

Biodiversity Analysis of four *Streptomyces* spp. Isolates Habitat in Soils

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Four *Streptomyces* spp. isolates from rhizosphere soils showing somaclonal variation were screened for morphological, antimicrobial, pigment, pectinase activity, isozymes and DNA-fingerprint. Based on visual observation by light and electron microscopes of four *Streptomyces* isolates were identified as *St. scab*, *St. viridiviolaceus*, *St. capillispirales* and *St. refuineus*. They showed different in mycelium and conidia spores as well as variable diffusible pigment in experimental medium. All isolates showed antimicrobial potentialities against tested microbial. The extend of antibiosis, the isolates were differed against microbial that exhibited a strong activity against +VG bacteria, yeast, a weak activity against -VG bacteria and fungi. DISC-PAGE of pectinase activity and isozymes appeared differences among four isolates in number and density 5, 5, 4, 5 isozymes of Streptomyces isolates, respectively. DNA-finger printing was used for molecular analysis of somaclonal variation among four Streptomyces isolates. Only three arbitrary primers (among ten tested) were chosen as producing polymorphic DNA fragments differentiating of four isolates. RAPD-PCR revealed polymorphism among four isolates, 2 polymorphic fragments (specific fragments) with 12.5%; 8 monomorphic fragments (common fragments) with 50% and revealed 6 unique fragments (genetic marker) with 37.5% (1327, 1018, 950 and 250 bp) of *St. scab*, (200 bp) of *St. viridiviolaceus*, (575bp) of *St. capillispirales* and (350 bp) of *St. refuineus*. The results showed that, the somaclonal variation among four Streptomyces isolates are close similarity within Streptomyces group depending on genetic variability.

Key words: *Streptomyces* spp., Biodiversity, pectinase, RAPD-PCR, DISC-PAGE.

Phylogenetically defined as a number of taxa within the high G+C subdivision of the gram positive phylum are involved in important processes in wide range of actinomycetes habitats¹¹. Many isolates lacking some of these morphological and physiological characteristics of typical Streptomyces have been obtained from soils^{1,20}. However, the application of criteria, morphological features such as sporangium formation in submerged liquid culture and chemical composition of certain cell constituents (such as cell wall) in the characterization of these a typical isolates is therefore not sufficient to identify these

isolates as members of the genus *Streptomyces*. Genetic and phenotypic diversity among *Streptomyces* isolates are well known²⁴ but ecological and systemic assessments have been problematic because readily used morphological, biochemical or molecular markers have largely been locking. Analysis of nucleotide sequence of RNA from the small ribosomal subunit (i.e 16sRNA) has gained rapid and wide acceptance in systemic, evolutionary and ecological studies of various microorganisms⁹. In general there are three types of gene markers could be used. Phenotype markers based on visible characters, biochemical markers based on the detection of protein or/ and isozymes are answers to gene expression that can affected by the environment²⁶ or stages of development and this analysis is time consuming. On the other hand DNA based molecular markers monitor the

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genotype directly are not affected by environment or differentiation and can be applied with representative RAPD-PCR².

The aim of this investigation is to analyze the biodiversity of four *Streptomyces* isolates which isolated from soils on the genetic basis of using pectinase isozymes and DNA fingerprint.

MATERIALS AND METHODS

Soil samples

Rhizosphere soil samples under cultivated plants were collected from different locations in Egypt. The collected samples were air-dried and ground then mixed with CaCO₃ and followed by sieving in 4 mm mesh screen. One gram of each prepared soil was stirred in 100 ml sterile water and serial dilution until 10⁻⁶ had been made. One ml of each dilution was spread on Petri-dish containing starch nitrate agar medium²². The dishes were incubated for 7 days at 28°C till *Streptomyces* colonies appeared.

The purification was achieved by picking up of unique single identical morphological *Streptomyces* colonies. Twenty purified *Streptomyces* isolates were sub-cultured on specific medium and stored at 4°C.

Antimicrobial activity

The antimicrobial activities were assessed by the cork-borer method as following⁸: Gram positive bacteria; (*Staphylococcus aureus* ATCC6538, *Bacillus subtilis* ATCC6633) and Gram negative bacteria (*Escherichia coli* ATCC7839, *Pseudomonas aeruginosa* ATCC9027) cultures (MARCIN, Faculty of Agriculture, Ain Shams University, Egypt) were inoculated in the form a loopful of each bacterial strain in 50 ml of nutrient agar medium and poured in sterilized plates and left to solidify. *Candida albicans*, ATCC10231 culture was inoculated in the form loopful in 50 ml of sabaroud agar medium and poured in sterilized plates and left to solidify. *Aspergillus flavus* ATCC16883 and *A. niger* TCC16404 (spore suspension) were inoculated in 50 ml of Dox agar medium and poured in sterilized plates and left to solidify. Plugs of each four *Streptomyces* test were cut out by the cork-borer and placed on the surface of the agarized medium seeded with test organisms. The plates were incubated at 37°C for 24 h (bacteria), 30°C for 48 h (yeast) and 25°C for 72 h (Fungi). This

method based on the observation of the microbial growth inhibition zone is an indication of the existence of a biologically active substance.

Spore chain morphology

Spore chain morphology was determined by light microscopy and described¹⁹ and spore print technique was applied copper grids coated carbon were gently pressed over the sporulating surface of starch nitrate agar cultures. The grids were observed and photographed using a JOEL-JEM-1010 transmission electron microscope (Fungal Center, Faculty of Science, Al-Azhar University, Egypt). *Streptomyces* isolates were inoculated into starch liquid medium and incubated at 28 ± 2°C for 2 weeks under stirred conditions²². The developed growths were harvested by centrifugation at 5000 rpm and determined cellular proteins⁴ using bovine serum albumin (BSA) as a standard protein.

Pectinase isozymes electrophoresis

Protein concentration was determined by using bovine serum albumin (BSA) is a standard¹⁶. Pectinase isozymes electrophoresis was performed among four *Streptomyces* isolates using one enzyme staining system. Polyacrylamide gel electrophoresis (DISC-PAGE) was performed in 10% (W/V) slab gel. The gel was stained after run¹⁴ for pectinase isozymes. The staining gel was incubated at 37°C in dark for complete staining.

Genomic DNA isolation

The pellets of four *streptomyces* isolates were added to 150µl of DNA-extraction buffer (200 mM Tris-HCl, pH8.5; 250 mM NaCl, 25 mM EDTA and 0.5% SDS) was added. The homogenate was transferred to a 0.5ml micro-centrifuge tube. After adding 75 µl of 3 mM potassium acetate, pH 5.2. The lysate was kept at -20°C for 10 min. After centrifugation, the supernatant was transferred to a new microcentrifuge tube. Nucleic acids were precipitated with on equal volume of isopropanol at room temperature for 30 min and pelleted by centrifugation at 15.000rpm for 5 min. The pellet was washed with 70% ethanol, dried and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). The concentration of template DNA was adjusted to about 1 mg/ml⁶.

RAPD-PCR analysis

DNA fingerprint was performed for four *Streptomyces* isolates using ten primers (Operon RAPD 10 mer kits). DNA (50 ng) of each isolate

was amplified by the polymerase chain reaction (PCR) using 25 μ l random mixtures under the following conditions: 200 mM of dNTPs, 1-2 mM $MgCl_2$; 10 mM Tris-HCl (pH 8.0); 50 mM KCl, 0.01% gelatin, 200 ng oligonucleotide primer and 2-5 unit Taq polymerase (promega Co. USA) and 1 x Taq polymerase buffer. Amplification was carried out in DNA thermal cycler (MWG-BIOTHCK primus) programmed²⁷ as follows: One cycle at 94°C for 4 min and then 40 cycles at 34°C for 20 sec., 5°C for 1 min and 72°C for 2 min and one cycle at 72°C for 5 min.

The PCR products were subjected to electrophoresis on 1% agarose in TAE buffer was prepared and a total volume 6 μ l of miniprep, 4 μ l d-H₂O and 1 μ l 6 x loading dye of each sample was loaded in each well. The gel was electrophoresed in 65 v for 1.5 h and then stained with ethidium bromide solution (10 mg/ml) for around 10-15 mins. DNA fractions visualized by a UV transilluminator. These fragments were scored as either + or - for their presence or absence across isolated and a binary matrix generated which was further analysis using the NISYS program.

RESULTS

Morphological characters

The four selected *Streptomyces* isolates out of twenty *Streptomyces* colonies were isolated based on cultural growth and morphological

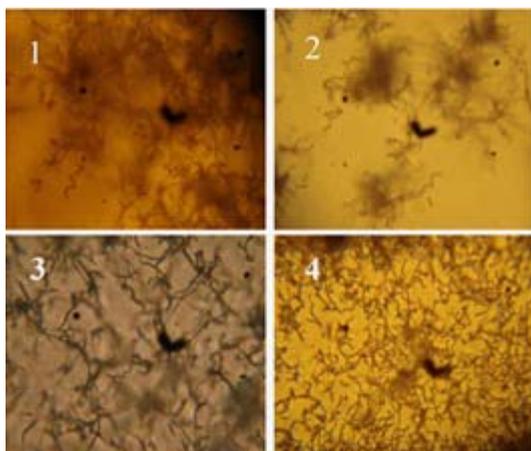


Fig. 1. Micographs of mycelium and spore chains of four isolates *St. scab* (St_1), *St. viridiviolaceus* (St_2), *St. capillspirales* (St_3) and *St. refuineus* (St_4) by light microscope 40X.

characters. Isolates were appeared variation in growth rate on nutrient agar medium (weak rate, moderate, strong and very strong growth) and color colonies (four isolates showed variable diffusible pigment in experimental medium).

Visual observations by light and electron microscopes of four isolates showed different in conediphores 5, 4, 6 and 7 μ m of St_1 , St_2 , St_3 and St_4 isolates respectively, spiral long (St_1) spiral short (St_2), spiral long (St_3) and spiral open long (St_4) (Fig. 1). In addition the pigments of spore mass were differed such as grayish, yellowish, brownish, and greenish respectively. The spore surface was differed between spiny and smooth among four isolates (Fig. 2).

Antimicrobial Activity

All four isolates, *St. scab*, *St. viridiviolaceus*, *St. capillspirales*, *St. refuineus* showed antimicrobial potentialities against tested microbial. Gram positive *St. aureus* was inhibited with 20, 50, 15, 15 mm; *B. subtilis* appeared the most sensitive one for all isolates with 30, 40, 30, 30 mm inhibition zone. An except Gram negative *Ps. aeruginosa* was inhibited with 12, 25 mm and *E. coli* with 15, 20 mm inhibition zone by *St. scab* and *St. capillspirales*, respectively. On the other hand, *C. albicans* was inhibited with 15, 20, 25, 18 mm inhibition zone, respectively. *A. flavus* and *A. niger* appeared low sensitive one for *St. scab* and *St. viridiviolaceus* only with (13, 16) and (12, 20) mm inhibition zone, respectively.

Somaclonal variation among four *Strptomyces* isolates was detected by protein

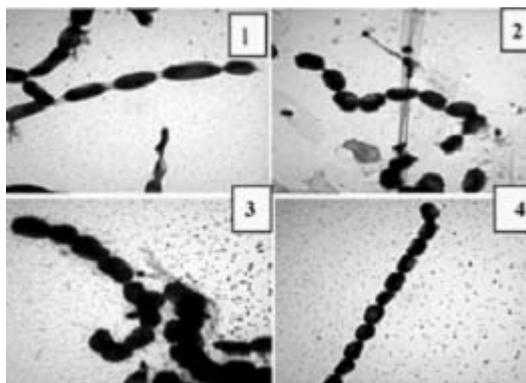


Fig. 2. Micographs of spore chain and spores surface of four isolates: *St. scab* (St_1), *St. viridiviolaceus* (St_2), *St. capillspirales* (St_3), and *St. refuineus* (St_4)

content, pectinase activity and isozyme numbers as well as DNA finger print. Protein content was determined in four isolates related to BSA (Table

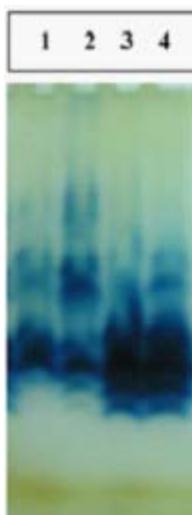


Fig. 3. DISC-PAGE (10%) illustrating pectinase isozymes of four isolates : *St. scab* (St₁), *St. viridiviolaceus* (St₂), *St. capillispirales* (St₃), and *St. refuineus* (St₄)

1). It is revealed that the protein content was varied among four isolates with 315, 270, 250 and 305 mg/g FW of St₁, St₂, St₃ and St₄ isolates, respectively.

Pectinase isozymes

Results of pectinase isozymes are shown in Fig. (3) and Table (2). The pectinase isozymes analysis basis of the number, intensity, relative mobility and reproducibility of bands among four *Streptomyces* isolates. Pectinase isozymes analysis displayed a total 7 bands whereas 2 bands of them common (monomorphic) variable in density and 5 bands variable (polymorphic) among four isolates. The number of isozymes of each isolates was 5, 5, 4 and 5 for St₁, St₂, St₃ and St₄ respectively. In addition the enzymes activity and specific activity were differed between four isolates. It was high and specific activity were higher in St₁ and St₄ followed St₄ and St₂ and St₃ isolates.

DNA fingerprint

Total DNA isolation is found crucial for RAPD-PCR. The DNA yield was determined spectrophotometrically as 7.5 µg/1.0 g mycelium tissues. The DNA purity as indicated by 260/280 was 1.5. It was found that DNA quality was a good

Table 1. Protein content and pectinase activity of four *Streptomyces* isolates

<i>Streptomyces</i> isolates	Protein content (mg/g Fw)	Total activity (U)	Specific activity (µ/mg)
<i>St. scab</i>	315	1520	4.83
<i>St. viridiviolaceus</i>	270	1075	3.98
<i>St. capillispirales</i>	250	970	3.63
<i>St. refuineus</i>	305	1250	4.09

Table 2. DISC-PAGE band patterns of pectinase isozymes of four *Streptomyces* isolates

Rf	Streptomyces isolates								Polymorphism
	St ₁		St ₂		St ₃		St ₄		
	Density	%	Density	%	Density	%	Density	%	
1.7	+	8	-	-	+	15	-	-	Polymorphic
1.9	-	-	+	15	-	-	+++	35	Polymorphic
2.5	+	13	-	-	++	40	+++	25	Polymorphic
3	+	25	++	25	++	25	++	12	Monomorphic
3.4	++	33	+++	45	++	20	+	15	Monomorphic
3.7	-	-	++	10	-	-	+	13	Polymorphic
4	+++	21	+	5	-	-	-	-	Polymorphic
Total bands	5		5		4		5		

Polymorphic= Specific polypeptide band, (-)= Absent fragment, (+++)= Strong fragment, Monomorphic= Common polypeptide band, (+)= Weak fragment, (++)= Moderate fragment

template per PCR sharp and clear amplification products. The polymorphism among four *Streptomyces* isolates were detected using 3 random primers by RAPD-PCR where gave the best results of amplification expressed on average number of fragments per primer. Out of 10 primers the three primers (operon random primer) were screened in RAPD analysis for their ability to produce sufficient amplification products. The three random primers were more stable and reproductive and gave sufficient polymorphism among four *Streptomyces* isolates. Therefore are focused our efforts on these primers are summarized in Fig. (4) and Table (3).

The RAPD-PCR analysis of DNA isolated from four *Streptomyces* isolates revealed 44 amplified fragments (17, 14 and 13) for primers respectively with different molecular weight ranged from 1327 to 200 bp. The DNA amplified fragments of four isolates were varied in number, density and molecular weight. The variability analysis among four isolates showed some DNA amplified fragments absent or/and present in some isolates (Table 3). RAPD-PCR revealed polymorphism among four isolates, 2 polymorphic fragments (specific fragments) with 12.5%; 8 monomorphic fragments (common fragments) with 50% and revealed 6 unique fragments (genetic marker) with

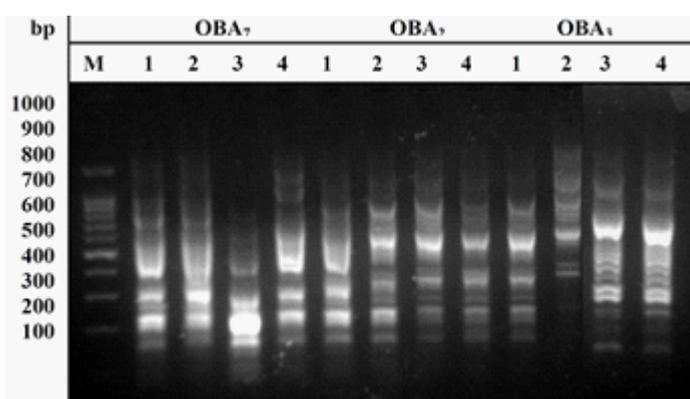


Fig. 4. Agarose gel 1% showing RAPD-PCR products amplified from DNA extracts of four isolates: *St. scab* (St_1), *St. viridiviolaceus* (St_2), *St. capillispirales* (St_3), and *St. refuineus* (St_4). M: DNA molecular weight marker (100bp ladder)

Table 3.

Primers	Polymorphism					Genetic markers of isolates			
	TAF	MAF	PAF	Unique	M.W(bp)	St_1	St_2	St_3	St_4
OBA ₇	6	3	1	2	1327	+	-	-	-
					575	-	-	+	-
					250	+	-	-	-
OBA ₂	6	2	1	3	1018	+	-	-	-
					950	+	-	-	-
					200	-	+	-	-
OBA ₃	4	3	-	1	350	-	-	-	+
					fragments	16	8	2	6
Percentage	-	50	12.5	37.5	43.75	25	6.25	6.25	6.25

St_1 (*St. scab*), St_2 (*St. viridiviolaceus*), St_3 (*St. capillispirales*) and St_4 (*St. refuineus*)

TAF = Total amplified fragments

MAF = Monomorphic amplified fragments (Common fragments)

PAF = Polymorphic amplified fragments (Specific fragments)

Unique fragments = Genetic marker

37.5% (1327, 1018, 950 and 250 bp) of *St. scab*, (200 bp) of *St. viridiviolaceus*, (575bp) of *St. capillspirales* and (350 bp) of *St. refuineus*. The results showed that, the somaclonal variation among the four isolates are close similarity within Streptomycetes group depending on genetic variability.

DISCUSSION

The four *Streptomyces* isolates were isolated from soil and purified on starch nitrate agar medium and identified according to cultural growth, morphological and pigment properties²³. The isolates can be identified as accordingly as *St. scab*, *St. viridiviolaceus*, *St. capillspirales* and *St. refuineus* to variation of morphological characters growth on media, presence or absence of sporangium, sporangiophore length, spore mass, spore surface, spore chain, spore shape and pigments¹⁰. The morphological variation among four isolates is due to different physicochemical characters of soils and different climatic factors of locations^{7,17}. This study aims to investigate the genetic diversity of four *Streptomyces* isolates selected on the basis of variation in morphological characters and antimicrobial potential⁵.

Several techniques of molecular biology for studies of genetic diversity between four *Streptomyces* isolates have been used^{2,8,13}. Electrophoretic protein banding pattern of an organism can be used to elucidate reliable biochemical genetic markers of this organism. It can also provide information about structural genes and their regulatory systems which control the biosynthetic pathways of that protein banding pattern³.

Gel electrophoresis is widely used tool in studies of genetic variability. The electrophoretic differences reflect the allelic variations of four *Streptomyces* isolates enzymes which might due to mutational events occurring in Streptomycetes under stress¹⁸ and affecting the loci controlling the synthesis of isozymes.

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the biosynthetic pathways of that protein banding pattern³.

An electrophoresis analysis was made on the homogenate of four *Streptomyces* isolates for pectinase isozymes in order to determine enzyme activity. Since four *Streptomyces* isolates were collected and from different locations, therefore separate electrophoresis runs were made for four *Streptomyces* isolates from each location. Pectinase isozymes are enzyme that characterized by their common activity on pectin substrate¹⁴. The group of pectinase isozymes is one of the largest and most complicated systems that have been intensively studied in many of microorganisms. Consequently, each band of pectinase activity reflects a structure of one polypeptide chain. Hence each band on the gel represents the end product of one locus (allele).

As revealed from pectinase zymograms, all bands were annelid migrated and their distributions were present in a certain *Streptomyces* isolates and were lacking in another. A total numbers of two bands were present in four *Streptomyces* isolates which seem to necessary ones for the enzyme constitution. Bands Rf 1.7 was observed of isolates St₁ and St₃, Rf 1.9 and 3.7 were observed in St₂ and St₄ while Rf 4.0 observed of St₁ and St₂, Rf 2.5 observed of St₁, St₃, St₄ and Rf 3.0, 3.4 observed in all the four isolates which could be a result of gene expressed under stress. The results indicates that there are allelic variants in this locus in all *Streptomyces* isolates, suggesting a polymorphic type of inheritance for this enzyme. The observed changes in protein enzyme patterns in the present study could be reasonably interpreted to be the result of gene mutation³. However, other investigators¹² traced such changes back to the induction of chromosomal abnormalities such as bridges breaks, laggards and micronuclei which can lead to loss of some of the genetic materials. Therefore some electrophoretic bands disappeared due to the electron of their corresponding bands.

Disappearance of some bands could also for explained on the basis of a mutational event at the regulatory genes²⁵ which are suppressed at transcription level. Meanwhile, the appearance of new bands could be explained on the basis of mutational event at the regulatory system of unexpressed gene(s) that activate them³.

Several factors may be considered or primary determinates of bands observed on a gel, including (1) The number of coding genes, (2) Their allelic states (homozygous or heterozygous) and (3) The quaternary of the protein products. The simplest case involves a single region of salinity with variant electromorphs (allozymes) observed in different individuals. Because allozymes are usually codominantly inherited, the presence and number of bands are depending on the number of polypeptide subunits contained in the active enzyme¹⁵.

The results obtained from RAPD-PCR presented the RAPD profiles generated by these primers, bands genetic markers with MW 1327, 250, 1018, 950 were determined in (St₁); 200 (St₂); 575 (St₃) and 350 bp (St₄) while were not seen in the other isolates. Fortunately, the averages of similarities among four isolates were 26, 24, 22 and 26% for St₁, St₂, St₃ and St₄ respectively. This are expect the similarity of genetic backgrounds of *S. scabes*, *St. viridiviolaceum*, *St. capillispirales* and *St. refuineus* isolates.

The interaction of RAPD-PCR technique has amplified the possibilities of polymorphisms analysis as it allowed the use of small arbitrary nucleotide segments without the need of a previous knowledge of genes and/or genomic sequences²⁸. Also, RAPD assay clearly has certain proactively advantages for detecting DNA variation. It is technically less demanding, cheaper and quicker than other molecular techniques²¹.

CONCLUSIONS

The present study demonstrated the utility of RAPD-PCR technique for the differentiation of *Streptomyces* isolates. Investigated isolates were selected on the basis of different habitat, antimicrobial agents by well characterized resistance, susceptibility phenotypes upon expose to *St. scabes*, *St. viridiviolaceum*, *St. capillispirales* and *St. refuineus*. Reproducible and inheritable stable polymorphic markers for *Streptomyces* isolates were identified with one out of the ten arbitrary primers tested. Several of the primers produced monomorphic fragments among the isolates or the polymorphism identified were not reproducible. The genetic diversity among the four *Streptomyces* isolates were evaluated either by RAPD-PCR fragments length or allozyme.

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