Original article

Phenotypic and molecular analysis of dominant occurring antibiotic active-producing Streptomyces soil flora in Northern Jordan

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A B S T R A C T
This investigation aimed to determine the relatedness of dominant occurring soil Streptomyces spp. in Northern Jordan based on their RAPD-PCR fingerprints, and to compare RAPD technique with the conventional phenotypic characterization of Streptomyces isolates. Fifty-eight white and gray color-bearing aerial mycelia antibiotic active-producing Streptomyces soil isolates along with three reference strains were genetically analyzed by RAPD-PCR. Polymorphisms between the isolates showed 1 to 10 bands per isolate and ranged from 200 to 3200 bp in size. Results revealed one common band of ~600 bp shared by ~85% of the isolates, and the observation of bands specific to some reference strains and some soil isolates. When RAPD patterns were analyzed with the UPGMA, results revealed clustering the tested isolates into two equal main super clusters (50% each). Super cluster I appeared to be homogenous and include the three reference strains. However, super cluster II was heterogeneous and not including any of the reference strains. The association of the antibiotic activity of the dominant white and gray aerial mycelium-bearing Streptomyces isolates to RAPD clustering is reported for the first time, and the RAPD-PCR fingerprints generated here deserve to be cloned, characterized and sequenced in future as Streptomyces species-specific DNA markers. The more random primers used in the analysis may add to RAPD technique a cost-effective, fast, precise result, and less labor work solution for analyzing the similarities and differences among the Streptomyces isolates.
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1. Introduction

Streptomycetes are widely distributed in soil and identification of members of the genus Streptomyces represents a great benefit, as they are rich source of most important pharmaceutical products (Chen et al., 2018; Janardhan et al., 2014; Li et al., 2019; Yoon et al., 2017; Yuan and Carwford, 1995) as well as for their activities as biological control and plant growth promoters (El-Tarabily et al., 2019, 2020; Kamil et al., 2018; Mathew et al., 2020; Saeed et al., 2017). Many classification schemes were proposed to record the vast number of Streptomyces species; majority of them based on a few subjectively chosen cultural properties as well as biochemical, nutritional and physiological characters (Küster and Williams, 1964; Shirling and Gottlieb, 1966). This analysis in addition to the failing or deficiency of taxonomists to find reliable and accurate test for the identification of Streptomyces as source of biotechnological products, has lead to many identification methods developed by researchers for these valuable industrial and pharmaceutical organisms (Williams et al., 1983b; Ochi, 1995; Beyazova and Lechvalier, 1993; Lebada, 1993; Mehling et al., 1995; Martin et al., 2000; Kong et al., 2001; Williams et al., 1983a).

The importance of Streptomyces in bio-industry emphasizes the application of more simple, accurate, and fast new identifying methods. Williams et al. (1983b) pointed out that there are no
simple and rapid procedures for objective identification of *Streptomyces*. Until now, conventional procedures for correct identification are still the only choice. Garaibeh et al. (2003) in their report emphasized that modern identification systems are still developing and mainly by using phage susceptibility, analysis of ribosomal protein sequence (Ochi, 1995), RFLP (restriction fragment length polymorphism) analysis (Beyazova and Lechvalier, 1993) and DNA-DNA hybridization (Lebada, 1993). However, all the methods that were used for the identification of *Streptomyces* suffered from time consumption, high expenses, efforts or uncertain results. The use of arbitrary primers to locate RAPD markers in *Streptomyces* was first done by Mehling et al., (1995). This identification system helped Yuan and Carwford (1995) to apply RAPD methodology in generating specific probes for mycetes was first done by Mehling et al., (1995). This identification efforts or uncertain results.

**Table 2**

Activity of the different *Streptomyces* isolates against different antibiotic-resistant bacteria.

| Color Series | No. of Isolates | No. of Active Isolates | Anti-bacterial Activity | S. aureus | E. coli |
|--------------|-----------------|------------------------|-------------------------|-----------|--------|
| White        | 89 (50.3%)      | 36 (40.45%)            | 29 (80.55%)             | 17 (47.22%) |
| Gray         | 88 (49.7%)      | 22 (25%)               | 18 (81.81%)             | 5 (22.72%) |
| Total        | 177 (100%)      | 58 (32.77%)            | 47 (81%)                | 22 (37.93%) |

* Isolate is considered active when the inhibition zone is 18 mm or more.

* Numbers in parenthesis represent the percent out of the total number of isolates.

**Table 3**

Phenotypical characterization of the white and gray antibiotic active-producing *Streptomyces* isolates.

| Color Series | No. of Isolates | Pigment Production | Reverse Side | Soluble | Melanin |
|--------------|-----------------|--------------------|--------------|---------|---------|
| White        | 36 (62%)        | 22 (38%)           | 17 (77%)     | 4 (18%) | 3 (14%) |
| Gray         | 22 (38%)        | 36 (62%)           | 17 (77%)     | 4 (18%) | 3 (14%) |

* Percentages between parentheses.
activity to produce inhibitory compounds. (Abussaud and Saadoun, 1991; Saadoun and Al-Momani, 1997a; Saadoun and Al-Momani, 1997b; Saadoun et al., 1998; Saadoun et al., 1999; Saadoun and Gharaibeh, 2002; Saadoun and Gharaibeh, 2003; Saadoun et al., 2000; Saadoun et al., 2007; Saadoun et al., 2008; Saadoun et al., 2017). These studies had demonstrated that white and gray bearing aerial mycelium \textit{Streptomyces} spp. were the most dominant aerial mycelia colors over the other ones. However, they did not include the clustering and association of the dominant occurring antibiotic-producing white and gray aerial mycelia bearing streptomycetes with RAPD analysis. Therefore, the work presented here aimed specifically to isolate the antibiotic-producing white and gray aerial mycelia \textit{Streptomyces} from soils in Jordan and to analyze the similarities and differences of these isolates according to their RAPD-PCR fingerprints, and to compare RAPD clustering with the conventional phenotypic grouping and characterization of \textit{Streptomyces} isolates.

2. Materials and methods

2.1. Sampling, isolation, characterization and detection of antibacterial activity

Collection of soil samples and isolation of streptomycetes were done according to procedure described before by Saadoun and Al-Momani (Saadoun and Al-Momani, 1997a). Characterization was carried out according to Williams et al. (1983a, 1983b). Ability of \textit{Streptomyces} isolates to produce inhibitory substances was assayed by the plate diffusion method (Bauer et al., 1966) towards 	extit{Escherichia coli} and \textit{Staphylococcus aureus}.

2.2. Bacterial cultures

Table 1 shows the different \textit{Streptomyces} cultures that were used in this study. \textit{Streptomyces lividans} ATCC 35287, \textit{S. halstedii}
ATCC 10897 and *S. violaceoruber* ATCC 3355 were used as positive controls. The white and gray antibiotic-producing *Streptomyces* strains (Table 1) were isolated from soils in Northern Jordan and proved to be active against several antibiotic-resistant pathogens (Odat, 2004). All tested bacteria that showed antibiotic resistant to several antibiotics were kindly provided by the clinical labs of some local hospitals in Northern Jordan.

2.3. Growth conditions

*Streptomyces* isolates that exhibited significant or unfamiliar antibiosis profile towards *E. coli* and *S. aureus* (Table 2) were cultured on tryptic soy broth (TSB) (Oxoid) (Hopwood et al., 1985) (per liter) 30 g, at 28 °C with shaking at 140 rev/min for 48 h. Purity of the *Streptomyces* cultures were tested by plating 0.1 ml from the broth on starch casein nitrate agar (SCNA) (Küster and Williams, 1964) plates and incubated at 28 °C for 72 h.

2.4. Extraction of genomic DNA from pure *Streptomyces* isolates

Extraction of genomic DNA was performed using Wizard Genomic DNA Purification Kit (Promega, USA) following manufacturer instructions.

2.5. Estimation of the purity and quantity of the extracted DNA

Purity and quantity of the isolated DNA was tested by spectrophotometric method (Saadoun et al., 2008).
2.6. RAPD analysis

Five random primers (Operon Technologies, USA), 10 mer long each, were separately used in the RAPD study. Three of the primers (OPA02, OPA09, and OPA10) were tested before in the literature (Garaibeh et al., 2003; Malkawi et al., 1999) and proved its success in determining the discrimination and reproducibility of each primer. Three trials of amplifications for each primer (OPA02: 5' TGCCGAGCTG-3'; OPA08: 5'-GTGACGTAGG-3'; OPA09: 5'- CGGTAAACGC-3'; OPA10: 5'-GTGATCGCAG-3' and OPA18: 5'- AGGTGACCGT-3') were conducted separately and the profile of each experiment was compared to the previous one (Arbeit 1994). Amplification reactions were performed according to Williams et al. (1990) in total volumes of 25 μl containing 2 μl of 0.5 μmol/1primer, 2.5 μl of 10X PCR Buffer (MgCl₂ free) (Finzyme, Finland), 1 μl of 50 mM MgCl₂ (Promega, USA), 0.25 μl of 10 mM

Fig. 1c. RAPD fingerprints profile of Streptomycetes isolates, on 1.5% agarose gel electrophoresis, amplified using primer OPA09. Lane M: HindIII/Φ X-Hae III molecular weight maker; N: PCR negative control; Lanes correspond to numbers in Table 1.
dNTPs mixture (Promega, USA), 0.5 µl of 2U/µl Taq DNA Polymerase (Finzyme, Finland), and 2.5 µl template DNA. Nucleases free water (Promega, USA) was added to a final volume of 25 µl.

PCR amplification was carried out in 0.2 ml, nuclease free PCR tubes (Treff Lab, Switzerland) using iCycler thermocycler (Bio-Rad, USA) with the following program: initial denaturation step at 94 °C for 5 min for 1 min for 45 cycles, followed by annealing at 33 °C for 30 s and extension at 72 °C for 1 min. Finally, extension at 72 °C for 7 min in which the reactions were held at 4 °C for direct processing or stored at −20 °C until needed.

2.7. Electrophoresis and photography

PCR products were checked for DNA profile by standard electrophoresis procedures (Saadoun et al. 2007) with 1.5% w/v agarose gel (Promega, USA) in 0.5 x TBE buffer. Fotodyne U.V. illuminator (Fotodyne Inc., USA) was used to view and photographed the gels using Polaroid MP4 Instant Camera System (Polaroid corp., USA).

2.8. Data analysis of RAPD profiles

An engineer’s disk with a magnifying lens was used to analyze the RAPD photographs. The binary matrix was generated based on the fingerprints for each primer which were reported as 1 and 0 binary form depending on the presence or absence of the DNA band (Caetano-Anollés and Gresshoff, 1994; Sneath and Sokal, 1973). The similarities and differences of the isolates were calculated based in the binary matrix using the simple matching coefficient of the SPSS software (www.spss.com) (Sneath and Sokal,
1973). The Unweighted Pair Group Mathematical Analysis (UPGMA) was used to generate the dendrograms based on (average linkage) and (single average, nearest neighbor) procedure as defined by Caetano-Anollés and Gresshoff (1994), Sneath and Sokal (1973), and Demeke and Adams (1994) were used to generate the dendrograms.

3. Results

3.1. Isolation, characterization, and detection of antimicrobial activity

During a survey on the dominant white and gray aerial mycelium-bearing Streptomyces biota in soils of Northern Jordan, 177 different Streptomyces isolates were recovered and characterized. Based on their antimicrobial activity, results indicated that 58 isolates were active against one or both tested bacteria and were distributed as 36 and 22 white and gray active isolates, respectively (Table 2).

3.2. Phenotypic relatedness to RAPD profile

All of the active 58 soil Streptomyces isolates showing an inhibition zones of 18 mm or more were further phenotypically characterized (Table 3) then genetically identified by the analysis of their RAPD fingerprints using five arbitrary primers. Each RAPD experiment was repeated three times with each primer and consistent
results were obtained. Based on that, typing scheme as reported by Arbeit (1994) was evaluated to be good, since the five primers showed good typeability, reproducibility and discrimination between the tested isolates.

The relatedness among the *Streptomycyes* strains was assessed by comparing the RAPD patterns produced by each primer. This comparison indicated that the various strains differed in the number of polymorphic bands (between 1 and 10) and size that ranged from 200 to 3200 bp (Fig. 1). Several bands were common among the tested isolates but there was one common band (600 bp) that appeared among 85% of the tested isolates (Fig. 1). In addition, 12 DNA bands ranged in size between 600 and 1350 bp were detected as the most common bands among the different isolates for each random primer used. Table 4 shows the size of these bands, the isolate belonging to, and the primer that generates this band. Moreover, bands specific to some reference strains were also observed.

UPGMA dendrograms were generated from the fingerprints produced from each primer. The dendrogram shown in Fig. 2 was based on joining the proximity matrices of the three primers into one matrix and obtaining the average of three proximity readings collected from each primer (Mangin et al., 1999). The dendrogram (Fig. 2) shows that the tested isolates dropped into two equal super clusters (50% each) (Table 5). Super cluster I appeared to be homogeneous and include the three reference strains. However, super cluster II was heterogeneous and but not including any of the reference strains. Super cluster I consists of two clusters (1 and 2). Cluster 1 includes 28% of the isolates in addition to *Streptomyces lividans* ATCC 35287. Cluster 2 includes 22% of the isolates in addition to *S. halstedii* ATCC 10897 and *S. violaceoruber* ATCC 3355. Super cluster II consists of two clusters. The first cluster (cluster 3) includes 15.5% of the tested isolates. The second cluster (cluster 4) includes 34.5% of the tested isolates and by itself consists of three sub-clusters representing the least similarity among other sub-clusters.

The data determined that when RAPD clustering was compared to phenotypical properties; most of the isolates within cluster 1 and 2 of super cluster I were unable to produce diffusible (21%, 17%) or melanin pigments (23%, 18.5%). However, they were able to exhibit distinctive reverse color (24.5%, 17%) (Table 5). Yet 17.5% and 10.5% of the isolates within cluster 1 of super cluster I, and 13.5% and 8.5% of the isolates within cluster 2 of super cluster I showed white and gray aerial mycelium color, respectively (Table 5). In the case of super cluster II, data showed similar trend to super cluster I, where most of the isolates within cluster 3 and 4 showed un ability to produce diffusible pigments (12%, 26%) or melanin pigments (15.5%, 27.5%). However, they were able to exhibit distinctive reverse color (14%, 27.5%) (Table 5). Data indicated that 10.5% and 5% of the isolates within cluster 3, and 20.5% and 14% of the isolates within cluster 4 showed white and gray aerial mycelium color, respectively (Table 5).

When the antibiotic activity of all dominant white and gray aerial mycelium-bearing *Streptomycyes* isolates where compared to RAPD clustering, results revealed that the dominant white and gray isolates where interestingly divided by 50% in each super-cluster with 16.38% and 16.39% of the isolates were active against both tested bacterial pathogens in super-cluster 1 and II, respectively (Table 6). However, when this activity was compared to the different clusters, data indicated that most of the dominant white and gray isolates belong to cluster 4 of super-cluster II (11.3%), and least abundant belong to cluster 3 (5.08%) (Table 6).

### 4. Discussion

Testing of the antimicrobial activity of the recovered 177 white and gray *Streptomycyes* isolates resulted in identification of 58 active-producing isolates with more potential for the white isolates than gray one and mainly towards *S. aureus*. Morphological characterization of these 58 active isolates revealed that the white isolates tend to be more producers to the three pigments; reverse side, soluble, and melanin, than the gray isolates. RAPD profiles for the 58 antibiotic-producing *Streptomycyes* strains along with three reference strains were evaluated by UPGMA dendrograms. This analysis provided a simultaneous sequence comparison between these isolates.

When the band patterns of this study were compared to the patterns obtained by Garaibeh et al. (2003), data revealed that the number of polymorphic bands generated for each isolate was between 1 and 10 with a size ranged between 200 and 3200 bp as compared to 1 and 18 with a size ranged between 250 and 2777 bp obtained by Garaibeh et al. (2003). This study revealed only one common band of 600 bp shared by approximately 85% of the tested isolates compared to three common bands of 2777, 800 and 250 bp shared by approximately (95%) of the isolates that were reported by Garaibeh et al. (2003). This difference between the two studies in band numbers, profile and even the size could be due to the location of isolation being restricted to the most humid and vegetative part of Jordan, or to that the analysis applied here is confined to only two color aerial mycelia groups (white and gray) of *Streptomycyes* isolates, or to the use here of more primers in RAPD analysis. Our results are also different from what was reported by Malkawi et al. (1999), Saadoun and Garaibeh (2003), Mehling et al. (1995); or Saadoun et al. (2007) as a result of using different analysis conditions like the thermo-stable DNA polymerase, thermo-cycler machine, and PCR reagents (Caetano-Anollés and Gresshoff, 1994; Power, 1996).

Five arbitrary primers were used to conduct the RAPD-PCR amplification, these primers showed the existence of common

### Table 4
The most common 12 DNA bands among the different isolates for each random primer used.

| Serial No. | Band (clone) No. | Size (bp) | Isolate | Primer |
|-----------|-----------------|-----------|---------|--------|
| 1         | 54              | 1350      | H116    | OPA02  |
| 2         | 55              | 870       | C15     | OPA08  |
| 3         | 44              | 870       | B10     | OPA09  |
| 4         | 20              | 600       | J133    | OPA09  |
| 5         | 56              | 870       | J17     | OPA10  |
| 6         | 42              | 1350      | H19     | OPA10  |
| 7         | 35              | 1350      | C12     | OPA10  |
| 8         | 48              | 1350      | S13     | OPA18  |
| 9         | 57              | 870       | A39     | OPA18  |
| 10        | 58              | 1000      | S144    | OPA18  |
| 11        | 50              | 1000      | H16     | OPA10  |
| 12        | 2               | 1000      | 21g     | OPA18  |
Fig. 2. UPGMA dendrogram derived from the combination of primers OPA02, OPA08, OPA09, OPA10 and OPA18 patterns. Numbers correspond to numbers in Table 1.
Distribution of antibiotic activity of all dominant white and gray aerial mycelium-bearing Streptomyces isolates when compared to RAPD clustering.

Table 5
Phenotypical characteristics of the RAPD clusters members. The reference strains were not included.

| RS   | DP   | MP   | AM   | Total |
|------|------|------|------|-------|
|      | W    | G    | W    | G     |
| **Super Cluster I (50%)** |      |      |      |       |
| Cluster 1 |      |      |      |       |
| Cluster 2 |      |      |      |       |
| Cluster 3 |      |      |      |       |
| Cluster 4 |      |      |      |       |
| **Super Cluster II (50%)** |      |      |      |       |
| Cluster 3 |      |      |      |       |
| Cluster 4 |      |      |      |       |

b Numbers in parenthesis represent the percent out of the total.

Table 6
Distribution of antibiotic activity of all dominant white and gray aerial mycelium-bearing Streptomyces isolates when compared to RAPD clustering.

| S. aureus | E. coli |
|----------|---------|
| White    | Gray    | White | Gray |
| **Super Cluster I (50%)** |      |      |      |       |
| Cluster 1 | 14.09% | 8.36% | 8.60% | 2.38% |
| Cluster 2 | 10.87% | 6.37% | 6.95% | 1.93% |
| **Total** | 24.96% | 14.63% | 15.55% | 4.31% |
| **Super Cluster II (50%)** |      |      |      |       |
| Cluster 3 | 8.46%  | 4.96%  | 4.03%  | 1.14%  |
| Cluster 4 | 16.51% | 9.68%  | 11.45% | 3.18%  |
| **Total** | 24.97% | 14.64% | 15.48% | 4.32%  |

6. Ethical disclosures

The authors announce that no experiments were performed on animals and no data were collected from patient in this research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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