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New Strains of the *Streptomyces* as Perspective Antagonists of Microbial Phytopathogens †

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Abstract: Biological protection is an important part of the strategy of modern environmentally safe protection of agricultural plants from phytopathogens. The most promising in this regard are soil microorganisms, in particular representatives of the phylum Actinomycetota. Actinomycetes are producers of biological compounds of various chemical structures with antibacterial, antifungal and antitumor effects. The authors have a collection of microorganisms (120 bacterial strains) isolated from soil and water sources. When analyzing the isolates according to the morphological features of cells and mycelium, 25 bacterial cultures were selected from the collection. Studies of the ability to antimicrobial activity in strains selected have been carried out. Five cultures were selected that effectively inhibit the growth of some phytopathogens. The bacterial strains were identified by the 16S rRNA gene, and their belonging to the *Streptomyces* genus was shown. The analysis of antibiotic resistance to 80 antibiotics showed that most antibiotics inhibited the growth of the studied strains of streptomyces. Data on the physiological characteristics of growth were obtained: the temperature optimum of growth for all strains is in the range of 24–30 °C, the optimum of NaCl concentration values for all strains is in the range of 0–3%, with the exception of the strain IPS92w, whose optimum range is wider and equal to 0–6%, the optimum of pH values is in the range of 6–8. The strains selected have biotechnological potential for the development of a biological product with antimicrobial activity against phytopathogenic microorganisms.

Keywords: *Streptomyces*; phytopathogens; biological protection; antimicrobial activity

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1. Introduction

Microbiological protection is the most important element of the strategy of modern, environmentally safe protection of crops against pests. It is based on the complex use of various physiological groups of microorganisms, as well as the use of products of their secondary metabolism with target properties. Soil microorganisms seem to be the most promising in this regard, in particular actinomycetes.

Actinomycetes are producers of biological compounds of various chemical structures with antibacterial, antifungal and antitumor effects. Currently, the attention of researchers is focused on the isolation and study of actinomycetes, which may be potential producers of new, not yet studied antibiotics. The method of metagenomic analysis shows that there

is a huge number of actinomycetes in natural sources, but the isolation of producers into pure culture is necessary to obtain new antibiotics [1,2].

Most antibiotics are isolated from actinomycetes of the widespread genus *Streptomyces*. Streptomycetes are fast-growing microorganisms, they are easily isolated from natural sources and are easy to cultivate [3–5]. Dahal et al. [6] noted that *Streptomyces* representatives are considered to be the main source of broad-spectrum antimicrobial agents.

In addition to antibiotics, streptomycetes also produce many other secondary metabolites with herbicidal [7], antifungal, antitumor and anthelmintic activity [8].

Streptomycetes are also widely known for their ability to synthesize antibiotics that allow them to inhibit plant pathogens [9–11]. Trejo-Estrada et al. [12] showed that *Streptomyces violaceusniger* YCED9 produces three antifungal compounds, including nigrecin, geldanamycin, and guanidylfungin A, which fight plant pathogens.

Antibiotic-mediated inhibition of pathogens is usually the main focus of plant disease suppression efforts. Yet, the various secondary metabolites produced by Streptomyces and other actinomycete species also has great potential to suppress fungal, bacterial, oomycete and nematode plant pathogens.

The aim of our work was to select strains belonging to the genus *Streptomyces* from the authors' collection, assess their ability to suppress the growth of test cultures, including phytopathogens, study their physiological and biochemical properties and phylogenetic position, and evaluate their prospects for further study in order to create biopreparations against phytopathogens.

2. Methods

Microscopic studies were conducted using a Nikon Eclipse Ci microscope (Nikon, Japan) with a phase contrast lens and with a ProgRes SpeedXT camera (Jenoptic, Jena, Germany).

Bacterial strains were cultured on the rich tryptone-soy medium for 24 h at 28 °C. Mineral medium without a carbon source was used to study the spectrum of utilizable substrates.

Genomic DNA was isolated from cells using the Fungal/Bacterial DNA Kit (Zymo Research, USA) according to the manufacturer's recommendation. The 16S rRNA gene was amplified by PCR using primers universal for 16S rRNA prokaryotes: 27f and 1492r [11]. The amplified DNA was purified using the Zymoclean Gel DNA Recovery Kit (ZymoResearch, Irvine, CA, USA). Sequencing of PCR DNA fragments was performed on an Applied Biosystems 3130 Genetic Analyzer automatic sequencer.

Primary phylogenetic screening of the obtained sequences was performed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast (accessed on)) and the EzBioCloud database (www.ezbiocloud.net (accessed on)). Phylogenetic tree constructed using partial 16S rRNA gene sequences by the neighbor-joining method with a bootstrap test of 1000 replicates was performed using MEGA 10.0 (https://www.megasoftware.net (accessed on)).

The temperature optimum was established by varying the temperature within the 6–45 °C range, and the effect of pH on growth was assessed within the 3.0–11.0 range. The medium pH was changed by adding 3 M NaOH or 1 M HCl. Halotolerance was determined by cultivation in the media containing 0–10% NaCl. Growth intensity was estimated by measuring the optical density at 590 nm (UV-1800 Shimadzu, Japan).

To determine the resistance to antibiotics, we used the disc diffusion method. The results were evaluated after 24 h of culture at 25 °C.

The assessment of antagonistic activity was carried out by two methods: the method of intersecting strokes and the disk-diffuse method. Studies of antimicrobial activity were performed using non-concentrated culture fluid of the studied strains. A collection of 12 strains of gram-positive and gram-negative bacteria and 9 strains of phytopathogenic fungi served as test cultures.

The antagonistic activity was evaluated by two methods: the method of intersecting strokes and the disk-diffuse method. Studies of antimicrobial activity were carried out using non-concentrated culture fluid of the studied strains. A collection of 12 strains of gram-positive and gram-negative bacteria and 9 strains of phytopathogenic fungi served as test cultures.

Antagonistic activity was assessed using two methods: the cross streak method and the disk-diffusion method. Studies of antimicrobial activity were performed using the non-concentrated culture liquid of the strains under study. A collection of 12 strains of Gram-positive and Gram-negative bacteria and 9 strains of phytopathogenic fungi served as test cultures.

3. Results and Discussion

For this work, we screened over 120 bacterial isolates from the authors' collection.

3.1. Screening of Bacterial Strains with Antimicrobial Activity

The studies were carried out in two stages: (1) the search for strains with typical actinomycete cell morphology and antimicrobial activity and (2) the study of their properties and the spectrum of antimicrobial victims. The first step was to use the cross streak method to obtain information about potential antimicrobial activity.

As a result of the first stage, five representatives of actinomycetes were selected, designated as strain Act25, strain ActVer, strain IPS92w, strain IPS92ro and strain Lzd4kr (Figure 1).

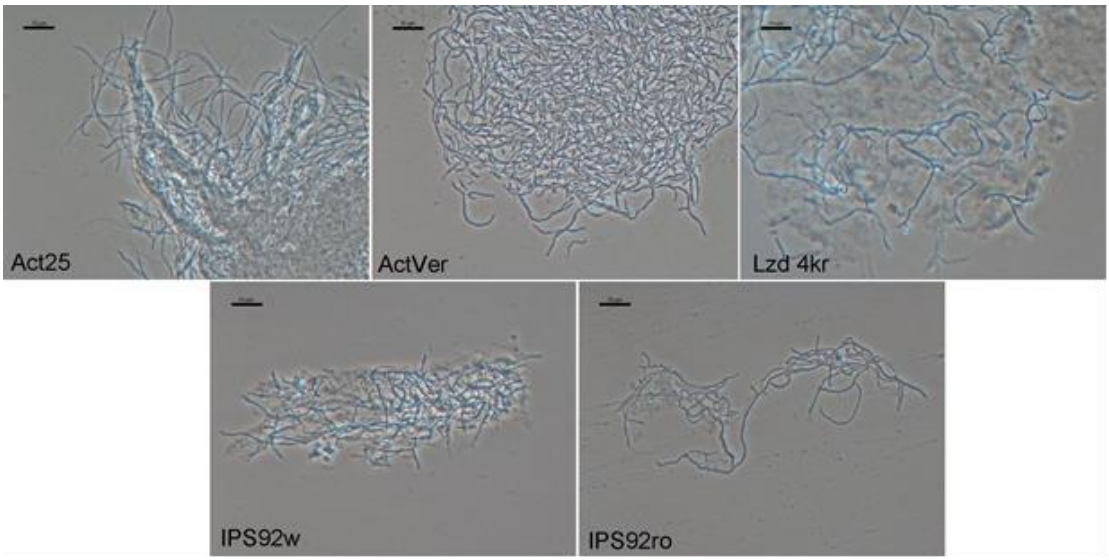

Figure 1. The new strains of actinomycetes group from different natural sources. Phase-contrast microscopy. Scale marker length 10 μm.

The sources of isolation were as follows: strain Act25—chernozem soils of Belgorod Region, strain ActVer—soil of Rostov Region polluted with heavy metals (territory of Sornoe Lake, sludge reservoir, rhizosphere of *Verónica* species), strains IPS92w and IPS92ro—silt from bottom sediments of Oka River, strain Lzd 4kr—the surface of the skin of the lizard *Lacerta agilis*.

The phylogenetic position of the selected strains was determined, analysis of the data obtained showed that the Act25 strain is closest to *Streptomyces tauricus*, the IPS92w strain to *Streptomyces anthocyanicus*, IPS92ro strain to *Streptomyces rubrogriseus*, the ActVer strain and Lzd 4kr strain belong to *Streptomyces* sp. (Figure 2).

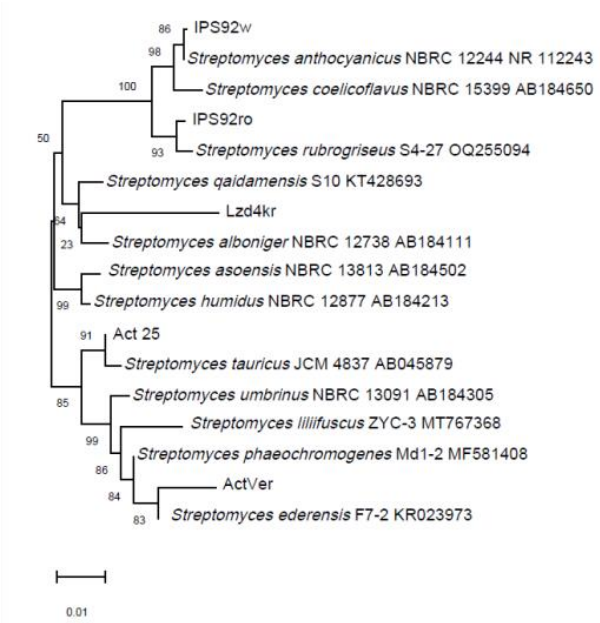

Figure 2. Phylogenetic tree of new *Streptomyces* strains. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences. Bar, 0.01 substitutions per nucleotide position.

3.2. Physiological and Biochemical Properties Study

The study of growth at different temperatures led to the conclusion that the optimum of growth for all strains lies in the 24–30 °C range, while the strains are also capable of slow growth at temperatures as low as 10 °C. All strains except ActVer showed good growth at temperatures up to 37 °C.

The IPS92w and Lzd4kr strains are moderate halophiles capable of growing at salt concentrations up to 8–9%. The ActVer strain was the most sensitive to salt concentration and can grow at salt concentrations of only up to 4%. Optimum concentration values for all strains were 0–3% NaCl, excepted of the IPS92w strain with an optimum range of 0–6%.

The strains are neutrophilic microorganisms whose optimum pH values lie in the range of 6–8. Nevertheless, for the Lzd4kr strain, the ability to grow at a slightly acidic pH of 5 was shown, and for the Lzd4kr, Act25 and IPS92ro strains, on the contrary, at slightly alkaline pH values of 9, and in the case of the IPS92w strain of 10.

The strains studied are able to grow using a number of carbohydrates and sugars as the only source of carbon and energy: sucrose, maltose, lactose, glucose and glycerol in concentrations of 2%. The liquid mineral medium containing one of these carbohydrates produced the greatest increase in biomass, the cultures released actively pigmented secondary metabolites into the medium, staining it: Act25 strain stained the medium pink, ActVer—yellow, IPS92w—blue, IPS92ro—red and Lzd4kr—light gray.

Conclusions about the nutrient requirements for the selected strains, both to increase the biomass yield and to enhance the production of secondary metabolites, were made on the basis of the studies conducted on the biochemical characteristics and substrates consumed by the new strains.

3.3. Antibiotic Resistance

All tested strains were sensitive to most of the 80 tested antibiotics: 50–88% of the antibiotics inhibited the growth of the studied streptomycetes. All studied strains are resistant to the following antibiotics: azactam (15 μg), bacitracin (10 units), oxacillin (1 and 10 μg), optochin (6 μg), saponin (750 μg), and cefixime (5 mg). The strains IPS92w and IPS92ro showed the highest resistance to the studied antibiotics, and the strain Act25 showed the highest sensitivity. The absence of antibiotic resistance in the studied strains

is a fundamental indicator when choosing the further use of strains in biotechnology as biological products or only as a producer of biotechnologically important products.

3.4. Antimicrobial Activity Assessment

The data obtained showed that the IPS92w strain has the highest activity, it was able to inhibit the growth of the Gram-negative bacteria *Aeromonas veronii*, *Pectobacterium carotovorum* B15 and *Pantoea agglomerans* ATCC 27155, Gram-positive bacteria *Bacillus subtilis* ATCC 6633, *Bacillus sphaericus* and *Kocuria rosea* VKM B-1236 and phytopathogenic fungi *Fusarium avenaceum* F-132 and *Pythium vexans* F-1193. The Lzd4Kr strain had an antimicrobial effect on the Gram-positive bacterium *Kocuria rosea* VKM B-1236 and the phytopathogenic fungi *Alternaria brassicicola* F-1864 and *Penicillium gladioli* F-2088. The ActVer strain suppressed the growth of the Gram-negative bacterium *Escherichia coli* C600 and the Gram-positive bacterium *Bacillus subtilis* ATCC 6633.

The results of the evaluation of antimicrobial activity led us to conclude that additional preparation of the culture liquid is necessary for a more effective study of its effect on the cells of test cultures.

4. Conclusions

Five strains belonging to the genus *Streptomyces* were selected from the authors' collection by direct observation, phase-contrast microscopy and 16S rRNA gene sequencing: *Streptomyces tauricus* Act25, *Streptomyces* sp. ActVer, *Streptomyces anthocyanicus* IPS92w, *Streptomyces rubrogriseus* IPS92ro, and *Streptomyces* sp. Lzd4kr.

The optimums of temperature, NaCl concentration, and pH values were determined, and the ability to utilize various carbohydrates and antibiotic resistance were evaluated.

The strains selected showed antimicrobial activity against a number of Gram-positive and Gram-negative microorganisms, including the phytopathogenic bacteria *Pectobacterium carotovorum* B15 and *Pantoea agglomerans* ATCC 27155, also suppressed the growth of phytopathogenic fungi *Fusarium avenaceum* F-132, *Pythium vexans* F-1193, *Alternaria brassicicola* F-1864 and *Penicillium gladioli* F-2088.

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Conflicts of Interest: The authors declare no conflict of interest.

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