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DNA FINGERPRINTING OF SOME HERBICIDE-TOLERANT *STREPTOMYCES* SPECIES AND PCR-ISOLATION OF *BAR* GENE

Saleh, E.A.[1]; Zaki M.M.[1]; Rahal, A.[2]; Mohamed Sonya H.[2,4]; Bahieldin A.M.[3] and A.S. Sadik[1,3,4]

[1]Dept. Agric. Microbiol., Fac. Agric., Ain Shams University, P.O. Box 68, Hadayek Shobra 11241, Cairo, Egypt, [2]Dept. Agric. Microbiol., Institute of Soil, Water and Environment Research, ARC, Giza, P.O. Box 12619, Egypt, [3]Agricultural Genetic Engineering Research Institute (AGERI), ARC, Giza, P.O. Box 12619, Egypt, [4]Department of Biology, Faculty of Science, Taif University, P.O. Box 888, Taif, Kingdom of Saudi Arabia

ABSTRACT

In this study, differentiation between five identified *Streptomyces* species was carried out on the basis of the nucleotide sequence of DNA using random amplified polymorphisms of DNA-polymerase chain reaction (RAPD-PCR) technique. Results revealed that these species differed in their DNA sequence as indexed by differences in numbers, sizes and unique amplified fragments. Such dissimilarities could be considered as an additional proof that identified isolates were different species of *Streptomyces*. In addition, the herbicide resistance (*bar)* gene was isolated by PCR technique in the DNA extracts of the five identified *Streptomyces* isolates (*Streptomyces albosporeus* subsp *abilomycaticus*, *S. herbaricolor* and *S. aureomonopodiales*) to be available for using as a selectable marker in the transformation of the transgenic plants using the Basta herbicide. It is the first evidence to detect this gene in local *Streptomyces* species in Egypt.

Key words: Identification, *Streptomyces*, RAPD-PCR, Herbicide-resistance (*bar*) gene.

INTRODUCTION

Several studies have documented the use of RAPDs for the typing of fungal as well as bacterial species and strains, on the basis of the standard RAPD as originally described by Williams *et al.*, (1990). For characterization of *F. graminearum* strains RAPD and restriction analysis of amplified fragments from the PCR were used (Ouellet and Seifert, 1993). They found that three out of 40 oligonucleotide primers were selected and used successfully to characterize an additional 17 strains of *F. graminearum.* They also noted a relatively low amount of genetic diversity among the *F. graminearum* strains and the RAPD and specific PCR profiles could be used for tracking strains of *F. graminearum* in field experiments. The genetic variability among 35 isolates from *Magnaporthe poae* was surveyed by Huff *et al.*, (1994) based on RAPD markers. They found that DNA amplification patterns of 23 out of 35 isolates were phenotypically unique. Kelly *et al*., (1994) distinguished 63 isolates of *F. oxysporum* f. sp. *ciceris* and 11 isolates of other fungi using RAPDs. RAPD markers were also used to detect genetic variation of *Erysiphe graminis* f. sp. *hordei* isolates collected from throughout Europe (McDermott *et al*., 1994). Schilling *et al*., (1994) used RAPD technique to

differentiate between the closely related species: *F. culmorum* and *F. graminearum.* They found that species-specific RAPD patterns were extremely useful to identify false classified isolates. In 1996, Woo *et al*., reported that, RAPD analysis could be utilized to distinguish nonpathogenic isolates of *F. oxysporum* from the pathogenic formae species.

The applicability of the RAPD technique for investigating genetic variation within and between bacterial species is further illustrated by work on *Haemophilus somnus* (Myers *et al*., 1993). A number of 26 strains of methicillin-resistant *Staphylococcus aureus*, were examined by RAPD analysis using three primers not all strains could be differentiated (Saulnier *et al.,* 1993). Recent publications reported the use of RAPD-PCR as a molecular tool for determining the DNA fingerprinting of actinomycetes (Mahfouz and Mohamed, 2002; El-Domyati and Mohamed, 2004; Selim *et al*., 2010 and Mohamed et al., 2012).

In this study, the differentiation between some *Streptomyces* spp. using the RAPD-PCR technique was aimed. A trail to isolate the herbicide resistant (*bar*) gene from the selected streptomycetes was also aimed.

MATERIALS AND METHODS

Extraction of total nucleic acids from actinomycete isolates: The total nucleic acids were extracted from the streptomycete cells using the Marmur method (1961) together with two modifications.

Modification No.I: The pulverized actinomycete cells were homogenized in 3.0ml of DNA extraction buffer (0.1M NaCl, 10mM Tris.HCl (8.0), 0.1 mM EDTA, 0.5% Triton X-100 and 1% SDS) followed by heating at 65°C for 15 min. An equal volume of phenol was added and the mixture was then vortexed for 30sec followed by centrifugation at 14000 rpm at room temperature (RT) for 15 min. The aqueous phase was treated with phenol/chloroform and chloroform as described before. The nucleic acids were precipitated from the aqueous phase as mentioned above.

Modification No.II: The pulverized actinomycete cells were homogenized in 3 ml of TE buffer (pH 8.0) containing 1% SDS followed by vortexing for 2 min. at RT. The mixture was treated with phenol/ chloroform twice and once with chloroform as mentioned before. To the aqueous phase, an equal volume of isopropanol and 1/10 volume of 3 M sodium acetate, pH 5.2 were added and the mixture were stored at -20°C for 2 hrs. The nucleic acids were precipitated, dried and resuspended in sterilized d.H_2O as mentioned above.

Purification of DNA extracts: The DNA extracts were purified from the RNA and protein molecules using the enzyme ribonuclease (DNA free RNase) and the enzyme proteinase K as described by Sambrook *et al*., (1989).

Measurement of DNA concentration: The concentration and purity of DNA extracts of the identified Streptomyces isolates were determined as recommended by Brown (1990) with a pure sample of DNA the ratio of the absorbance at 260 nm and 280 nm (A_{260}/A_{280}) is 1.8. DNA concentration was adjusted to 10ng/µl.

Primers used for RAPD-PCR: A number of nine primers of Operon G kit were used with the nucleotide sequences (5` to 3`) of 1:CTA CGG AGG A; 2:GGC ACT GAG G; 3:GAG CCC TCC A; 4:AGC GTG TCT G; 5:CTG AGA CGG A; 6:GTG CCT AAC C; 7:GAA CCT GCG G; 8:TCA CGT CCA C; 9:CTG ACG TCA C and 10:AGG GCC GTC T.

RAPD-PCR: RAPD-PCR was carried out according to the procedure given by Williams *et al.*, (1990) with minor modification. Amplification reaction was carried out in a volume of 50 µl. Each reaction mixture contained 100 ng genomic DNA (as a template), 0.5 µM decamer oligonucleotide primer from OPERON Technologies, Alameda, CA. (Kit G), 2 units of *Taq* DNA polymerase (Promega Corp., Madison, WI, USA), 5 µl of 10x buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0) and 1% Triton X-100], 3 mM $MgCl_2$, 0.2 mM dNTPs (dATP, dCTP, dTTP, dGTP) and deionized dd H_2O. The reaction was overlaid with a drop of mineral oil. PCR amplification was performed in a Perkin-Elmer\DNA Thermal cycler 480 (Norwalk, CT) for 40 cycles after initial denaturation for 3 min at 94° C. Each cycle consisted of denaturation at 94°C for 1 min, annealing temperature at 36°C for 1 min, extension at 72°C for 2 min, and then the final primer extension segment was extended to 5 min at 72°C.

RAPD analysis: The banding patterns generated by RAPD-PCR analysis were compared to determine the genetic relatedness of *Streptomyces* species. The amplified fragments were scored wither as present (1) or absent (0). Bands of the same mobility were scored as identical. The similarity coefficient (F) between two isolates was defined by the formula of Nei and Li (1979). A dendrogram was derived from the distance by the unweighted paired-group method, arithmetic mean (UPGMA) algorithm contained in the computer program package NTSYS 1.5 (Rohlf, 1990).

PCR isolation of the herbicide resistance gene (*bar*): Hundred nano-grams of the total DNA extract (crude and diluted) of each of *S. albosporeus* subsp *Labilomycaticus*, *S. herbaricolor* and *S. aureomono-podiales* as a template for isolation of the herbicide resistance (*bar*) gene were used. The PCR reaction was conducted in a volume of 50µl containing as mentioned before using two oligonucleotides with the nucleotide sequences of A:5'AGG TAT CAG TAA GCC AGT AG3'and B:5'ATG CGG AAT CTC TGC ACG TA3' in final concentration of 50 ng per reaction. PCR amplification was performed for 35 cycles after initial denaturation for 4 min at 94°C. Each cycle consisted of denaturation at 94°C for 1 min, annealing temperature at 50°C for 2 min, extension at 72°C for 2 min, then the final primer extension segment was extended to 5min at 72°C.

Gel electrophoresis analysis: All electrophoresis of DNA extracts of

the identified *Streptomyces* isolates or PCR amplified products were carried out in a 1.2% agarose at 60 volts for 2½ hr and 1 hr with 1x TAE, respectively, as described by Sambrook *et al.*, (1989). Nucleic acids or PCR products were visualized by staining gel in ethidium bromide (0.5µg/ ml) and photographed under UV light using a Polaroid camera.

RESULTS AND DISCUSSION

Topp *et al.*, (2004) showed that numerous atrazine-degrading micro-organisms have been isolated, and there is much known about the genetics and biochemistry of atrazine biodegradation. A variety of studies have explored the utility of atrazine-degrading bacteria for soil bioremediation.

Differentiation between identified *Streptomyces* species using RAPD-PCR: Some investigators carried out trials to identify some Streptomyces isolates (strains) using the random amplified polymorphic DNA RAPD-polymerase chain reaction (PCR) (Mehling *et al.*, 1995; Mohamed, 1998; Mohamed *et al.*, 2001 and Abdel-Fattah, 2005).

To obtain highly purified DNA of *Streptomyces* species needed for the present study, extraction of DNA of *S. rectiviolaceus* as a test organism was carried out by Marmur method (1961) together with two modifications. Results in Table -1 showed that modification I was the most effective for obtaining high purity ($A_{260/280}$=1.8) and high concentration (1905.8µg/ml) of nucleic acid extract. Therefore, this modification was superior to the other modifications and hence was employed for extraction of the genomic DNA of the five *Streptomyces* species. Data presented in Table -2 clearly indicate high purity of DNA extracted from the five *Streptomyces* species using modification I since purity of DNA was found to be around 1.8. Furthermore, the nucleic acid extracts were subjected to additional purification by treating them with proteinase K and RNase to exclude the protein and RNA, respectively.

Results of random amplified polymorphic-polymerase chain reaction (RAPD-PCR) for the differentiation between the five *Streptomyces* species are given in Figure-1 and Table-3. Data reveal that no amplified bands were observed in any of the negative controls indicating that the reaction mixtures were free from any strange DNA contamination. For each isolate, number of amplified fragments differed with different primers which are expected. On the other direction, the number and size of amplified fragments differed from one species to another for the same primer used. Moreover, each species was characterized by unique band(s) with primer used (Table 4). However, some bands were common for all tested species. A statically analysis of RAPD polymerphisms as given in Figure -2 and Table -5 revealed a degree of dissimilarities between these species ranging between 40.4-56.6%. The present data therefore confirms those of identification studies that the five isolates were different *Streptomyces* species. The data of the present experiment could be considered as an additional proves that tested isolates are different species of the genus *Streptomyces*. This is clear since they differed in their DNA sequence as indexed by differences in numbers, sizes and unique amplified fragments together with clear dissimilarities between them.

Results of this study agree with that reported by Mahfouz and Mohamed (2002); El-Domyati and Mohamed (2004); Selim *et al.*, (2010) and Mohamed

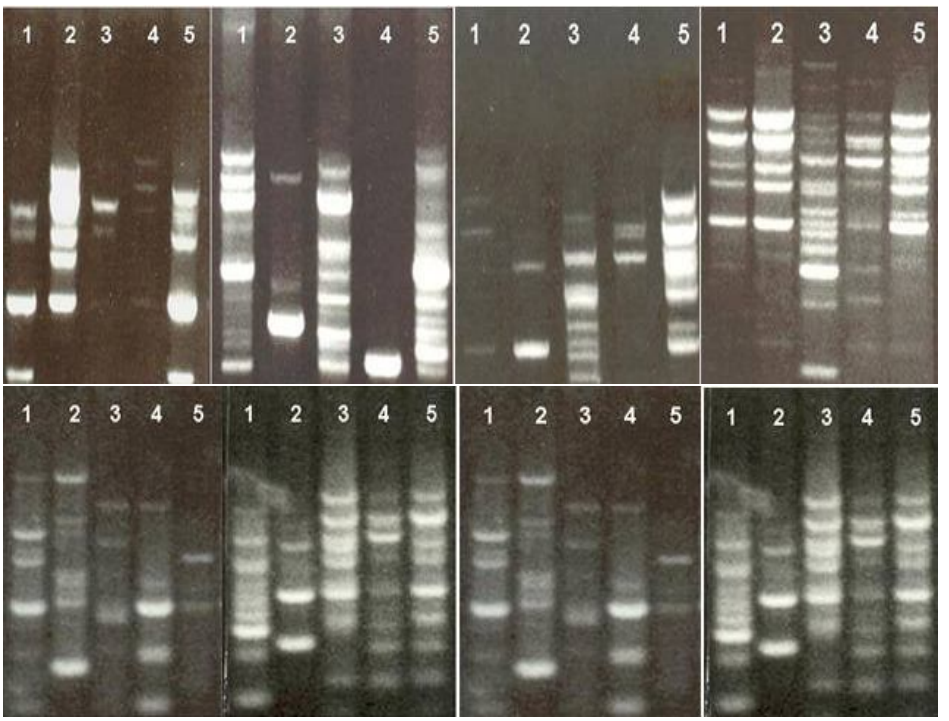
et al., (2012), who recommended the use of RAPD-PCR as a molecular tool for determining the DNA fingerprinting of actinomycetes.

Table-1: Comparison between three different methods for nucleic acids extraction of *Streptomyces retiviolaceus*.

Methods	Wavelength (nm)		Purity	DNA concentration
	A_{260}	A_{280}	($A_{260/280}$)	(µg/ml)
Marmur (1961)	0.594	0.358	1.66	1188.2
Modification I	0.953	0.541	1.76	1905.8
Modification II	0.676	0.441	1.53	1352.8

Table-2: Purity and concentration of DNA of identified *Streptomyces* species.

Streptomyces species	Wavelength (nm)		Purity	DNA concentration
	A_{260}	A_{280}	($A_{260/280}$)	(µg/ml)
S. rectiviolaceus	0.199	0.114	1.8	497
S. roseolus	0.096	0.054	1.8	240
S.albosporeus subsp. Labilomycaticus	0.198	0.115	1.7	495
S. herbaricolor	0.337	0.201	1.7	842
S. aureomonopodiales	0.122	0.067	1.8	305



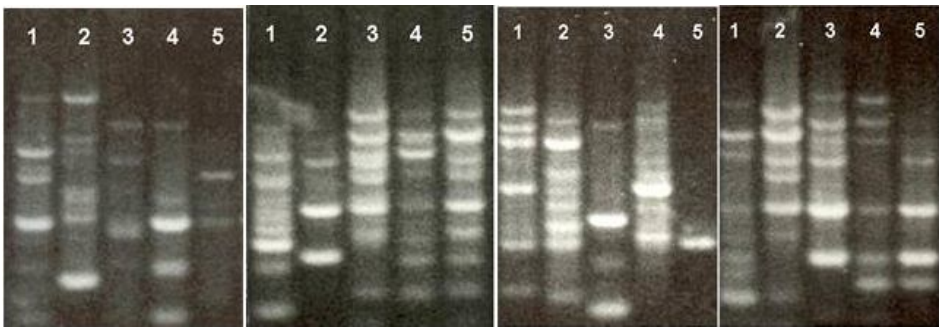


Figure-1: RAPD-PCR polymorphisms of NA of *Streptomyces* species using primers, G01, G02, G04, G10, G06, G07, G08, and G09, respectively, in 1% agarose gel in TAE buffer. M: DNA marker. Lane 1: *S. rectiviolaceus*; Lane 2: *S. roseolus*; Lane 3: *S. albosporeus* subsp *Labilomycaticus*; Lane 4: *S. herbaricolor*; Lane 5: *S. aureomonopodiales.*

Table-3: Number of amplified fragments of *Streptomyces* species using nine primers using RAPD-PCR technique.

Primers No.	No. bands	Number of fragments				
		S. rectiviolaceus	*S. roseolus*	*S. albosporeus* subsp Labilomycaticus	*S. herbaricolor*	*S. aureomonopodiales*
G01	10	4	5	2	2	4
G02	17	7	3	9	1	10
G04	12	4	2	4	1	5
G06	14	2	5	3	6	5
G07	15	7	3	8	4	8
G08	15	4	6	6	6	4
G09	16	6	8	4	7	1
G10	23	7	9	13	9	9

Table-4: Unique amplified fragments for each *Streptomyces* species using nine primers.

Primers No.	No. bands	Unique amplified fragments				
		S. rectiviolaceus	*S. roseolus*	*S. albosporeus* subsp Labilomycaticus	*S. herbaricolor*	*S. aureomonopodiales*
G01	10	0	1	2	1	1
G02	17	1	2	0	1	2
G04	12	2	2	1	1	2
G06	14	1	1	2	2	1
G07	15	4	0	2	0	0
G08	15	0	1	1	3	3
G09	16	1	2	3	2	0
G10	23	0	2	8	3	0

Table-5: Dissimilarities between the five identified *Streptomyces* species based on RAPD analysis.

Isolates	Relationship between the five *Streptomyces* isolates (%)				
	S. rectiviolaceus	*S. roseolus*	*S. albosporeus* subsp Labilomycaticus	*S. herbaricolor*	*S. aureomonopodiales*
S. rectiviolaceus	00.00				
S. roseolus	41.40	00.00			
S.albosporeus subsp Labilomycaticus	42..20	57.40	00.00		
S. herbaricolor	44.10	41.90	55.60	00.00	
S. aureomonopodiales	44..90	45.60	50.00	49.30	00.00

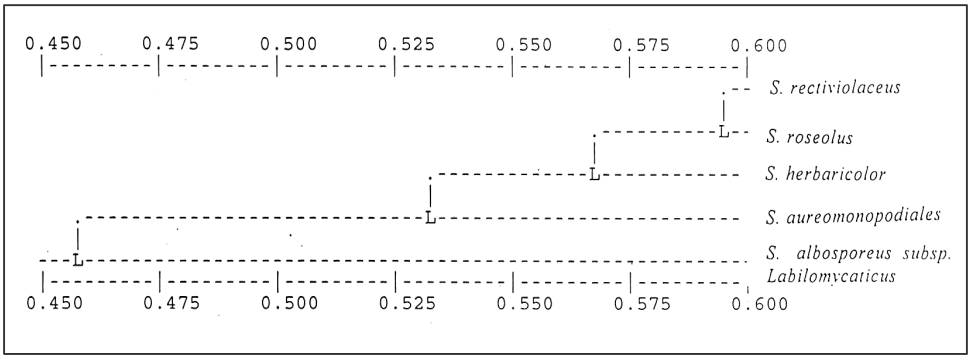


Figure-2: Dendrogram showing relationship between the five identified *Streptomyces* species based on RAPD analysis.

Isolation of herbicide resistance gene (*bar*): The herbicide resistance gene (*bar*) which encodes an enzyme that inactivates phosphinothricin (glufosinate ammonium) - the active ingredient of bialaphos (Basta) was first isolated from *Streptomyces hygroscopicus* (Kobets, 1989**)**. This *bar* gene is widely used for producing transgenic plants tolerating the toxicity of bialaphos (Basta) herbicide (Quinn, 1990 and Rathus *et al.*, 1996).

Extracted and purified DNA of these species was separately used as templates with two specific primers complementary to the nucleotide sequence flanking the herbicide resistant gene. Results of PCR detection revealed the presence of the *bar* gene with a size of 400 bp (Figure 3) in the three tested *Streptomyces* species. This is the first evidence to detect this *bar* gene in locally isolates *Streptomyces* species namely, *S. albosporeus* subsp *abilomycaticus, S. herbaricolor* and *S. aureomonopodiales* in Egypt. It should be kept in mind that these three species tolerated Basta and Sencor herbicides and were also active in utilizing Sencor as a sole source of nitrogen. Moreover, these three species were active in degrading Sencor herbicide in 3 (*S. albosporeus* subsp *abilomycaticus*), 4 (*S. herbaricolor*) and 5

(and *S. aureomonopodiales*) products. Further studies should be carried out to proof the sequence and expression of the bar gene from these local strains and use it for production of transgenic plants instead of importing this gene from abroad. Mulbry *et al*., (2002) characterized 2 DNA fragments containing the triazine chlorohydrolase gene, trzN was conducted using oligonucleotides derived from the N-terminal sequence of triazine chlorohydrolase from Nocardioides sp. strain C190, which degrades atrazine residues in the soil. The results of this study could be supported by that of Vaishampayan *et al*., (2007), who reported that atrazine is one of the most environmentally prevalent s-triazine-ring herbicides. The widespread use of atrazine and its toxicity necessitates search for remediation technology. They also showed that presence of trzN gene indicates possible presence of bacterial community with more efficient and novel enzymatic capabilities.

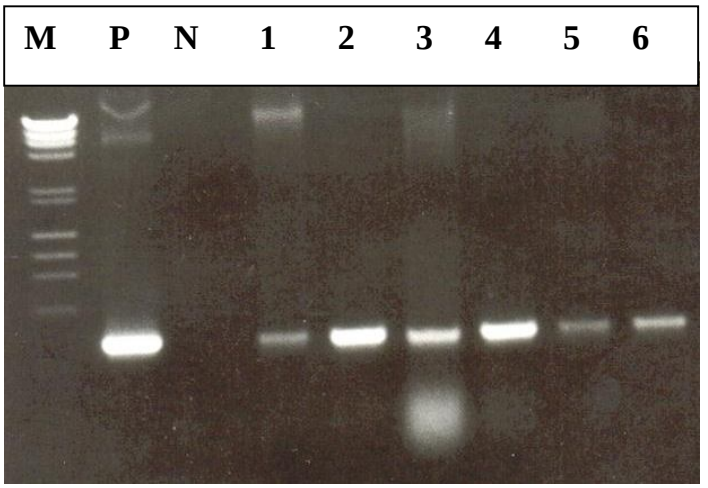


Figure-3: Agarose gel (1.2%) in TAE buffer stained with ethidium bromide showing the PCR products of *bar* gene of *Streptomyces* species using two DNA dilutions. M: DNA marker. P: Positive control. N: Negative control. Lanes 1, 2: *S. albosporeus* subsp *abilomycaticus.* Lanes 3, 4: *S. herbaricolor*. Lanes 5, 6: *S. aureomonopodiales.*

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