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SCREENING OF ANTIMICROBIAL ACTIVITY AND GENES CODING POLYKETIDE SYNTHETASE AND NONRIBOSOMAL PEPTIDE SYNTHETASE OF ACTINOMYCETE ISOLATES

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ABSTRACT

The aim of this study was to observe antimicrobial activity using agar plate diffusion method and screening genes coding polyketide synthetase (PKS-I) and nonribosomal peptide synthetase (NRPS) from actinomycetes. A total of 105 actinomycete strains were isolated from arable soil. Antimicrobial activity was demonstrated at 54 strains against at least 1 of total 12 indicator organisms. Antifungal properties were recorded more often than antibacterial properties. The presence of PKS-I and NRPS genes were founded at 61 of total 105 strains. The number of strains with mentioned biosynthetic enzyme gene fragments matching the anticipated length were 19 (18%) and 50 (47%) respectively. Overall, five actinomycete strains carried all the biosynthetical genes, yet no antimicrobial activity was found against any of tested pathogens. On the other hand, twenty-one strains showed antimicrobial activity even though we were not able to amplify any of the PKS or NRPS genes from them. Combination of the two methods showed broad-spectrum antimicrobial activity of actinomycetes isolated from arable soil, which indicate that actinomycetes are valuable reservoirs of novel bioactive compounds.

Keywords: Actinomycetes, antimicrobial activity, PKS-I gene, NRPS gene

INTRODUCTION

Actinomycetes are Gram-positive bacteria, having high G+C (>55 %) content in their DNA **(Pandey *et al.*, 2004)**, most common producers of secondary metabolites **(Kekuda *et al.*, 2010; Nanjwade, *et al.*, 2010)**, that are one of the largest communities of microbial population present in the soil **(Basilio *et al.*, 2003; Ceylan *et al.*, 2008; Oskay *et al.*, 2004)**. In the genomes of actinomycetes exist widely polyketide synthases (PKS-I) and nonribosomal peptide synthetases (NRPS) that are biosynthetic systems involved in the synthesis of a large number of important biologically active compounds **(Ayuso-Sacido and Genilloud, 2005; Zhao *et al.*, 2011)**. These structurally diverse metabolites include among others antibiotics, antifungals, antitumor agents, antihelmintics and immunosuppressive agents **(Ayuso-Sacido and Genilloud, 2005; Gao and Huang, 2009)**. Antibiotics are the most important products of actinomycetes. One of the first antibiotics used is streptomycin produced by *Streptomycin griseus* **(Nanjwade *et al.*, 2010).** Nearly 80 % of known antibiotics are produced by *Streptomyces* **(Kieser *et al.*, 2000; Pandey *et al.*, 2004; Şahin and Uğur, 2003)**. Screening of actinomycete cultures has for decades yielded novel industrially important products and pharmaceuticals. Only a small fraction of microbes have been described. It can be expected that the majority of pharmaceutically and industrially important molecules of microbial origin remain to be discovered **(Oskay *et al.*, 2004; Şahin and Uğur, 2003; Wawrik *et al.*, 2005)**. Many pathogenic bacteria have become resistant to antibiotics in common use. This resistance is serious subject of study and it is necessary to search new antibiotics **(Mellouli *et al.*, 2003; Velho-Pereira and Kamat, 2011)**. Research studies are nowadays oriented towards looking for new *Streptomyces* species from different soil and water samples **(Mellouli *et al.*, 2003)**.
The objective of our study was to investigate the antimicrobial activity of actinomycetes isolated from arable soil using agar plate diffusion method and screening genes coding polyketidesynthetase (PKS-I) and nonribosomal peptide synthetase (NRPS).

MATERIAL AND METHODS

Soil samples were collected from the location of Research and experimental base in Dolná Malanta (48°19´S, 18°07´V), Slovak Agricultural University in Nitra. The location of the research base has the character of a plane with a slight slope to the south. Altitute reaches 175-180 m. It is located in a very hot area with average daytime temperatures above 10 °C. Maize was cultivated in this plot. The samplings were realized by random choice at the beginning of June 2012 from four different parts of this area to the deep 0, 20 m. The soil samples belonged to the soil type brown earth according to the basic soil conditions. Soil texture was loam with pH 6.5. Soil samples were air dried at room temperature and sieved through a 2 mm sieve. The sieved soil was used for pure actinomycete cultures isolation.

Isolation of Pure Cultures of Actinomycetes

A complete of 168 actinomycete strains were isolated and obtained as pure culture by using standard microbiological methods. Serial dilutions (10^{-3}, 10^{-4}) were made from 1g of soil sample and sterile saline solution. The dilutions were inoculated to the surface of Pochon medium **(Korzeniewska, *et al.*, 2009)** in triplicate. Plates were incubated at 28 °C in thermo box (TS 606 CZ/4 – Var; WTW) and monitored for 7 days. Suitable colonies those showed actinomycetes like appearance (rough, chalky) were cultivated several times for purity using medium ISP2 (yeast-malt extract agar) from the International *Streptomyces* Project (ISP) **(Shirling and Gottlieb, 1966)**. The cultivation lasted 7 days at 28 °C. The cultural and morphological characteristics, including morphology and colour of aerial mycelium, characteristics of colonies on the plate, spore mass colour, colour of diffusible pigments and sporophore and spore chain morphology, following the directions given by Bergey's manual of systematic bacteriology **(Holt *et al.*, 1994)** and International *Streptomyces* project (ISP) **(Shirling and Gottlieb, 1966)**, indicated that 105 actinomycete strains belonged to the genus *Streptomyces.* Analysis of 16S rRNA of monitored strains showed also high similarity with the 16S rRNA gene of *Streptomyces*. These strains were subsequently screened for antimicrobial activity analysis and screening genes coding polyketide synthetase (PKS-I) and nonribosomal peptide synthetase (NRPS).

Screening of Antimicrobial Activity of Pure Isolates

The agar plate diffusion method (method of agar blocks) was applied in the screening tests of antimicrobial activity. We used 12 test-microorganisms (*Bacillus subtilis* (DSM 347), *Brevibacterium flavum* (ATCC 21529),

Escherichia coli (DSM 498), S*phingopyxis terrae* (IFO 15098T), *Candida glabrata* (DSM 6425), *Candida utilis* (DSM 70167), *Fusarium graminearum* (IFA 77), *Pseudallescheria boydii* (FCB 395), *Aspergillus fumigatus*(ZB 221), *Botrytis cinerea* (FCB 69), *Alternaria tenuissima* (E.68–34–015), *Aspergillus flavus* (37 M). The test-microorganisms were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany), from Interuniversitäres für Agrarbiotechnologie, Tulln, from American typeculture collection and from the collection of the Department of Microbiology in the Slovak University of Agriculture in Nitra. The culture media applied for test-microorganisms were Mueller Hinton medium (MH) **(Hawke *et al*., 2000)** and peptone-yeast extract medium (NA) in the case of bacteria and yeasts. The tests microscopic fungi were cultivated on malt agar (MA). The total amount of 100 µl solution of mentioned indicator organisms were inoculated to the surface of Petri dishes with prepared broth using L-stick. Cylindrical pieces were cut out from well grown and sporulated culture of the actinomycetes strain cultivated 10 days at 30 °C on ISP2 medium. The blocks were placed on the Petri dishes inoculated with test-microorganisms in 2 replicates. The cultures stayed at 30 °C in aerobic conditions. The antagonism of bacteria, yeast and fungi was detected by formation of an inhibition zone measured using Haloes Caliper, IUL Instruments. The antibacterial activity was measured after 24 h and the antifungal activity after 72 h.

Detection and Analysis of PKS-I and NRPS

DNA was acquired from pure actinomycete strains cultivated 15 days at 30 °C in aerial conditions in thermo box (TS 606 CZ/4; WTW). Isolation was performed according to **Sambrook *et al*. (1989)**. The quality of isolated DNA was determined electrophoretic on 2 % agarose gel and quantified using Gel Logic 212 PRO Imaging System (Carestream Health, Inc., USA).
Degenerate primers PKS-I and NRPS, targeting genes encoding polyketide synthases (PKS-I) and nonribosomal peptide synthetase (NRPS) in actinomycete sequences were used to screen the biosynthetic potential of the isolates **(Ayuso–Sacido and Genilloud, 2005)**: NRPS: A3 (5'–GCSTACSYSATSTACACSTCSGG–3'), A7R (5'–SASGTCVCCSGTSCGGTAS–3'), PKS: K1 (5'–TSAAGTCSAACATCGGBCA–3'), M6R (5'–CGCAGGTTSCSGTACCAGTA–3'). The PCRs were made in total volume of 50 µl. Each reaction contained 5 µl of 10 × DreamTaq Green PCR buffer, 5 µl of 2 mmol.dm^{-3}dNTP, 2 µl of each 10 µmol.dm^{-3} primer, 0,3 µl Taq DNA polymerase and 0,5 µl of template DNA (approximately 20 ng). The reactions ran in thermo cycler Biometra T Personal. PCR conditions were as follows: 3 min at 95 °C followed by 44 cycles of 45 s at 95 °C, 1 min at 56 °C, and 2 min at 72 °C. A 10 min extension step at 72 °C was applied at the end of the PCR program. The quality of PCR products was determined electrophoretic on 2 % agarose gel and quantified using Gel Logic 212 PRO Imaging System (Carestream Health, Inc., USA). The presence of screened genes was confirmed, if fragment of equivalent size had been synthetized (1200 – 1400 bp for PKS–I and 700 – 800 bp for NRPS).

RESULTS AND DISCUSSION

We isolated 168 strains that showed morphological characteristics of actinomycetes. According to their colour on media and morphological characteristics, described in Bergey's manual of systematic bacteriology **(Holt *et al*., 1994)** and International *Streptomyces* project (ISP) **(Shirling and Gottlieb, 1966),** we selected 105 isolates, which belonged to the genus *Streptomyces* for further analysis. That, these strains belong to genus *Streptomyces* was confirmed with the analysis of 16S rRNA gene. These strains were subsequently screened for antimicrobial activity analysis using agar plate diffusion method and screening genes coding polyketide synthetase (PKS-I) and nonribosomal peptide synthetase (NRPS).

Assessment of antimicrobial activity using agar plate diffusion method

In our study, 54 out of the total 105 strains (51 %) showed antagonistic activity against at least one of the 12 indicator organisms (Table 1). Similar results have also other authors. **Nedialkova and Naidenova (2004-2005)** found 60 % and **Rahman *et al*. (2011)** found 53.3 % actinomycete strains with antimicrobial activity. The total of 49 % of the investigated strains did not possess any activity against the tests. The diameter of inhibition zone ranged most frequently from 10 to 20 mm.
The active isolates exhibited different inhibitory patterns against the tested organisms. We found the highest antibacterial activity potential against *Bacillus subtilis* (52 strains out of total 105). Similar results reported also **Nedialkova and Naidenova (2004-2005)** and **Thakur *et al*. (2007)**. **Pandey *et al.* (2004)** found only 31 actinomycetes of the total 106 active against *Bacillus subtilis*. We recorded the biggest antibacterial activity against *Bacillus subtilis* at strain MDM 126 (27 mm). This strain showed expressive antibacterial activity also against gram-negative bacteria *Sphingopyxis terrae* (25 mm). None strain of actinomycetes inhibited the growth of *Brevibacterium flavum*, and only two of the studied strains (MDM 104 and MDM 124) suppressed the growth of *Escherichia coli*. **Nedialkova and Naidenova (2004-2005)** found only one actinomycete strain that suppressed the growth of *E. coli* from total forty studied strain. Conversely, **Rahman *et al.* (2011)** found 62.5 % actinomycete isolates from soil used for wheat cultivation and **Pandey *et al.* (2004)** 17 of the 106 screened actinomycetes, that inhibited the growth of *E. coli*. We found higher percentage of inhibition against Gram-positive bacteria while Gram-negative test bacteria were less inhibited. The same result recorded also **Pandey *et al.* (2004), Şahin and Uğur (2003) and Thakur *et al*. (2007)**.
Antifungal activity was recorded in the case of strain MDM 126, that was the most effective (23 mm) against yeast *Candida utillis*. The activity against *Candida glabrata* showed 5 actinomycete strains. Actinomycete strain MDM 221 most suppressed the growth of *Aspergillus flavus* and *Aspergillus fumigatus*. **Ayari *et al*. (2012)** found moderate activity against *A. fumigatus*, but did not reveal any activity against *A. flavus*. The growth of *Fusarium graminearum* was suppressed by strains MDM 165, MDM 191 and MDM 230 (24 mm). *Pseudallescheria boydii* was most inhibited by strain MDM 161 with inhibition zone diameter 19 mm. The best antifungal activity against *Botrytis cinerea* showed strain MDM 099 (13 mm) and against *Alternaria tenuissima* strain MDM 150 (15 mm).

Screening genes coding PKS-I and NRPS

In this study, we assessed the biosynthetic potential of the 105 isolates by amplifying the genes encoding polyketide synthases (PKS-I) and nonribosomal peptide synthetase (NRPS) using sets of degenerate primers designed for actinomycetes. The total of 61 out of the 105 strains carried at least one of the biosynthetic enzyme genes (Table 1). The number of strains with PKS-I and NRPS gene fragments matching the anticipated length were 19 (18%) and 50 (47%) respectively. **Ayuso–Sacido and Genilloud (2005)** and **Pathom-aree *et al.* (2006)** observed also the extensive distribution of NRPS sequences (79.5 %) among the actinomycete taxa tested, whereas PKS-I genes were concentrated in fewer genera (56.7 %). But according to **Wawrik *et al.* (2005)** contain many actinomycetes PKS gene clusters which are of potential pharmaceutical relevance. We found total eight strains that carried all the biosynthetical genes. According to **Gao and Huang (2009)** the broad distribution of PKS and NRPS genes and their high numbers even in a single actinomycete complicate their use.

Agar plate diffusion method versus screening PKS-I/NRPS genes

Strains MDM 083, MDM 089, MDM 123, MDM 194 and MDM 231 carried all the biosynthetical genes, yet it did not have antimicrobial activity against any of tested pathogens. A total of 21 strains showed antimicrobial activity even though we were not able to amplify any of the PKS or NRPS genes from them. Antimicrobial activity against more than half of the test microorganisms had strains MDM 076 (positive to 6 test organisms out of total 12), MDM 099 (8), MDM 113 (6), MDM 124 (9), MDM 191 (7) and MDM 230 (7). But we demonstrated the presence of both NRPS and PKS-I genes only by strain MDM 113. The presence of gene sequences were not present by the strain MDM 099. The remaining strains showed the presence of only one of the monitored genes. Also **Zhao *et al*. (2011)** found strain (SAUK6023, most similar to *Streptomyces aurantiacus*), that did not show any antimicrobial activity, yet it could amplify the PKS-NRPS genes from it. For this phenomenon there are several plausible explanations. According to **Zhao *et al*. (2011)** it could be because the antimicrobials of this strain are effective against pathogens not tested by them, or it produced it in quantities too low to inhibit pathogens. It may also be due to the fact that the PKS-NRPS genes of this strain were silent or the PKS-NRPS gene clusters of the strain were incomplete. Possibly, the code genes of antimicrobial products were not PKS or NRPS synthetical genes, or it may be due to the primers amplifying PKS-NRPS genes were not suitable to these strains **(Zhao *et al*., 2011).**

CONCLUSION

We confirmed antimicrobial activity of actinomycetes isolated from arable soil using agar plate diffusion method (51 %) and screening PKS-I and NRPS gene fragments (58 %). These results indicated that arable soils could be an interesting source of antimicrobial bioactive substances. Further investigations are needed in order to determine the active metabolites of these isolates.

Table 1 Antimicrobial activity and presence of PKS-I and NRPS genes in selected strains of actinomycetes

Isolates	Test microorganisms												PKS–I / NRPS
	1	2	3	4	5	6	7	8	9	10	11	12	
MDM 065	+ +	–	–	+ +	–	–	+ +	+ +	–	–	–	–	–/–
MDM 069	+ +	–	–	+ +	–	+ +	–	–	–	+ +	+ +	–	–/+
MDM 076	+ +	–	–	+ +	–	+ + +	+ +	+ +	–	+ +	–	–	–/+
MDM 084	+ + +	–	–	+ +	–	–	+ +	–	+ +	–	–	–	–/–
MDM 092	+ +	–	–	–	–	–	+ +	–	+ +	+ +	–	–	–/+
MDM 095	–	–	–	–	–	–	+ +	–		–	–	–	–/–
MDM 099	+ +	–	–	+ +	–	–	+ +	+ +	+ +	+ +	+ +	+ +	–/–
MDM 100	+	–	–	–	–	–	–	–	+ +	+ +	–	+ +	–/–
MDM 103	+ +	–	–	–	–	–	–	+ +	–	–	–	–	–/–
MDM 104	+ + +	–	+ +	+ +	+ +	–	–	–	–	–	–	–	–/+
MDM 107	+ + +	–	–	+ +	–	–	+ +	+ +	–	+ +	–	–	–/+
MDM 113	+ +	–	–	+ +	–	–	+ +	+ +	–	+ +	–	+ +	+/+
MDM 124	+ + +	–	+ +	+ + +	+ +	+ +	+ +	+ +	+ +	+ +	–	–	+/–
MDM 126	+ + +	–	–	+ + +	–	+ + +	–	–	–	–	+ +	–	–/+
MDM 127	+ + +	–	–	–	–	–	–	–	–	–	–	–	–/–
MDM 128	+ +	–	–	+ +	–	+ +	–	–	–	–	–	–	–/+
MDM 129	+ + +	–	–	+ + +	–	–	–	–	–	+ +	–	–	–/+
MDM 132	+ +	–	–	+ +	–	–	–	–	+ +	–	–	–	–/+
MDM 136	+ +	–	–	–	+ +	–	–	–	–	–	–	–	–/+
MDM 142	+ +	–	–	–	–	+ +	–	–	–	–	–	–	+/–
MDM 150	+ +	–	–	–	–	–	+ +	–	+ +	–	–	+ +	–/–
MDM 152	+ +	–	–	–	–	–	–	–	+ +	+ +	–	–	–/+
MDM 158	–	–	–	–	–	–	–	+ +	–	–	–	–	–/–
MDM 161	+ +	–	–	+ +	–	–	+ +	–	+ +	+ +	–	–	+/–
MDM 164	+ + +	–	–	+ + +	–	+ + +	+ +	–	–	+ +	–	–	–/+
MDM 165	+ + +	–	–	+ +	–	–	–	+ +	+ +	–	–	–	–/–
MDM 166	–	–	–	–	–	–	+ +	+ +	+ +	+ +	–	+ +	–/–
MDM 176	+ +	–	–	+ +	–	–	–	–	+ +	–	+ +	–	–/–
MDM 181	+ +	–	–	–	–	–	+ +	–	+ +	–	–	–	–/+
MDM 184	+ +	–	–	+ +	–	–	–	–	+ +	–	–	+ +	–/–
MDM 186	+ +	–	–	–	–	–	–	–	–	–	–	–	–/–
MDM 191	+ + +	–	–	+ +	–	+ +	+ +	–	+ +	+ +	–	+ +	–/+
MDM 196	+ +	–	–	–	–	–	+ +	–	–	–	–	–	–/+
MDM 221	+ + +	–	–	–	–	+ +	+ +	+ +	+ +	–	–	–	–/–
MDM 222	–	–	–	–	–	+ +	–	–	–	–	–	–	–/+
MDM 228	+ +	–	–	+ +	–	–	+ +	–	+ +	–	–	–	–/–
MDM 230	+ +	–	–	+ +	–	+ +	+ +	–	+ +	+ +	–	+ +	+/–
MDM 237	+ +	–	–	+ +	–	–	+ +	+ +	–	–	–	–	–/–

Legend: Zone of inhibition: + (< 10 mm); + + (10 – 20 mm); + + + (21 – 30 mm); + + + + (> 30 mm); – (without inhibition);
1 – *Bacillus subtilis*, 2 – *Brevibacterium flavum*, 3 – *Escherichia coli*, 4 – *Sphingopyxis terrae*, 5 – *Candida glabrata*, 6 – *Candida utilis*, 7 – *Aspergillus flavus* (37 M), 8 – *Aspergillus fumigatus*, 9 – *Fusarium graminearum*, 10 – *Pseudallescheria boydii*, 11 – *Botrytis cinerea*, 12 – *Alternaria tenuissima*
+, presence of PKS-I/NRPS gene; –, PKS-I/NRPS gene not determined
MDM – isolates of actinomycetes

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