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Acidotolerant Actinomycetes of the Genus *Micromonospora* Are Producers of Antibiotic Compounds †

Olga N. Sineva *, Olga P. Bychkova, Larisa P. Terekhova

Gause Institute of New Antibiotics, ul. Bolshaya Pirogovskaya, 11, Moscow 119021, Moscow, Russia; olrika@mail.ru (O.P.B.); terekhova@lst.ru (L.P.T.)

* Correspondence: olga.sineva@yandex.ru

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Abstract: Actinomycetes are the most important group of microorganisms as producers of various valuable secondary metabolites, such as antibiotics, antifungal, antitumor agents [1]. There are potentially many actinomycetes of rare genera that can be producers of new antibiotics, but special conditions are required for their isolation. Different selective methods have been developed to facilitate the frequency of isolation of rare Actinobacteria from untapped ecological niches, leading to the identification of novel strains with new natural antibiotic compounds [2,3]. This study aims to establish effective methods for selective isolation of rare acidophilic actinomycetes - producers of new antibiotics. The selective isolation approach established here is robust for isolating various acidophilic actinomycetes. At list 82 strains were isolated from fresh soil samples using an acid supplement (lemon juice at concentrations of 30% and 50%). A taxonomic analysis based on the morphological, physiological, biochemical, and molecular investigation of selected strains revealed that 11 isolates were affiliated to Micromonospora genus. All strains were resistant to low pH = 2-2,5, and 6 cultures could grow on liquid media for 7 days at pH = 2. According to the literature, actinomycetes of the genus Micromonospora are sensitive to low pH, so in our study, acid-tolerant representatives of this genus were identified. An assessment of antimicrobial proprieties of the nine strains showed moderate to strong antimicrobial activities against fungi and bacteria: Staphylococcus aureus INA 00985, S. aureus INA 00761 (MRSA), Micrococcus luteus ATCC 9341, Bacillus subtilis ATCC 6633, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Saccharomyces cerevisiae ИНА 01042, Candida albicans ATCC 14053, Aspergillus niger ATCC 16404. Isolated acidotolerant Micromonospora strains can be considered as potential producers of new antibiotics.

Keywords: : actinomycetes, antibiotics, antibiotic activity, Micromonospora

1. Introduction

Micromonospora strains were isolated in general from the soil. Also, they have been found in plant tissues, in water samples, sediments, and aquatic organisms such as ascidians, sponges, coral [4]. Members of this genus are Gram-positive, sensitive to pH below 5.0 and have optimal temperatures between 20 and 40 °C, aerobic Actinobacteria that can form single spore on substrate hyphae. Colonies of genus Micromonospora have carotenoid pigments: yellow, orange, red, purple, brown, or black [5]. Representatives of genus Micromonospora are produsers of antibiotics as gentamicin, sisomicin, rosamicin, rapamycin, fortimicin and etc [4].

2. Methods

The sample of sod-podzolic soil were air-dried at room temperature for 2 weeks, 100 mg of soil sample was added 9,9 ml sterile water, this suspension was pretreatment lemon juice at concentrations of 30% and 50% and incubated for 30 minutes at room temperature. The sample dilution was vigorously shaken for 5 min on a vortex mixer. Aliquots of soil suspensions were sown on media Gause №2 modified (tripton - 3,0 g/l, peptone - 5,0 g/l, glucose - 10,0 g/l, NaCl - 5,0 g/l, agar – 20,0 g/l, distillated water) and cultivated of the thermostat at temperature 280C for 14 days. Experiments with lemon juice at concentrations of 30% and 50% were conducted on liquid media Gause №2, time of cultivation strain of Micromonospora was 7 days. Lemon juice was sterilized using bacterial filters d=0,22 mkm. Antibiotic activity was determined against test-organisms: Staphylococcus aureus INA 00985, S. aureus INA 00761 (MRSA), Micrococcus luteus ATCC 9341, Bacillus subtilis ATCC 6633, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Saccharomyces cerevisiae ИНА 01042, Candida albicans ATCC 14053, Aspergillus niger ATCC 16404. The cultivation was carried out on liquid media:1) 11654 (soy flour – 20,0 g/l, glucose - 30,0 g/l, NaCl – 3,0 g/l, CaCO3 – 3,0 g/l, distilled water); 2) A4: soy flour – 10,0 g/l, glucose - 10,0 g/l, NaCl – 5,0 g/l, CaCO3 – 2,5 g/l, distilled water); 3) 6613 (starch – 20,0 g/l, KNO3 – 4,0 g/l, NaCl – 5,0 g/l, CaCO3 - 5,0 g/l, distilled water);4) special sucrose medium (SSM): sucrose – 20,0 g/l, soy flour– 10,0 g/l, KNO3 – 2,0 g/l , NaCl – 3 g/l, CaCO3 – 3,0 g/l, distilled water); 5) 2663: glycerol – 30,0 g/l, soy flour – 15,0 g/l, NaCl – 3,0 g/l, CaCO3 – 3,0 g/l, distilled water). Light microscopy OLYMPUS BX-41 observed the morphology of hyphae and spores. Biomass for the chemotaxonomic and molecular systematic studies was obtained after cultivating strains on liquid media Gause №2, cultivated for 7 days at temperature 280C. The isomers of cell-wall diaminopimelic acid and reducing sugars were determined using thin-layer chromatography (TLC) [6, 7]. Genomic DNA was extracted using a Power Soil DNA Isolation Kit (MO BIO, USA). The 16S rRNA gene of was amplified using the universal primers 27 F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492 R (5′-TACGGYTACCTTGTTACGACTT-3′) (Sintol, Russia). The «Thermo Fisher Scientific» (USA) was used for PCR amplification. The sequencing reaction was performed on an automatic amplifier 2720 Thermal Cycler (Applied Biosystems, USA). Sequencing was performed on an automatic capillary sequencer 3500 Genetic Analyzer (Applied Biosystems, USA) using reagents BigDye Terminator v3 Cycle Sequencing Kit (Applied Biosystems, USA), under the manufacturer's recommendations. The spectrum of antimicrobial activity was determined by agar well diffusion method.

3. Results

A total of 82 actinomycete strains were isolated in the experiments; 11 strains were determined based on the morphological, physiological, biochemical, and molecular features as representatives of the genus Micromonospora. The inducing effect of lemon juice on the isolation of actinomycetes of the genus Micromonospora was shown. Only one strain was isolated in control, three strains and eight strains were isolated when lemon juice was added to the medium at concentrations of 30% and 50%, respectively (Table 1).

Table 1. Number of isolated actinomycetes of the genus *Micromonospora*.

Lemon Juice Concentration		
Control (without Lemon Juice)	30%	50%
Micromonospora sp. 11-L	*Micromonospora* sp. 57-L *Micromonospora* sp. 58-L	*Micromonospora* sp. 65-L *Micromonospora* sp. 71-L *Micromonospora* sp. 77-L *Micromonospora* sp. 81-L *Micromonospora* sp. 84-L *Micromonospora* sp. 93-L *Micromonospora* sp. 94-L *Micromonospora* sp. 99-L

Six strains (Micromonospora sp. 11-L, Micromonospora sp. 57-L, Micromonospora sp. 58-L, Micromonospora sp. 71-L, Micromonospora sp. 84-L, Micromonospora sp. 93-L) was able to grow for 7 days in liquid medium Gause №2 with lemon juice and was resistant to the influence of pH=2-2,5. Two strains (Micromonospora sp. 94-L, Micromonospora sp. 99-L) was able to grow for 7 days on medium with lemon juice on concentration 30% and had no abundant growth in medium with lemon juice on concentration 50%. Also two strains (Micromonospora sp. 77-L, Micromonospora sp. 81-L) had poor growth in the medium with lemon juice on concentration 30% and had no growth with lemon juice on concentration 50%. One strain (Micromonospora sp. 65-L) did not grow on the medium with lemon juice.

We have tested the isolated strains for antibiotic activity against gram-positive, gram-negative microorganisms and fungal. The results showed that the strains Micromonospora sp. 11-L, Micromonospora sp. 77-L, Micromonospora sp. 93-L, Micromonospora sp. 94-L, Micromonospora sp. 99-L had a high antibiotic activity against gram-positive and gram-negative microorganisms and fungal. The strains Micromonospora sp. 58-L, Micromonospora sp. 71-L, Micromonospora sp. 81-L, Micromonospora sp. 84-L had moderate antibiotic activity and two strains had antibiotic activity against only P. aeruginosa ATCC 27853. The best results are shown when cultivated on different medium (Table. 2,3).

Table 2. Antibiotic activity of strains of the genus *Micromonospora* against gram-positive and gram-negative microorganisms (*n/a – no activity).

Number	Media	Areas of Suppression of Test - Organisms, mm					
		***M. luteus* ATCC 9341**	***S. aureus* INA 00985**	***S. aureus* INA 00761 MRSA**	***B. subtilis* ATCC 6633**	***E. coli* ATCC 25922**	***P. aeruginosa* ATCC 27853**
11-L	11654	21,5±0,3	21,3±0,3	n/a	n/a	22,5±0,4	20,3±0,29
77-L	A4	18,1±0,2	23,8±0,4	n/a	14,1±0,35	n/a	25,4±0,4
93-L	2663	18,2±0,26	23,9±0,35	n/a	12,0±0,3	11,9±0,3	14,3±0,4
	A4	16,1±0,2	18,1±0,35	14,9±0,45	11,6±0,49	n/a	12±0,41
94-L	SSM	30,1±2	26,5±0,3	n/a	14,6±0,32	n/a	21,2±0,36
	A4	23,8±0,26	23,7±0,3	n/a	16,2±0,26	21,2±0,3	21.6±0,43
99-L	11654	30,2±0,36	14,1±0,2	n/a	n/a	16±0,3	11,1±0,35
57-L	A4	n/a	n/a	n/a	n/a	n/a	22±0,2
58-L	11654	n/a	n/a	n/a	n/a	12,1±0,2	n/a
65-L	A4	n/a	n/a	n/a	n/a	n/a	13
71-L	A4	n/a	21,7±0,3	n/a	n/a	n/a	15,1±0,46
81-L	A4	n/a	n/a	n/a	n/a	n/a	21,8±0,4
84-L	A4	10,1±0,19	n/a	n/a	11,2±0,39	n/a	25,4±0,4

Table 3. Antifungal activity of strains of the genus *Micromonospora.*

Number	Medium	Areas of Suppression of Test - Organisms, mm		
		***Sac.cerevisiae* INA 01042**	***C. albicans* ATCC 14053**	***A. niger* ATCC 16404**
11-L	11654	25,2±0,26	11,1±0,35	17,2±0,26
99-L	2663	20,2±0,39	n/a	14,5±0,32
58-L	6613	n/a	n/a	16,3±0.3
71-L	2663	n/a	n/a	26±0,43
77-L	6613	n/a	n/a	13,9±0,35
	A4	15,2±0,4	n/a	n/a
81-L	A4	n/a	n/a	10,2±0,26
84-L	A4	10,3±0,3	n/a	n/a
93-L	2663	21,8±0,26	n/a	n/a
94-L	6613	14±0,29	n/a	10,5±0,32
	A4	25,9±0,45	n/a	n/a
99-L	11654	n/a	n/a	20,4±0,32

SSM	25,3±0,3	n/a	16,1±0,35
6613	n/a	21,2±0,26	n/a

The study showed that lemon juice at concentrations of 30% and 50% was stimulated growth Micromonospora isolates. The addition of lemon juice to medium in these concentrations can be recommended for the isolation of acidotolerant strains from Micromonospora. These strains showed antibiotic activity against test - organisms, so they can be potential producers of new antibiotics.

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