKM F-16866, *A. tenuissima* ONU F-24, *Aspergillus niger* UKM F-16706, *A. flavus* var. *oryzae* UKM F-3023, *A. terreus* UKM F-16718, *Cladosporium cladosporioides* UKM F-2235, *Fusarium oxysporum* UKM F-54201, *Paecilomyces variotii* UKM F-424, *Penicillium expansum* UKM F-575, *Rhizoctonia cerealis* ONU F-30 – on potato-dextrose agar (Potato Dextrose Agar Biolife Italiana S.r.l.).

To obtain agar blocks of the strain, ONU 561 was grown on agar media Gauze 1, Gauze 2, and OA with sea salt (2%), at a temperature of 30 °C for 10 days (Korotaeva et al., 2021). After that, on the surface of the Petri dish previously inoculated with culture of the test strain (10^9 cells/mL) were placed blocks of the 10-day culture of the strain ONU 561.

The results were estimated after incubation at temperatures optimal for each group of microorganisms after 1 day (for bacteria), 2 days (for *C. albicans*), and 10 days (for mycelial fungi), measuring the sizes of the zones of no growth of the test strains around the blocks with ONU 561 (Korotaeva et al., 2021).

Susceptibility to antibiotics was determined by the disc diffusion method on SCA medium; using standard paper discs with antibiotics: penicillin (10 μg/disc), ampicillin (10 μg/disc), erythromycin (15 μg/disc), oleandomycin (15 μg/disc), tetracycline (30 μg/disc), streptomycin (300 μg/disc), kanamycin (30 μg/disc), neomycin (30 μg/disc), gentamicin (10 μg/disc), amikacin (30 μg/disc), chloramphenicol (30 μg/disc), rifampicin (5 μg/disc), nystatin (80 units).

After 3 and 5 days of cultivation at 28 °C, the sensitivity of the strain to antibiotics was determined and the results were evaluated. A strain was considered resistant if the zone of no growth was less than 2 mm.

Isolation and sequencing of genomic DNA. Bacteria were grown on TSB medium (pancreatically hydrolyzed casein – 17.0 g/L; soy peptone – 3.0 g/L; sodium chloride – 5.0 g/L; dipotassium phosphate – 2.5 g/L; glucose – 2.5 g/L) and SG (glucose – 20,0 g/L; yeast extract – 5.0 g/L; soy peptone – 10.0 g/L; calcium carbonate – 2.0 g/L). Biomass was precipitated from 25 mL of culture by centrifugation, resuspended in 1 mL of GET (50 mM glucose, 25 mM EDTA, 25 mM Tris, pH 8.0) on ice, 50 µL of lysozyme (100 mg/mL) and 5 µL of RNase (10 mg/mL) were added, incubated for 1 h at 37 °C, 75 µL of 10% SDS was added and incubated for 1 h at 65 °C, 1 mL of phenol/chloroform 1:1 was added, centrifuged, the supernatant was transferred to an Eppendorf tube and 750 µL of 100% EtOH was carefully added mixed and centrifuged. The precipitate was resuspended in 100 µL of MilliQ water. Sequencing was performed on an Illumina HiSeq 4000, 2x150 and MinIon Flongle FLG-001. Genome assembly was conducted with flye v2.9 and PILON, using both Illumina and ONT reads. Genome annotation was done using NCBI PGAP.

The presence of clusters of PKS I, PKS II and NRPS in the genome was additionally verified by PCR with primers K1F and M6R targeting PKS-I KS and methylmalonyl-CoA transferase domains; KSαF and KSαR target the KSα subunit of PKS-II, and the primer pair A3F and A7R targeting conserved parts A3 and A7 in the NRPS adenylation domain (Ghashghaei et al., 2023).

16S rRNA gene sequence analysis was performed using the EzBioCloud database (Yoon et al., 2017), Silva (Quast et al., 2012), and NCBI BLAST.

The phylogenetic tree was constructed by pairwise comparisons of the 16S rRNA gene sequences using the Genome to Genome Distance Calculator (GGDC) web server (Meier-Kolthoff et al., 2022).

Multiple comparison was performed with Clustal W algorithm software in MEGA 11 (Tamura et al., 2021). The phylogenetic tree was constructed using the NJ method (Saitou & Nei, 1987). The ITOL server (https://itol.embl.de) was used to visualize the resulting tree.

The Type (Strain) Genome Server (TYGS) was used to determine the degree of DNA-DNA hybridization (DDH) between genomes (Meier-Kolthoff et al., 2022).

Biosynthetic gene clusters were predicted using antiSMASH 7.0 (Blin et al., 2023).

Extraction of secondary metabolites. Streptomycete inoculation culture was grown on a TSB medium with glass beads for 72 h at 28 °C on a rotary shaker at 180 rpm. SG medium was inoculated with the seed culture and cultured at 28 °C on a rotary shaker at 180 rpm for 7 days until the stationary phase of growth. The culture liquid was separated from the biomass of actinobacteria by centrifugation at 10,000 g for 10 min at a temperature of 18 °C. Exometabolites of actinobacteria were extracted from the culture liquid with ethyl acetate in a 1:1 ratio with gentle stirring for 2 hours at room temperature. The extractant with metabolites was carefully separated and the ethyl acetate was evaporated at 40 °C under a stream of nitrogen gas under a pressure of up to 2 psi using The Techne Sample Concentrator FSC 400D with DB 100/3 Dri-Block Heater (Cole-Parmer Ltd, United Kingdom) (Paulus et al., 2017).

Analysis of exometabolites spectra. The extracted metabolites were dissolved in dimethylsulfoxide. Sample analysis was performed using ThermoFischer Dionex UltiMate 3000 BioRS UPLC System coupled with a maXis II Bruker Daltonics mass spectrometer (4G hr-ToF). A Waters BEH C18 column (100 mm x 2.1 mm, 1.7 µm) was used at a temperature of 45 °C and a flow rate of 0.5 mL per min. Solvents – A: 0.1% formic acid in water; B: 100:20 acetonitrile + formic acid 0.1%. Gradient: 1 min 5% B, 5% B to 95% B in 18 min, 95% B – 1 min at 100%, 95% B to 5% B in 1 min, 2 min equilibration at 5% B. Mass detection occurred in the range of 150–2000 m/z optical absorbance in wavelength range 200–600 nm. Identification of compounds was carried out using the electronic version of the "Dictionary of Natural Products" (Buckingham, 1997).

Results

The isolated strain according to the character of growth on nutrient media, ability to form the substrate and aerial mycelia was assigned to the phylum Actinomycetota with the number ONU 561.

Morphological properties. The isolated strain forms separate flat small colonies with a diameter of up to 3.0 mm during surface cultivation on agar media NA, OA, SCA, Gauze 1, and Gauze 2 at a temperature of 28 °C for 5 days (Fig. 2).

On ISP-6 (Peptone Yeast Extract Iron Agar), but not on ISP-7, actinobacteria synthesize brown melanoid pigments that diffuse into the medium. The physiological and biochemical properties of the strain ONU 561 are summarized in Table 1.

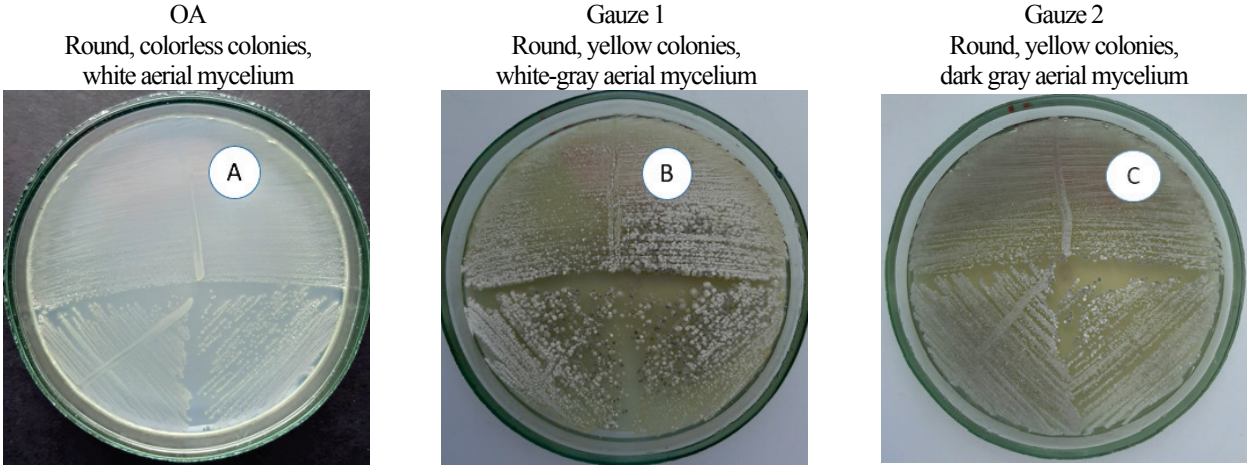

Fig. 2. The growth of the strain ONU 561 on agarized media: *A* – OA, *B* – Gauze 1, *C* – Gauze 2

The morphology of the spore chains and mycelium corresponds to the Spirales type, however, the bends of the long chains resemble those characteristic of *Retinaculum-Apertum* (Fig. 3). The studied strain is an aerobe; the optimal temperature for growth is 28 °C, but it grows in the temperature range from 10 to 37 °C; also grows well at a concentration of NaCl in the medium from 1% to 5%, with an increase in the concentration of sodium chloride, growth is inhibited. With varying intensity, this strain metabolizes such carbon sources as arabinose, galactose, glucose, xylose, lactose, maltose, rhamnose, sucrose, glycerol, mannitol, sorbitol; does not utilize fructose. The strain has catalase activity, oxidase activity is not clearly expressed, it grows on a medium with gelatin, but does not dilute it, coagulates but does not peptonize milk, hydrolyzes starch, decomposes amino acids with the formation of hydrogen sulfide, is not capable of anaerobic respiration using nitrate, does not have lipase activity, synthesizes cellulase and urease (Table 1). The dominant fatty acids included in the composition of the membranes are C15:0anteiso (33.67%), C15:0 iso (14.89%), and C16:0 (19.24%) (Table 2), which is significantly different from type strain *S. ambofaciens* ATCC 23877. Dominant fatty acids of the latter are C16:0 iso (20.50%), C16:0 (16.50%),C15:0 anteiso (14.50%), C18:0 (13.20%).

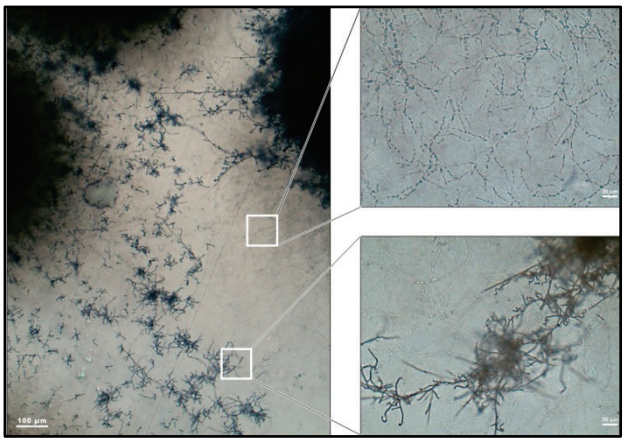

Fig. 3. Mycelium with sporangia of the strain ONU 561 on medium Gauze 1: microscope Carl Zeiss AxioScope.A1; image processing software: ZEN1

Table 1
Physiological and biochemical properties of strain ONU 561 in comparison with the type strain of the most closely related species *Streptomyces ambofaciens*

Test/substrate	Strain ONU 561	*Streptomyces ambofaciens* ATCC 23877
Growth temperature range, °C	10–37	Nd
Temperature optimum, °C	28	28
NaCl (%)	1–5	Nd
Catalase	+	Nd
Oxidase	±	Nd
H_2S production	+	+
Amylase	+	Nd
Caseinase	±	Nd
Gelatinase	–	+
Lipase	–	+
Urease	+	+
Cellulase	+	+
Nitrate reduction	–	Nd
Carbon source		
L-arabinose	+	+
D-galactose	+	Nd
D-glucose	+	+
D-xylose	+	+
Lactose	+	Nd
Maltose	+	Nd
Rhamnose	+	+
Sucrose	+	+
Fructose	–	+
Mannitol	+	+
Glycerol	+	Nd
Sorbitol	+	Nd

Note: Nd – information is absent; "+", "–", "±" – respectively, positive, negative, and intermediate test results.

Resistance to antibiotics. Determination of the sensitivity of the strain ONU 561 to antibiotics showed that the strain is sensitive to streptomycin, kanamycin, tetracycline, gentamicin, amikacin, neomycin, and chloramphenicol, resistant to benzylpenicillin, ampicillin, erythromycin, oleandomycin, rifampicin, and nystatin.

Antagonistic activity. Determination of the antagonistic activity of the strain ONU 561, isolated from shells of mussels showed that the strain inhibited the growth of almost all tested test strains of pro- and eukaryotic microorganisms. Note that the activity of the strain, that is, the intensity of its influence on the tested cultures, depended both on the test strain of microorganisms and on the culture medium of the strain ONU 561 (Fig. 4, 5).

The spectrum of antagonistic activity of the strain ONU 561 to opportunistic human pathogens and mycelial fungi was found to be broad. It demonstrated the ability to significantly (with a diameter of the zone of growth inhibition not less than 13 mm) inhibit the growth of bacteria and yeast of the following strains: *Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* ATCC 4698, *Kocuria rhizophila* DSM 348, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 6896, *Salmonella enterica* NCTC 6017, *Klebsiella pneumoniae* ATCC 131, *Pseudomonas putida* KT 2440, and *Candida albicans* ATCC 18804.

Table 2
Fatty acid profile of the strain ONU 561

Fatty acid (FA)	FA, %
11:0 anteiso	0.10
12:0	0.18
13:0 iso	0.43
13:0 anteiso	0.45
13:0	0.10
14:0 iso	1.87
14:0	1.09
15:0 iso	14.89
15:0 anteiso	33.67
15:1 B	0.16
15:0	2.03
16:1 iso H	0.99
16:0 iso	7.26
16:1 cis 9	5.93
16:0	19.24
16:0 9-methyl	3.21
17:1 anteiso c	2.81
17:0 iso	3.43
17:0 anteiso	9.46
17:1 cis 9	0.32
17:0 cyclo	0.34
17:0	0.58

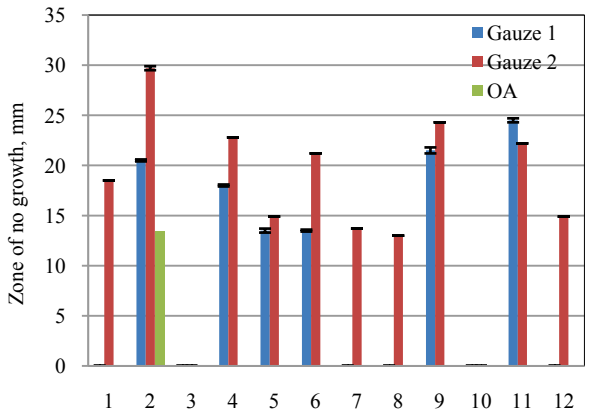

Fig. 4. Antagonistic activity of the strain ONU 561 against indicator strains of microorganisms: 1: *Staphylococcus aureus* ATCC 25923, 2: *Micrococcus luteus* ATCC 4698, 3: *Enterococcus faecalis* ATCC 29212, 4: *Kocuria rhizophila* DSM 348, 5: *Bacillus subtilis* ATCC 6633, 6: *Escherichia coli* ATCC 25922, 7: *Proteus vulgaris* ATCC 6896, 8: *Salmonella enterica* NCTC 6017, 9: *Klebsiella pneumoniae* ATCC 131, 10: *Pseudomonas aeruginosa* ATCC 27853, 11: *P. putida* KT 2440, 12: *Candida albicans* ATCC 18804; error bar indicates standart deviation

Interestingly, after cultivation on OA, the antagonistic activity remained only against *Micrococcus luteus*, and on Gauze 1 it disappeared against *Staphylococcus aureus*, *Proteus vulgaris*, *Salmonella enterica*, and *Candida albicans*. Insensitive to the antibiotic action of the strain ONU 561 on all media were only *Enterococcus faecalis* and *Pseudomonas aeruginosa*.

Antagonistic activity of the strain ONU 561 in relation to mycelial fungi was more pronounced than to bacteria and yeast.

Antitumor effect of exometabolites of the Black Sea actinobacteria ONU 561 was determined by the index of cytotoxic activity on the model of passaged cultures of human malignant cells – RD and Hep-2 by indicators of morphological changes of cells, cell death and destruction of the monolayer. The results of the study are presented in Figure 7.

It was established that the secondary metabolites of the studied strain at concentrations of 25.0–500.0 μg/mL showed a cytotoxic effect on the

culture of human rhabdomyosarcoma cells *in vitro*. Already after 24 hours of exposure, morphological changes of RD cells were detected, including rounding of cells, vacuolization of cytoplasm, wrinkling, and pyknosis of nuclei, destruction of the monolayer (Fig. 7).

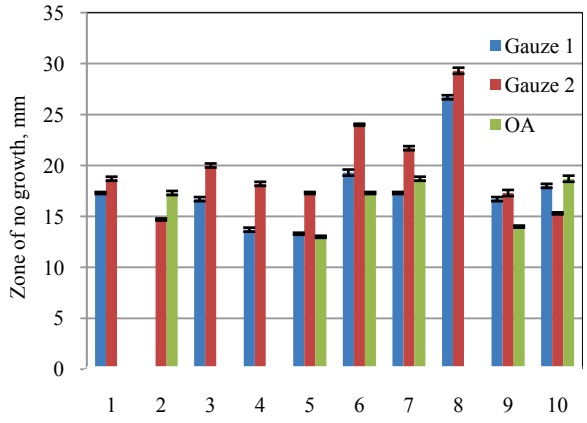

Fig. 5. Antagonistic activity of the strain ONU 561against mycelial fungi: 1: *Alternaria alternata* UCM F-16866, 2: *A. tenuissima* ONU F-24, 3: *Aspergillus niger* UCM F-16706, 4: *A. flavus* var. *oryzae* UCM F-3023, 5: *A. terreus* UCM F-16718, 6: *Cladosporium cladosporioides* UCM F-2235, 7: *Fusarium oxysporum* UCM F-54201, 8: *Paecilomyces variotii* UCM F-424, 9: *Penicillium expansum* UCM F-575, 10: *Rhizoctonia cerealis* ONU F -30; error bar indicates standard deviation

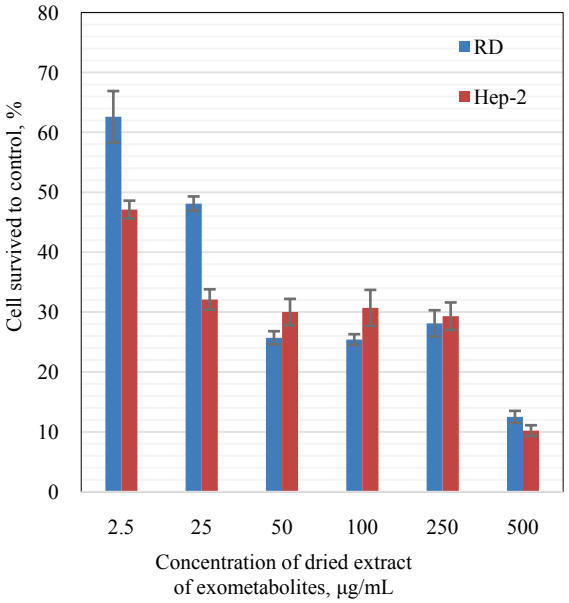

Fig. 6. Influence of exometabolites of the strain ONU 561on cell viability in a monolayer of cultures RDand Hep-2 (time of exposure 24 h), in the percentage of viable cells to control with DMSO (by the index of optical density); error bar indicates standard deviation

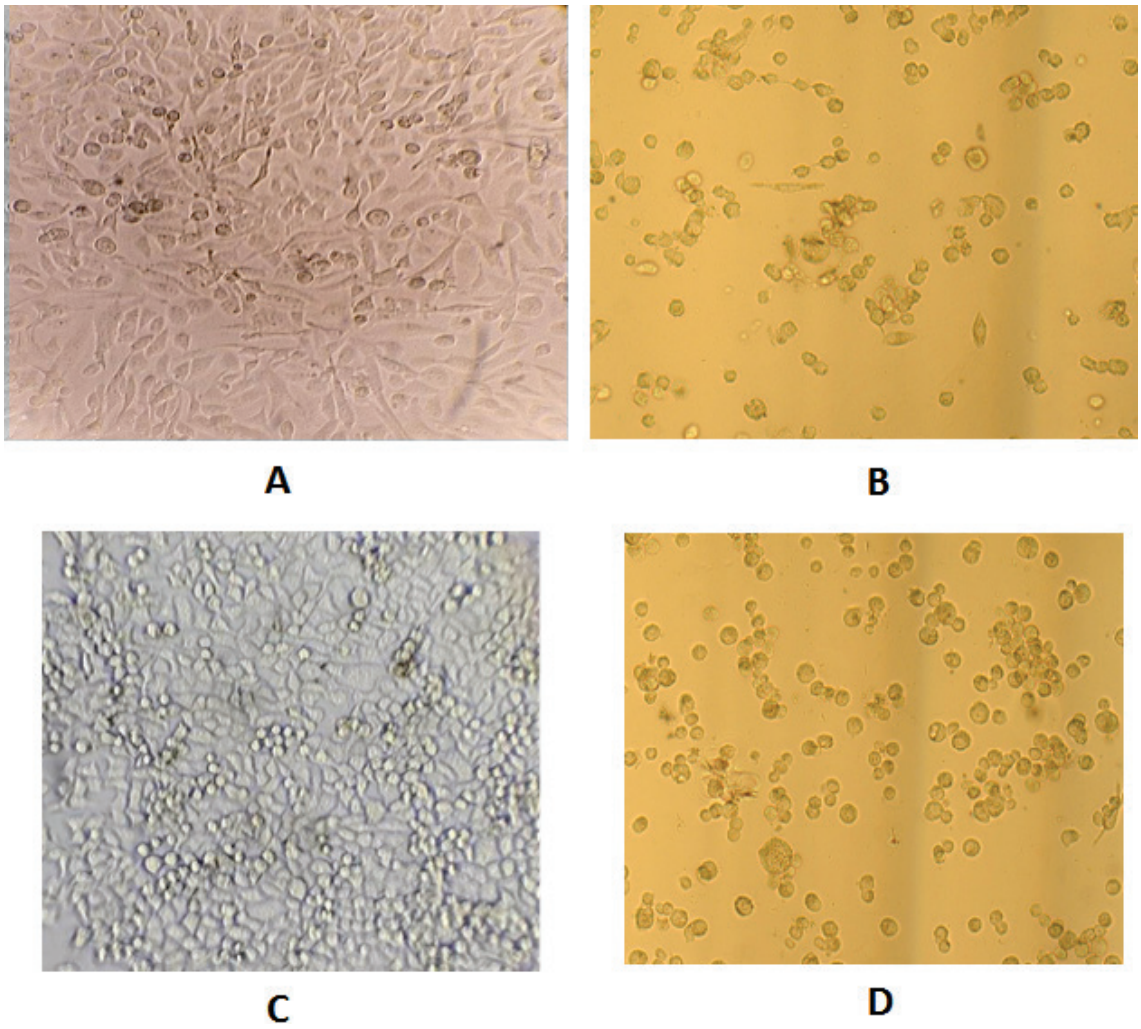

Fig. 7. Morphological changes of cells and destruction of the monolayer of passaged cultures of RD and Hep-2 cells under the action of exometabolites of actinobacteria: exposure time 24 hours: magnification ˣ100, microscope Zeiss AxioScope.A1; A – RD cell culture, control with DMSO; B – RD cell culture, exometabolites of the strain ONU 561, 100,0 µg/mL; C – Hep-2 cell culture, control with DMSO; D – Hep-2 cell culture, exometabolites of the strain ONU 561, 100 µg/mL

From the data presented in Figure 7, it can be seen that exometabolites of actinobacteria ONU 561 were highly toxic to human RD cell culture at concentrations of 25–500 μg/mL. Already after 24 h of exposure, they caused the death of 51.9–87.5% of cells and almost complete destruction of the monolayer. At a concentration of 2.5 μg/mL exometabolites of actinobacteria ONU 561 showed an average level of toxicity to RD cell culture – the number of viable cells in the monolayer decreased to 62.6 ± 4.3% compared to the DMSO control.

In the culture of Hep-2 exometabolites of the strain ONU 561, showed an acute cytotoxic effect after 24 h of exposure in all tested concentrations (25–500 μg/mL) – the number of non-viable monolayer cells reached 67.9–89.8%. Even at a minimum concentration of 2.5 μg/mL, the metabolites of these bacteria had a pronounced antitumor activity – they caused the death of 52.9% of monolayer cells.

The results of our research indicate that actinobacteria of the strain ONU 561 is a producer of cytotoxic compounds and can be recommended for further in-depth studies of its antitumor activity.

Genomics and molecular phylogenetics. The genome of actinobacterium ONU 561 has a size of 8 359 197 bp. GC content – 71.59%. Both parameters are typical for representatives of this genus.

In total 7,427 genes were found in the genome, of which 7,337 are protein-coding ORFs, 318 pseudogenes, 3 rRNA operons, 69 tRNA genes, 1 tmRNA, and 3 small non-coding RNAs.

According to the results of molecular phylogenetic analysis of 16S rRNA, strain ONU 561 was identified as belonging to genus *Streptomyces* and turned out to be the most closely related to *S. ambofaciens* strains isolated from the soil of Algeria (*S. ambofaciens* TG30) and China (*S. ambofaciens* 173589) (Fig. 8), and also type strain of this genus.

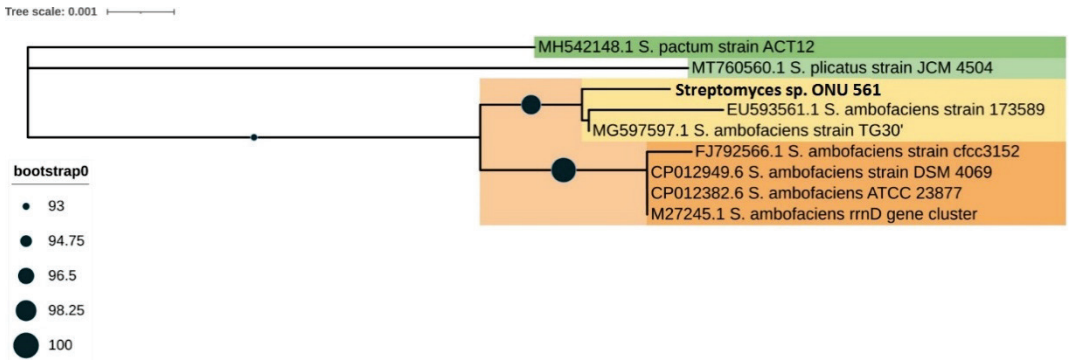

Fig. 8. Neighbour-joining phylogenetic tree, based on 16S rRNA genesequences using 1000 repeats of bootstrap

The index of digital DNA-DNA hybridization (according to the d4 formula) with ONU 561 for none of the type strains, the genomes of which were sequenced and placed in the database, does not exceed 39.4%. That is significantly lower than the threshold of 70% for the discrimination of species.

The orthoANI index for a pair of strains *Streptomyces ambofaciens* ATCC 23877 – *Streptomyces* sp. ONU 561 is high (90.05%), but also below the species delimiting threshold of 95%. Together with the difference in the content of GCof about 0.61% and the value of dDDH, these results indicate that the strain may belong to a species new to science (Fig. 9).

Genome sequence analysis of *Streptomyces* sp. ONU 561 using the antiSMASH 7.0 server helped to detect 35 biosynthetic clusters. For four of them, it was not possible to identify homologs among known clusters (Table 3).

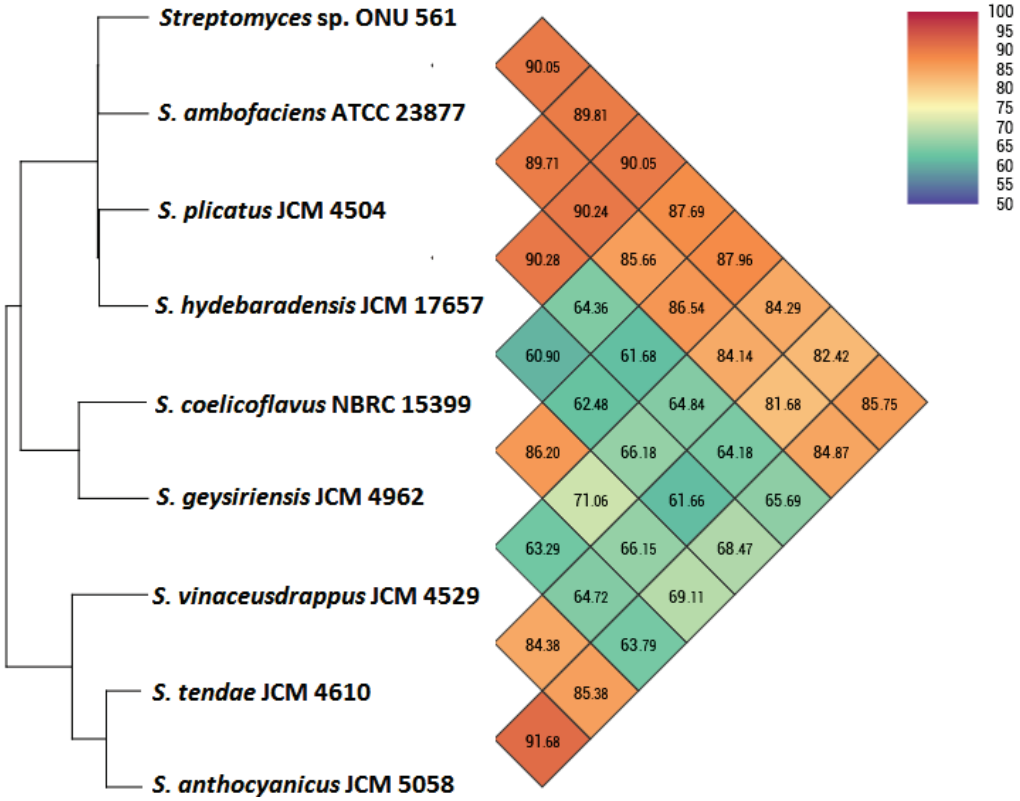

Fig. 9. Heatmap of OrthoANI values between *Streptomyces* sp. ONU 561 and other strains of *Streptomyces* genus

Table 3
Biosynthetic gene clusters in the genome of *Streptomyces* sp. ONU 561

Cluster	Type	from	to	The most similar known cluster	Similarity, %
Region 1	NRPS,T1PKS,other,T3PKS,butyrolactone	48 422	184 373	Polyoxypeptin	48
Region 2	T1PKS,NRPS-like	197 408	352 171	Candicidin	95
Region 3	RiPP-like	362 302	372 381	Unknown	–
Region 4	Lanthipeptide-class-iii	509 529	532 201	SapB	100
Region 5	Indole	599 247	620 374	5-Dimethylallylindole-3-acetonitrile	100
Region 6	Terpene*	662 476	686 527	Isorenieratene*	75
Region 7	T1PKS	827 948	921 592	Vicenistatin	75
Region 8	Lanthipeptide-class-v	975 920	1 018 165	Tetronasin	5
Region 9	NRPS-like	1 190 126	1 230 850	Meridamycin	10
Region 10	Terpene	1 367 846	1 388 217	Xiamycin A	9
Region 11	Ectoine*	1 973 937	1 984 335	Ectoine*	100
Region 12	Melanin*	2 883 159	2 891 484	Istamycin*	4
Region 13	NI-siderophore*	2 977 988	2 988 914	Desferrioxamin B/desferrioxamine E*	83
Region 14	Melanin	3 503 337	3 513 711	Bagremycin A / bagremycin B	44
Region 15	T3PKS	5 201 241	5 242 425	Naphthomycin A	9
Region 16	Terpene*	5 360 325	5 379 820	Albaflavenone*	100
Region 17	T2PKS*	5 418 930	5 489 629	Spore pigment*	66
Region 18	NI-siderophore	5 956 181	5 966 827	Unknown	–
Region 19	Other,T1PKS,butyrolactone	6 065 471	6 117 822	Tambjamine BE-18591	17
Region 20	RiPP-like	6 260 811	6 270 254	Unknown	–
Region 21	Terpene*	6 283 055	6 302 537	Geosmin*	100
Region 22	NI-siderophore	6 434 260	6 447 428	Paulomycin	9
Region 23	T2PKS	6 504 376	6 576 918	Enterocin	90
Region 24	Lanthipeptide-class-i	6 746 586	6 771 242	Unknown	–
Region 25	NRPS-like	6 780 434	6 821 030	Streptothricin	87
Region 26	Lanthipeptide-class-i	6 831 829	6 858 288	Borrelidin	5
Region 27	NRPS,transAT-PKS,NRPS-like	6 952 272	7 048 605	Inthomycin B	100
Region 28	Terpene*	7 219 162	7 245 916	Hopene*	100
Region 29	T1PKS	7 617 910	7 661 141	Rifamorpholine A/B/C/D/E	11
Region 30	RiPP-like	7 756 084	7 766 299	Informatipeptin	42
Region 31	Phenazine	7 816 991	7 837 434	Oxalomycin B	9
Region 32	NRP-metallophore, NRPS	8 012 447	8 070 902	Coelichelin	100
Region 33	T1PKS	8 142 197	8 188 473	Depsibosamycin B/C/D	9
Region 34	Butyrolactone	8 198 589	8 209 575	Griseoviridin/fijimycin A	11
Region 35	Other, NRPS, T1PKS	8 211 367	8 311 460	Polyoxypeptin	48

Note: * – clusters, common both for *Streptomyces* sp. ONU 561 and *S. ambofaciens* ATCC 23877.

Metabolomic analysis data are weakly correlated with the set of identified genomic clusters. 89 compounds were found in the exometabolome, of which 52 were identified. The detected compounds can be divided into two conventional categories: series and singletons. The series include: 2,5-diketopiperazines (DKPs), germicidins, phenazines, and fatty acid derivatives.

Discussion

A complex of phenotypic traits of the strain ONU 561 shows its similarity to the species *Streptomyces ambofaciens*. The morphology of the spore chains and mycelium corresponds to the *Spirales* type, however, the bends of the long chains resemble that characteristic of *Retinaculum-Apertum* (Fig. 3), which is a trait of *S. ambofaciens* (Shauner et al., 1999; Kämpfer, 2015).

Formula d_4 was chosen due to its tolerance to the difference in the size of the compared genomes, which is important in the context of the lack of complete sequences for many type strains of the genus *Streptomyces* (Meier-Kolthoff et al., 2013). Based on the fact that, according to the comparison of the 16S RNA gene and a set of morphological and physiological features, the closest type representative of *Streptomyces* to the studied strain is *S. ambofaciens* ATCC 23877, its genome was chosen for further comparisons. Additional factors leading to this choice were: 1) specificity of the structure of the 16S rRNA gene in *S. ambofaciens*, which makes false identification with it less possible (Stackebrandt et al., 1991); 2) the fact that the genomes of all alternative identifications, suggested by the TYGS system (*Streptomyces geysiriensis*, *S. plicatus*, and *S. vinaceus* drappus) are not completely assembled, so full-value comparisons using whole-genome indexes are not yet possible. In 2021, these species names were reduced to synonyms of *Streptomyces rochei* (Oren & Garrity, 2021). To the best of our knowledge, even partial sequence of type strain *Streptomyces rochei* ATCC 43682 is also absent in public databases. Common with the type strain of *S. ambofaciens* ATCC 23877 in *Streptomyces* sp. ONU 561 are mainly clusters responsible for vital functions. These are clusters encoding the synthesis of melanin, hopene, ectoine (an osmotic pressure-compensated solute), siderophores of various types (desferrioxamine, coelichelin, bagremycin, and two unidentified clusters), surface-active class III lanthipeptide SapB, and the terpene geosmin. The terpene antibiotic albaflavenone, together with geosmin and 2-methylisoborneol, belongs to the most widespread terpenes in actinobacteria (Yamada et al., 2015).

A characteristic difference between the genome of *Streptomyces* sp. ONU 561 and the genome of *S. ambofaciens* ATCC 23877 is the presence of a large number of clusters of specific non-ribosomal peptide synthetases and polyketide synthetases; along with those that are reliably identified (inthomycin, streptothricin, and enterocin synthases), some are possibly responsible for the production of analogs of known compounds (e.g. vicenistatin) and clusters whose products cannot be predicted at all (clusters 3, 18, 20, and 24). The presence of both types of NRPS, PKS I, and PKS II biosynthetic clusters in the genome was confirmed by PCR. Note the presence of two clusters that are similar to clusters of biosynthesis of cytotoxic lipopeptides of the azinothricin-polyoxypeptin group (Zhang et al., 2023).

An interesting finding is the discovery of two rare clusters. First, there is PKS II, which is responsible for the synthesis of enterocin, a polyketide antibiotic that was discovered in 1976 and began to be actively studied only in recent years due to its ability to undergo spontaneous non-enzymatic rearrangements (Salim et al., 2020). In the records for both previously sequenced *S. ambofaciens* strains in antiSMASH-DB, this cluster is absent. Similarly, in the compared genomes, the PKS I cluster of inthomycin is missing – an interesting oxazole-containing polyketide, which is a stable structural component of the class of a rare type of polyketides – oxazolomycin (Oleksak et al., 2020; Mu et al., 2021).

The most represented group of metabolites is DKPs, which are cyclic dipeptides, most of which in this strain are not modified or have minimal chemical modifications. DKPs are known to be prone to spontaneous

formation from proteins, especially at high temperatures, but in this case, the nature of the detected compounds makes it possible to rule this out. The predominance of nonpolar amino acids as their components and a low proportion of proline-containing cyclic peptides is obvious, which is not typical for spontaneously formed DKPs (Bojarska & Wolf, 2020; Canu et al., 2020). Only cyclo(isoleucylprolyl) (active against *Vibrio anguillarum*) and cyclo(methionylprolyl), an inhibitor of angiogenesis, have known antibiotic activity among the reliably detected DCPs.

Germicidins are signaling polyketides of the alpha-pyrone group, which, as can be assumed, are synthesized with the help of one of the two PKS III, which are found in the genome of *Streptomyces* sp. ONU 561. Germicidins inhibit the germination of own spores of streptomycetes. Also in the genome of *Streptomyces* sp. ONU 561, a cluster of phenazine biosynthesis was found, which may correspond to the phenazine compounds found in the exometabolome of this strain – saphenic acid and its methyl ester.

The non-cytotoxic compound medelamine A was discovered, which was previously known for the endophytic isolate *Streptomyces* sp. YIM 66142, together with its cytotoxic derivative medelamine C, which showed cytotoxicity against rat kidney cells (Zhang et al., 2014). In the metabolome of *Streptomyces* sp. ONU 561, in addition to the ion with m/z 214.2528, which corresponds to protonated medelamine A, an ion with m/z 272.2581, which may correspond to medelamine C, was also detected, but no adduct was detected for the latter. Another reliably identified cytotoxic compound is enteroxin. Its identification is supported by detection in the genome of *Streptomyces* sp. ONU 561 of the corresponding biosynthetic cluster (Table 3). It has a wide range of biological activities, which include antibacterial (against *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, and several more gram-positive bacteria, but has no activity against *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Candida albicans*). Strains of the last two species of bacteria were inhibited by metabolites of *Streptomyces* sp. ONU 561. This compound also has moderate cytotoxic activity (Xu et al., 2015).

Identification of all other compounds requires additional confirmation. Here we can note 2-allyloxyphenol – a rare compound with antimicrobial activity, which is already known for marine *Streptomycetes* (Arumugam et al., 2010). This compound has a spectrum and extent of antibacterial activity that can be compared with those of phenol and at the same time is devoid of cytotoxicity. However, due to the absence in the culture of *Streptomyces* sp. ONU 561 of the characteristic smell of this compound, it can be considered that its role as an antagonism agent was secondary at most.

Based on the above, it is possible to postulate the specificity of the biosynthetic potential of the strain *Streptomyces* sp. ONU 561, which corresponds to its separate phylogenetic position. In particular, its pronounced cytotoxic activity can be associated with only two identified moderately active compounds – enterocin and medelamine C, which cannot explain the cytotoxic effect that persists at high dilutions of the extracts. The powerful antibacterial and antifungal effects cannot be explained either because of any identified compound in the metabolomics spectra of the strain ONU 561. Antifungal activity could be explained by the ability of *Streptomyces* sp. ONU 561 to synthesize candicidin, a cluster of which was detected in this genome, but this compound could not be detected in extracts of its culture liquid. In the metabolome of *Streptomyces* sp. ONU 561, using ESI-MS+ were detected 9 adducts [M+H] of undefined compounds with relative molecular masses from 401 to 663 Da, and 6 adducts [M+H] of undefined compounds by ESI-MS- in the range 143–619 Da. This serves as a final argument in favor of the fact that *Streptomyces* sp. ONU 561 is capable of biosynthesis of a large number of unidentified compounds with high antibiotic activity, which is subject to further active study (Otsuka et al., 2000; Zhang et al., 2023).

The results of genotypic and phenotypic identification of an antagonistically active strain of *Streptomyces* sp. ONU 561, isolated from the shell of the mussel *Mytilus galloprovincialis*, indicates its affinity with the species *S. ambofaciens* (Kämpfer, 2015). However, the value of the indices of whole-genome comparison and the set of biosynthetic clusters indicate the difference of the studied strain of actinobacteria from all strains of the genus *Streptomyces*, the sequenced genomes of which have been published, and indicate the probability of its separation into a new species in the *Streptomyces* genus. Refutation or confirmation of the classification of *Streptomyces* sp. ONU 561 as a representative of a new species needs an additional study.

The wide spectrum and high level of antibiotic activity of *Streptomyces* sp. ONU 561 cannot be explained based on the set of compounds identified in its exometabolome. The presence of numerous biosynthetic clusters, a significant part of whose genes do not match among the known ones, the large number of peaks with high m/z values in the mass spectrum and resistance to several antibiotics, may indicate the ability of the strain *Streptomyces* sp. ONU 561 to synthesize previously unknown structural analogs of known compounds, or molecules with new structural motifs (Nodwell, 2007).

Conclusion

The actinobacterial strain *Streptomyces* sp. ONU 561, isolated from the shell of the mussel *Mytilus galloprovincialis* from the Black Sea in Odesa Bay, with a broad-spectrum antibiotic activity against opportunistic bacteria, yeast, mycelial fungi, and human tumor cells is promising for further research as a potential representative of a new species of the genus *Streptomyces*, related to *S. ambofaciens*. The results of metabolomic and genomic analysis indicate the potential ability of actinobacterium *Streptomyces* sp. ONU 561 to synthesize new previously unknown exometabolites with antimicrobial and cytotoxic activities, and they also confirm our assumption that the Black Sea microbiota have biotechnological potential.

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