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Metabolic Profiling of *Streptomyces* Strains from Different Types of Tatarstan Soils Using GEN III OmniLog System.

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ABSTRACT

Isolation and evaluation of the ability of various actinomycetes to produce bioactive secondary metabolites is a long-term and expensive procedure. Many species of actinomycetes have similar morphological characteristics, therefore phenotyping of these microorganisms requires methods of metabolic profiling to be applied. Analysis of metabolic pathways can help in both the taxonomic identification of a microorganism and the assessment of its ability to produce a variety of secondary metabolites. In this paper, we first carried out metabolic profiling of *Streptomyces* isolates from different types of Tatarstan soils using GEN III OmniLog system. We also performed a phylogenetic analysis of strains. We assigned strains to the genus *Streptomyces*, and identified species such as *Streptomyces fimicarius*, *Streptomyces badius*, *Streptomyces mirabilis* and *Streptomyces violaceoruber*. We have shown that the system GEN III OmniLog® II Combo Plus is a powerful tool to get an overview of the active metabolic pathways of actinomycetes and can be used for high-performance analysis. It can also provide useful information to clarify the phylogenetic position of a microorganism. We can use both the profiles of substrate utilization, growth, secondary metabolites, and anti-microbial profiles, obtained by using the GEN III OmniLog® II Combo Plus system, in the microbial drug development programs.

Keywords: *Streptomyces*, 16S rDNA, metabolic profiling, GEN III OmniLog system

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INTRODUCTION

Streptomyces are aerobic gram-positive filamentous bacteria of *Actinobacteria* genus. In terms of quantity and diversity of the identified species, *Streptomyces* represent one of the major taxonomic units being identified in the class of *Actinobacteria*. They feature a high guanine and cytosine content in their DNA, more than 70% [1,2]. *Streptomyces* occur widely in terrestrial and aquatic ecosystems, particularly in soil, and play an important role in the degradation of biomaterials and the humus formation due to their unique metabolic pathways [3].

Many species have become known as activators of plant growth, the biological remedy for plant pathogens and the producers of a wide range of biologically active secondary metabolites. They are the main source of clinically important antibiotics, most of which are too complex to be synthesized by combinatorial chemistry methods [4,5].

Global resistance dictates the demand for new antimicrobials, so actinomycetes can continue to meet this demand. For this purpose, there is an application of methods of combinatorial biosynthesis using data on known genomes of actinomycetes, and search for new strains in marine sediments and unexplored soil [6].

Most species *Streptomyces* have similar morphological properties, therefore searching for their unique features requires much time and efforts [7-9]. GEN III OmniLog® II Combo Plus system is based on multisubstrate testing and can be used for the rapid identification and characterization of actinomycetes. Each plate contains various sources of nitrogen and carbon, each microorganism in turn has a certain substrate consumption profile, depending on its metabolic pathways activity [10].

In this paper we described the metabolic profile of different species of *Streptomyces* using GEN III OmniLog® II Combo Plus system, and performed phylogenetic analysis of these strains.

MATERIALS AND METHODS

Microbial strains isolation and culture medium

We obtained five isolates from different types of soil of the Republic of Tatarstan (RT): sod-podzolic soil of Elabuga forestry (S.749), black earth soil of Cheremshanskii district of RT (S.549), grey forest soil of Baltasinskii district of RT (S.130), buried soil of leached chernozem of Kama-Ustinskii district of RT (S.909) and grey forest soil of Baltasinskii district (S.187).

For isolation and differential count of actinomycetes we used method of seeding from the diluted soil suspensions in solid nutrient media such as Gause and glycerol nitrate agar [2, 11]. During seeding from soil we used air-dried soil samples. We added antibiotic Nystatin into the medium, which limits the growth of microscopic fungi, for a more complete identification of actinomycetes. We incubated inoculations in the temperature-regulated ovens at 28°C for 28 days. The isolates were assigned to the genus *Streptomyces* based on the analysis of their morphological characteristics [12-17].

Nucleic acid extraction and 16S rDNA amplification

DNA extraction was performed by the method of enzymatic lysis with further phenol-chloroform extraction [18].

The amplification of 16S rDNA was performed with universal bacterial primers [19]:

fD1 – 5'-AGAGTTTGATCCTGGCTCAG-3'
rP2 – 5'-TACGGCTACCTGTTACGAC-TT-3'.

Polymerase chain reaction (PCR) of DNA of pure cultures was performed in a final volume of 20 µl under the following conditions:

94°C – 5 min;
 94°C – 30 sec, 57°C – 30 sec, 72°C – 90 sec (30 cycles);
 72°C – 5 min;

The size and purity of the product was checked by electrophoresis in 1.5% agarose gel.

The sequences obtained from sequence analysis were compared with sequences of an international databank NCBI by using BLAST software package (<http://www.ncbi.nlm.nih.gov/blast>).

Phylogenetic analysis

Nucleotide sequences were aligned in clustal X2 (Version 2.1.0.0). The aligned sequences were used to construct a phylogenetic tree using MEGA 6.0 program, the cluster method Neighbor Joining, and the algorithm Kimura 2 parameters. Statistical significance of branching was evaluated using a bootstrap-analysis of 500 alternative trees. The resulting nucleotide sequences of 16S rDNA gene fragments of pure cultures were deposited with the GenBank database under the numbers: KF251104 - KF251107.

Substrates utilization patterns

We performed metabolic profiling using GEN III OmniLog® II Combo Plus system (Biolog, Inc., Hayward, USA) on GEN III microplates. These plates allow us to perform 94 phenotypic test simultaneously: 71 tests for utilizing different sources of carbon and 23 test for chemosensitivity (pH, antibiotic sensitivity, osmotolerance). The plates were inoculated with *Streptomyces* strains in triplicate according to the manufacturer's protocol, and incubated at 28°C [3]. Briefly, a pure culture of the microorganism grew on BUG agar (Biolog Universal Growth Agar), and 100 µl of microbial suspension (98 ± 3% transparency at 590 nm) was added to each well of the plate. Every 24 hours we incubated the plates at 28°C and determined the optical density (OD) at 590 nm by reduction of tetrazolium violet that responds to the oxidation of substrates. Table 2 provides a list of the substrates used. The plate was evaluated as positive if at least two wells had OD₅₉₀ above 0.4 [10]. Index of similarity (SIM), which correlates with the genetic distance (DIS), was calculated for each strain and 10 the most close to it species in the database. We compared metabolic profiles of the strains with each other and with typical species from the database using cluster analysis program Biolog UPGMA.

RESULTS AND DISCUSSION

Morphological observations during 28 days showed that the strains have the properties typical of the genus *Streptomyces*. Strains form a well-developed aerial mycelium, which does not fall into fragments during subsequent development cycle. Sporangia consist of straight or twisted spiral chains of resting spores. Spores are formed at the ends of hyphae.

Table 1: Cultural properties of the strains *Streptomyces*

Strains	Culture media	Growth (28°C)	Aerial mycelium	Substrate mycelium
S.187	MA*	+	lilac grey	blue grayish
	GNA*	+	lilac grey	blue grayish
	OA*	+	lilac grey	blue grayish
S.130	MA	+	pinkish grey	dark blue
	GNA	+	bluish grey	dark blue
	OA	+	pinkish grey	dark blue
S.549	MA	+	white, scanty	colorless to pale yellowish brown
	GNA	+	whitish	yellowish white
	OA	+	white, scanty	yellowish brown
S.749	MA	+	brownish grey	greyish brown
	GNA	+	grey	dark brown
	OA	+	brownish grey	greyish brown
S.909	MA	+	cream to green-yellow-brown	yellow-brown
	GNA	+	cream to green-yellow-brown	yellow-brown
	OA	+	cream to green-yellow-brown	yellow-brown

*MA - mineral agar, GNA - glycerol nitrate agar, OA - oat agar.

As can be seen from Table 1, the investigated strains grow well on mineral, oat and glycerol-nitrate agar at 28^oC. Color of aerial and substrate mycelium varies depending on the culture medium. Strains produce no pigments, diffusing into the environment. Strains grow well at 1 and 8% NaCl concentration and at pH 6 (Table 2). Some physiological properties of the strains *Streptomyces* are presented in Table 2.

16S rDNA analysis

According to the literature, PCR amplification of 16S rDNA with fD1 and rP2 primers creates a fragment of about 1.5kb [19]. We determined the structure of the nucleotide sequences of 16S rDNA (about 1300 base pairs) in all strains, which had a percentage of similarity (96-100%) to DNA of bacteria from different natural ecosystems. We have found that strains belong to the phylum *Actinobacteria*, family *Streptomycetaceae*, genus *Streptomyces*. To construct a phylogenetic tree we chose sequences with the strongest similarity (Figure 1).

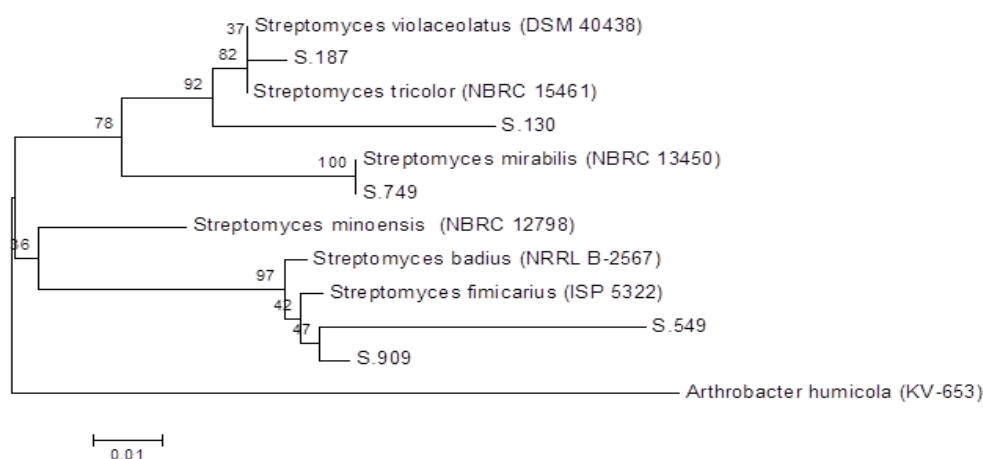


Figure 1: Phylogenetic position of the strains of genus *Streptomyces* based on the analysis of 16S rDNA nucleotide sequences; grouping by neighbor-joining method. Scale - one replacement for every 100 nucleotides. We used the nucleotide sequence of the gene 16S rDNA *Arthrobacter humicola* (KV-653) as an external group.

The nucleotide sequence of a strain S.749 had 100% similarity with the sequence of a strain *Streptomyces mirabilis* NBRC 13450. The sequence of a strain S.549 had 97% similarity with the strain *Streptomyces fimicarius* ISP 5322. The sequence of a strain S.909 had 99% similarity with the strain *Streptomyces badius* NRRL B-2567. The nucleotide sequence of a strain S.187 had 99% similarity with the sequence of a strain *Streptomyces violaceoruber*. The sequence of a strain S.130 had 96% similarity with the strain *Streptomyces violaceolatus* DSM 40438. According to morphological, physiological and biochemical features, we assigned the strains to the genus *Streptomyces*, and conducted the phylogenetic analysis that confirmed the belonging of the strains to this genus.

The analysis of the nucleotide sequences of 16S rDNA and the data of physiological and biochemical identification also allowed us to assign strains S.749, S.909, S.549 and S.187 to the species *Streptomyces mirabilis*, *Streptomyces badius*, *Streptomyces fimicarius* и *Streptomyces violaceoruber*, respectively. Strain S.130 cannot be assigned to one of the known species based on the analysis of 16S rDNA, and its further identification requires analysing other conserved regions.

Metabolic profiling by GEN III OmniLog system

We conducted biochemical analysis of 5 closely related strains of actinomycetes using GEN III OmniLog[®] II Combo Plus system, which is a simple screening test and allows you to quickly get the metabolic profile of strains. Figure 2 shows a comparative map of the metabolic profiles of strains *Streptomyces*, cultured in GEN III plates. The transition from blue to red indicates an increase in the intensity of growth and substrate consumption. As can be seen from the map, each species has a strictly specific profile of substrate consumption. Digital values of the map are shown in Table 2.

Table 2: Physiological and biochemical properties of the strains *Streptomyces*

Substratum	S. 187	S. 130	S. 549	S. 749	S. 909
NaCl, 1%	212 +	205 +	165 ±	269 +	215 +
NaCl, 4%	134 ±	56 –	71 –	120 ±	104 ±
NaCl, 8%	228 +	205 +	214 +	262 +	237 +
pH 5	120±	60–	50–	253+	68–
pH 6	185±	161±	151±	268+	173±
Polysaccharides					
Dextran	219+	215+	213+	90–	215+
Pectin	211+	211+	148 ±	213+	200+
D-maltose	208+	129±	100±	155±	237+
D-trehalose	243+	242+	255+	142±	194±
D-cellobiose	175±	247+	221+	147±	228+
Gentiobiose	235+	238+	254+	214+	243+
Saccharose	221+	226+	231+	183±	179±
D-turanose	233+	223+	229+	216+	225+
Stachyose	190±	228+	206+	108±	187±
D-raffinose	189±	223+	225+	177±	175±
α-D-lactose	210+	210+	202+	83–	178±
D-melibiose	191 ±	226+	211+	103±	221+
Monosaccharides					
α-D-glucose	209+	232+	231+	174±	252+
D-mannose	182 ±	209+	228+	161±	217+
D-fructose	189 ±	206+	194±	166±	225+
D-galactose	154 ±	192±	212±	153±	227+
D-fucose	175 ±	202+	173±	93–	198±
L-fucose	154 ±	163±	124±	95–	185±
L-rhamnose	172 ±	194±	192±	114±	203+
Glycosides					
β-methyl-D-glycoside	158 ±	201+	190±	167±	177±
D-salicin	194 ±	206+	198±	195±	169±
N-acetyl-D-glucosamine	199 ±	217+	223+	132±	237+
N-acetyl-β-D-mannosamine	182 ±	187±	194±	95–	226+
N-acetyl-D-galactosamine	210+	223+	196±	90–	167±
Amino acids					
D-serine	64–	46–	50–	200+	152±
L-alanine	168 ±	203+	192±	174±	196±
L-arginine	90–	204+	188±	136±	175±
L-histidine	231+	222+	214+	175±	181±
L-serine	169 ±	200+	199±	207+	173±
L-aspartate	237+	218+	200+	164±	195±
Alcohols					
D-sorbitol	187 ±	198±	224+	126±	188±
D-mannitol	177 ±	180±	166±	126±	173±
D-arabitol	181 ±	163±	159±	129±	166±
Glycerol	135 ±	193 ±	137 ±	142 ±	162 ±
Myoinositol	180 ±	213+	182 ±	136 ±	178 ±

Figure 3 shows a dendrogram reflecting the relationship between the metabolic profiles of the strains studied after 36 hours of incubation in GEN III microplates. Strain S.749, identified as *S. mirabilis*, differs greatly from the rest in the consumption of substrates and forms a separate cluster. It shows a low capacity for the consumption of some carbon sources, in particular polysaccharides, particularly dextran, α-D-lactose and D-melibiose, monosaccharides, particularly L-rhamnose, D- and L-fucose, glycosides, especially N-acetyl-β-D-mannosamine and N-acetyl-D-galactosamine. At the same time, the strain S.749 actively consumes pectin, gentiobiose, turanose, D- and L-serine, and practically does not use compounds from alcohols, monosaccharides and glycosides as a source of carbon.

The second cluster includes the remaining 4 strains and is divided into 2 subclusters. The first subcluster includes strain S.909, belonging to the species *S. badius*, and strain S.187 allocated to the species *Streptomyces violaceoruber*. The strains are similar in the decomposition intensity of polysaccharides, such as dextran, pectin, gentiobiose, D-turanose, stachyose, D-raffinose, consumption of certain glycosides, amino acids, and

almost all alcohols in the plate. Thus, we may assume that the strain S.187 is similar in its biochemical characteristics to the species *S. badius*.

Figure 2: Comparative map of the metabolic profiles of species *Streptomyces*, cultured in GEN III plates. The transition from dark blue to red indicates an increase in the intensity of growth and substrate consumption.

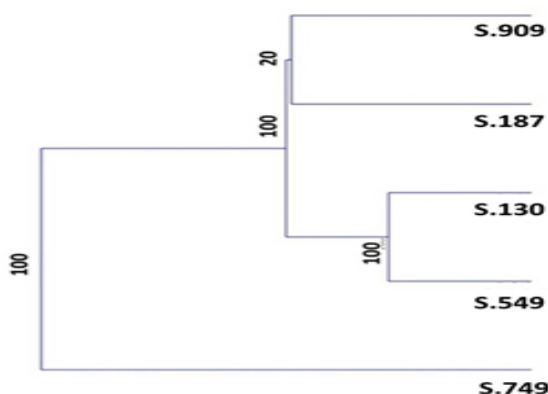
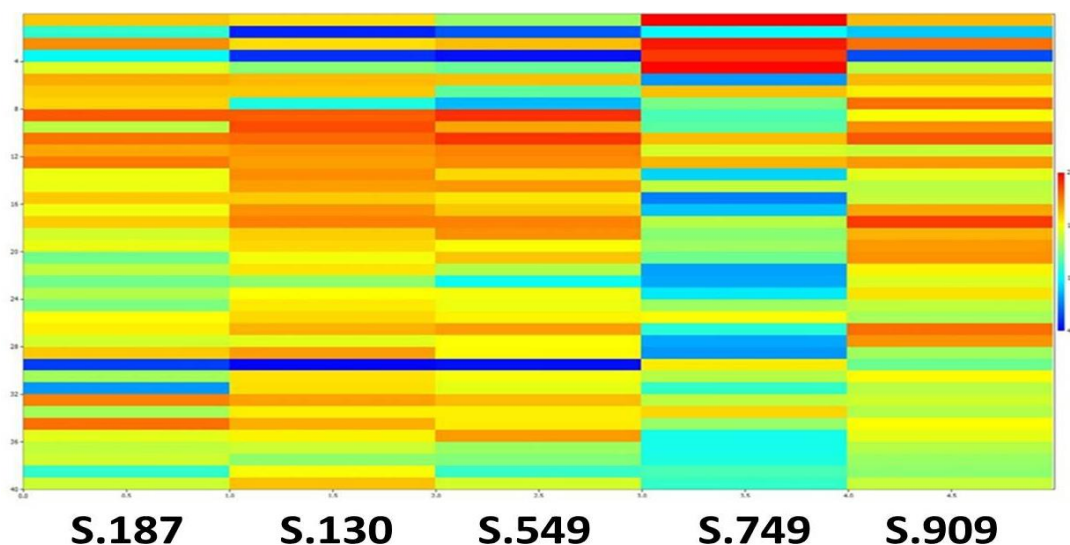


Figure 3: Dendrogram showing the relationship between the metabolic profiles of the strains studied after 36 hours of incubation. The dendrogram was built using cluster analysis program Biolog UPGMA.

The second subcluster includes strains S.130 and S.549, identified as *S. violaceolatus* and *S. fimicarius*, respectively. These strains utilize well dextran, D-trehalose, D-cellobiose, gentiobiose, saccharose, D-turanose, stachyose, D-raffinose, α -D-lactose, D-melibiose, α -D-glucose, D-mannose, D-fructose, D-galactose, N-acetyl-D-glucosamine, L-histidine, L-aspartate, and D-sorbitol.

Based on findings of phylogenetic analysis, we assigned the strains S.549 and S.909 to the same clade, while they differ greatly from each other in their biochemical properties. They especially differ in the consumption of certain polysaccharides and amino acids, and their osmosensitivity.

In this paper, we first performed biochemical characteristics of five strains of *Streptomyces* isolated from different types of Tatarstan soils and show that GEN III OmniLog® II Combo Plus system is a powerful tool to get an overview of the active metabolic pathways of actinomycetes and can be used for high-performance analysis [3, 10]. It can also provide useful information to clarify the phylogenetic position of a microorganism. We can use both the profiles of substrate utilization, growth, secondary metabolites, and anti-microbial profiles, obtained by using the GEN III OmniLog® II Combo Plus system, in the microbial drug development programs. It can be used in biotechnologies to identify feeding preferences of microorganisms and optimize their cultivating environment.

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REFERENCES

- [1] Raja A., Prabakaran P., 2011. Actinomycetes and drug – An overview. *American Journal of Drug Discovery and Development*. Vol.1. - P. 75 – 84.
- [2] G.M. Zenova. *Soil actinomycetes of rare genus*. M.: Publishing house of Moscow University. 2000. p. 81
- [3] Garland J.L, Mills A.L., 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Appl Environ Microbiol*; 57:2351–9.
- [4] Olano C., Méndez C and José A. Salas, 2009. Antitumor Compounds from Marine Actinomycetes. *Mar. Drugs*. – Vol. 7 - P. 210-248.
- [5] Kavita Tiwari, Rajinder K. Gupta, 2012. Rare Actinomycetes: Potential Storehouse for Novel Antibiotics. *Guru Gobind Singh Indraprastha University, Delhi, India Crit Rev Biotechnol*, 32(2):108-32.
- [6] Takahashi Y, Omura S., 2003 Jun. Isolation of new actinomycete strains for the screening of new bioactive compounds. *J Gen Appl Microbiol.*; 49(3):141-54.
- [7] Kim S.B., Goodfellow M., 2002. *Streptomyces thermospinisporus* sp. nov., a moderate thermophilic carboxydrotrophic streptomyce isolated from soil .*Int. J. Syst. Evol. Microbiol*. V. 52. № 4. P. 1225–1228.
- [8] Narayana KJP, Vijayalakshmi M. 2009. Chitinase production by *Streptomyces* sp. ANU 6277. *Brazilian Journal of Microbiology*. 4:P.725-733.
- [9] Ventura M., Canchaya C., Tauch A., Chandra G., Fitzgerald GF., Chater KF., 2007. Genomics of Actinobacteria: Tracing the evolutionary history of an ancient phylum. *Microbiol Mol Biol Rev*. V.3. P. 495.
- [10] Verschuere L., Fievez V., Van Vooren L., Verstraete W., 1997. The contribution of individual populations to the Biolog pattern of model microbial communities. *FEMS Microbiol. Ecol*. 24: 353–362.
- [11] *Methods of soil microbiology and biochemistry* / ed. D.G. Zviagintsev. M.: Publishing house of Moscow University, 1991. p. 303
- [12] S.T. Williams, V. Sharpe, J.A. Holt, 1989. *Bergey's Manual of Systematic Bacteriology*. Baltimore ets. Williams and Wilkins V. 4. 2648 p.
- [13] Stackebrandt E., Rainey F.A., Ward-Rainey N.L., 1997. Proposal for a new hieraric classification system, *Actinobacteria classic nov*. *Int. J. Syst. Bacteriol*. V. 47. № 2. P. 479–491.
- [14] R. Locci, *Streptomyces* and related genera, in: S.T. Williams, M.E. Sharpe, J.G. Holt (Eds.), *Bergey's Manual of Systematic Bacteriology*, Williams & Wilkins, Baltimore, 1989, pp. 2451–2493.
- [15] R.E. Buchanan, N.E. Gibbons (Eds.), 1974. *Bergey's Manual of Determinative Bacteriology*. Eighth ed., Williams & Wilkins, Baltimore, pp. 747–829.
- [16] Kim B., Sahin N., Minnikin D.E., Zakrzewska-Czerwinska J., Mordarski M., Goodfellow M., 1999. Classification of thermophilic streptomyces, including the description of *Streptomyces thermoalcalitolerans* sp. *Int. J. Syst. Bacteriol*. V. 49. P. 7–17.
- [17] Hadi Maleki, Alireza Dehnad, Shahram Hanifian, Sajjad Khani. 2013. Isolation and Molecular Identification of *Streptomyces* spp. With Antibacterial Activity from Northwest of Iran. *BiolImpacts*, 3(3), P.129-134.
- [18] Wen-Jun Li, Yi Jiang, Reiner M. Kroppenstedt, Li-Hua Xu, Cheng-Lin Jiang., 2003. *Nocardia alba* sp. nov., a novel actinomycete strain isolated from soil in China. *System. Appl. Microbiol*. V. 27. P. 308-312.
- [19] Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*. V. 173. P. 697-703.