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Molecular Detection of *Streptomyces griseus* Isolated from Isfahan Soil

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Abstract: The aim of the present study was to detect *Streptomyces griseus* from Isfahan soil using biochemical, morphological and molecular techniques. Soil samples were collected from different regions (compost, 50 year old garden, river bed, clove, wheat farms and domestic yards) of Isfahan in two different seasons. One gram of each sample was serially diluted and cultivated in a primary isolation medium. Morphological shapes of colonies and vegetative hyphae were initially used to separate the grown bacteria of the samples. Various biochemical tests based on Bergey's manual of systemic bacteriology were performed to separate the *Streptomyces* from other related Genera. In order to further separate *Streptomyces grisesus*, Polymerase Chain Reaction (PCR) was used to detect the presence of aphE and strA genes in isolated colonies. Biochemical and morphological results, showed the presence of several species of *Streptomyces* in the collected samples. The expected band of 924 bp belonging to strA gene was observed in the positive control bacterial DNA (PTCC 1125). Several other bands were also observed in the positive control sample. From the total of 10 colonies that undergone molecular detection for the presents of strA gene, 6 colonies (W_1, W_3, W_5, F_4, F_5, F_{26}) showed only one band at 750 bp and four (W1, W_3, F_5, F_{26}) showed an extra band of approximately 924 bp band as well. The expected band of 671 bp belonging to aphE gene was detected in positive control bacterial DNA (PTCC 1125). None of the soil samples, however, showed the presence of the aphE gene (data not shown).

Key words: *Streptomyces grisesus*, molecular detection, resistance genes, aphE, strA

INTRODUCTION

Streptomyces sp. are aerobic, gram positive filamentous bacteria within the order of actinomycetes (Muth *et al*., 1998). Their major habitat is soil, though they can be found in sediments, compost, fodder and almost all kinds of environment (Williams *et al*., 1989). Ever since 1944, when the first public announcement on streptomycin production was made by Waksman, Streptomycetes become the centre of focus for industrial microbiology and biotechnology researchers since a vast number of antibiotics (about 70%) are produced by these species. Among different type of Streptomycetes, *Streptomyces griseus*, the main producer of streptomycin, has been well studied. In addition to streptomycin, *Streptomyces griseus* also produces novobiocin, cyclohexamide and A factor, a regulator hormone in streptomycin biosynthesis pathway (Ja, 2003; Yamazaki *et al*., 2000).

Although the development of various molecular and non-molecular methods have improved the isolation, classification and detection of *Streptomycets*, but there is still no universal single criteria that makes selectively isolation of *Streptomyces* from environmental samples possible or arranged them in a well-defined taxonomical classification (Anderson and Wellington, 2001). The most sensitive way for streptomycetes detection is polymerase chain reaction (PCR) method (Zhou *et al*., 2000). Genes belonging to resistance enzymes against antibiotics produced by *Streptomyces* sp. are good target for PCR detection. A major group of resistance enzymes are aminoglycoside phosphotransferase (aph) which belongs to protein kinase family (Daigle *et al*., 1999). Among seven classes of aminoglycoside, phosphotransferase aph(3) and aph(6) have been shown to exist in streptomycin producing strain, i.e., *S. griseus* (Heinzel *et al*., 1988; Huddleston, 1997). Aph(6) or strA is clustered with other antibiotic biosynthesis genes whereas aph (3) or aphE is located outside the cluster (Cundliffe, 1992; Huddleston, 1997).

The aim of this study was to screen Isfsahan soil for isolating *S. griseus*, by the aim of both traditional (morphological and biochemical) and new molecular methods. A series of characteristics were utilized in order

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to identify unknown soil species. PCR technique was used to detect for the existence of aphE and strA genes in our colonies.

MATERIALS AND METHODS

Soil sample preparation: Streptomycetes came from twenty soil samples which were collected in two seasons; autumn and winter 2004. Ten samples were composts, gathered from different parks in Isfahan, Iran, others from cultivated farms in city outskirt and one sample from a Zayanderoud river bed.

Isolation and identification of streptomycetes: Soil suspensions were prepared by heating 10 g of soil at 45°C for 2 h to reduce the proportion of other bacteria. The samples were serially diluted up to 10^{-2} and 10^{-3} g of soil per mL of Ringers solution (Huddleston, 1997). Aseptically, 0.1 mL of each dilution was spread on casein-starch agar medium containing nystatine (50 μg mL^{-1}) and streptomycin (5 μg mL^{-1}). After 2 weeks incubation at 30°C, the colonies resembling actinomycetes were isolated. For further identification up to genus, slide culture method was used as described by Benson (2002). A thin square part of agar was cut from poured plate of inorganic salts-starch agar and placed on a microscopic sterile slide and inoculated from four angles and incubated in a moist chamber for 5 day in 30°C. The cover slip was transferred on a new slide and stained with methylen-blue (Benson, 2002). The identification was based on the appearance of hyphae and spore chains.

Biochemical tests: Biochemical tests were carried out to identify streptomycetes up to cluster level using the instruction provided by Bergey's Systematic Bacteriology (Williams *et al*., 1989), except for antibiosis test: streptomyces broth culture in GMP (Glucose-Meat Extract-Peptone) medium was swapped at the center of YEME (Yeast Extract-Malt Extract) medium plate and incubated for 5 days at 30°C. One night old broth culture of Candida albicans ATCC 10231, *Bacillus subtilis* ATCC12711, *Aspergillus niger* ATCC 16404 and *Saccharomyces cerevisiae* in Sabouraud Glucose Broth were swapped on cross lines by sterile swap for each strain. The plates were incubated at 25°C for 2 days as described by Egorov (1985).

DNA extraction: Samples isolated from soil were cultivated in GMP medium at 30°C for 24 h. After centrifugation at 3000 g for 5 min the precipitates were resuspended in 200 μL of PBS. Lysozyme (10 mg mL^{-1} in Tris-HCl, pH 8.0) was then added and the samples were incubated for 30 min at 37°C. The nucleic acid solutions were pooled and the DNA was separated using High Pure PCR Template Preparation Kit (Roche, Germany) as recommended by the manufacturer.

Polymerase chain reaction: The PCR reaction systems consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM of each of the four deoxynucleotide triphosphates (dNTPs), 2.5 μM of each oligonucleotides primer, 5 units of Taq DNA polymerase (Sinagen, Iran) and 10 ng of DNA in a final volume of 50 μL.

Two pairs of primers were designed for PCR amplification experiments. The first pairs based on the strA gene (5' ATGAGTTCGTCGGACCACAT 3' and 5' TCAGGGCTTCGCCAGCGCTT 3') were previously described by Huddleston in s. griseus (ATCC 11429) (Huddleston, 1997). The second pairs of primers were based on aphE gene (5' CGGTACGCCAAGTG 3' and 5' AGGTAGAAGCGCAGC 3'). All primers were designed by primer premier® V.5 and dnasis® V.6.2 and synthesized by Cinagen (Tehran, Iran).

The amplifications were carried out in Genius thermal cycler (Techne, UK) With an initial cycle of denaturation (10 min at 95°C), followed by 35 cycles of a 1 min denaturation of 94°C, a 1 min annealing step (57°C) and a 2 min extension step (72°C). Annealing temperature was raised from 57 to 65°C and then to 68°C for primers amplifying aphE gene.

RESULTS

Non-molecular detection: From total of 50 colonies that had the appearance of actinomycetes, 19 showed microscopic features of streptomyces. The criteria used to isolate the *Streptomycetes* sp. were based on the length of spore chain and its shape, size of conidia and substrate and aerial mycelium as described by Locci (1989). Figure 1 shows microscopic view of six samples differentiated as streptomyces. Figure 1A and B show a hook-like spore chain that is seen with sample W8. Figure 1C and D and E represent the picture of the some soil samples such as F5, F3 and F26, respectively.

Samples that were distinguished as streptomyces genera by morphological features were further screened using biochemical tests. In order to identify streptomyces to genera level, each sample was compared to Major and Minor cluster as described previously by Locci (1989) and shown in Table 1. Using these criteria we found some soil samples (F_7, F_{29} and F_{38}) that do not fall within this category. All winter (W_1, W_2, W_3, W_5 and W8) and fall

Table 1: Morphological and biochemical and Mipleculcharacteristics of the isolated *Streptomyces* sp. According to percentage positive probability matrix for *Streptomyces* sp. Defined as major cluster (Bergey's manual of systematic Bacteriology)

			Other characteristics			Utilization of Nitrogen sources					Utilization of carbon sources								
Cluster	Probable identity	Spore mass colour	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Strain
15	*S. lydicus*	W	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	W_1
2	*S. anulatus*	W	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	-	W_2
4	*S. exfoliatus*	Gy	-	+	-	-	+	+	-	+	-	+	-	±	-	+	-	+	W_3
2	*S. anulatus*	Y	-	+	-	+	+	+	+	+	-	-	+	+	-	+	-	+	W_5
11	*S. cyaneus*	Gy	-	-	-	±	-	+	+	+	-	+	-	-	-	+	+	-	W_8
1	*S. albiflavus*	Y	-	-	+	+	+	+	+	+	-	-	+	+	-	+	+	+	F_1
11	*S. cyaneus*	P	-	+	+	+	-	+	+	+	-	-	+	+	-	-	+	-	F_4
21	*S. antibioticus*	Gy	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	F_5
11	*S. cyaneus*	CR-W	-	-	-	+	-	+	+	+	-	+	±	±	+	-	±	-	F_{10}
3	*S. halstedii*	Gy	-	-	-	+	+	-	±	-	+	+	+	+	+	+	+	+	F_{20}
12	*S. diastaticus*	W	-	+	+	-	-	+	±	+	-	+	-	±	+	-	+	+	F_{24}
1	*S. albiflavus*	W	-	-	+	+	-	+	-	±	+	±	+	+	±	+	+	-	F_{11}
8	*S. chromofuscus*	Gy	-	-	-	-	-	+	+	+	-	±	+	±	±	-	+	-	F_{12}
6	*S. fulvissimus*	R-B	-	-	+	-	-	-	+	+	-	-	±	-	-	-	±	+	F_{11}
8	*S. chromofuscus*	P	-	-	-	+	-	±	±	+	-	+	+	+	+	+	+	±	F_{16}

+: Positive, ±: Weakly positive, -: Negative, W: Colonies isolated in winter, F: Colonies isolated in autumn, R: Red, Gy, Grey; p: Pink, R-B: Reddish brown, Y: Yellow; W: White, CR-W: Creamy white. 1 = aphE, 2 = strA, 3 = Antibiosis activity, 4 = L-hydroxyproline, 5 = L-histidine, 6 = L-phenylalanine, 7 = L-valine, 8 = L-cystein, 9 = Xylitol, 10 = D-Melibiose, 11 = Adonitol, 12 = Raffinose, 13 = L-Rhomnose, 14 = Meso-Inositol, 15 = Mannitol, 16 = Sucrose

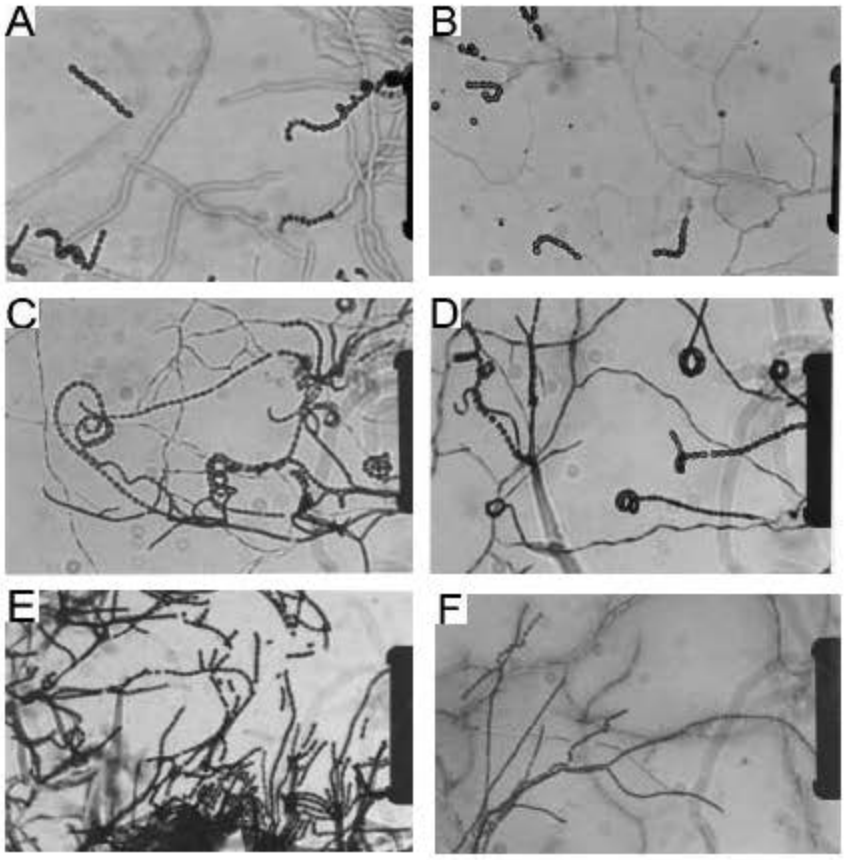

Fig. 1: Microscopic view of some streptomycetes isolates. (A) and (B) show a hook-like shape isolates that was seen in some soil samples such as W8. (C) shows a short and flexuous spore chain that was seen in sample F5. (D) shows spiral spore chain of sample F_3, (E) shows long spore chain of sample F_{24} and (F) shows long spore chain of sample F_{20}. Pictures were taken using 100x magnification

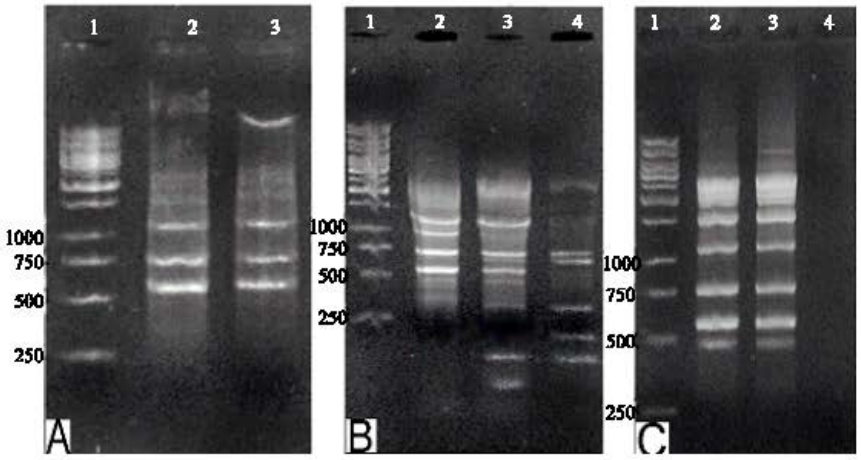

Fig. 2: PCR amplification of 671 bp aphE gene of *S. griseus* (PTCC 1125). PCR amplification of the aphE gene carried out under different $MgCl_2$ concentrations and annealing temperatures. (A) Lane 1: 250 bp molecular WM; Lane 2: aphE with 2 mM of $MgCl_2$; Lane 3: aphE with 4 mM of $MgCl_2$. (B) Lane 1: 250 bp molecular WM; Lanes 2, 3 and 4 are amplified aphE gene at annealing temperatures of 59, 62.6 and 65°C, respectively. (C) Lane 1: 250 bp molecular WM; Lanes 2, 3 and 4 are amplified aphE gene at annealing temperature of 58, 59 and 62.6°C, respectively

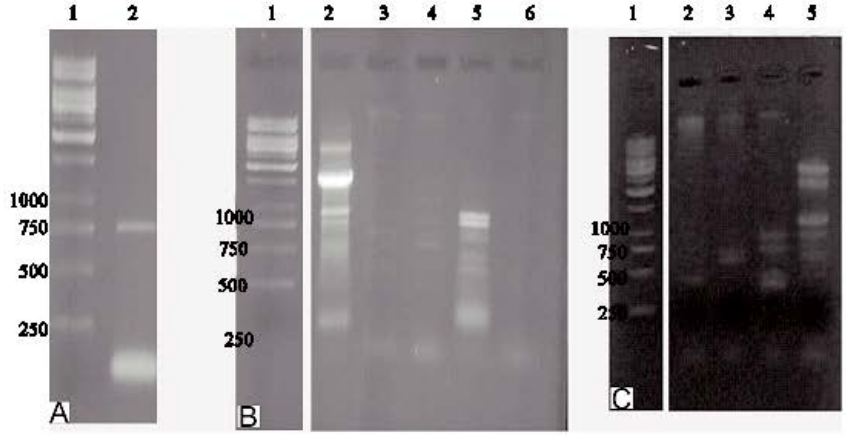

Fig. 3: PCR amplification of 924 bp strA gene of *S. griseus* (PTCC 1125). (A) Lane 1: 250 bp molecular WM; Lane 2, srtA with 1.5 mM of $MgCl_2$. (B) Lane 1: 250 bp molecular WM; Lane 2: *S. griseus* (PTCC 1125); Lane 3: sample W_1; Lane 4, sample W_3; Lane 5: sample W_5 Lane 6: sample W_8. (C) Lane 1: 250 bp molecular WM; Lane 2: sample F_3; Lane 3, sample F_4; Lane 4: sample F_5; Lane 5: sample F_{26}

(F_3, F_4, F_5, F_{26}) samples that matched with the major s. anulatus cluster or showed antimicrobial activity were screened for the presence of strA and aphE genes using PCR.

Molecular detection: Using s. *griseus* (ATCC 11429/PTCC 1125) as positive control, the presence of aphE gene and strA genes were examined in various soil samples. Amplification of aphE gene in control bacteria was carried out under different magnesium concentrations and annealing temperatures. As shown in Fig. 2A, the expected band 671 bp belonging to aphE gene was observed in control bacteria at both 2 mM and 4 mM $MgCl_2$ (lanes 2 and 3). Lower concentrations of magnesium did not give any bands (data not shown).

Amplification of aphE gene in presence of 2 mM $MgCl_2$ and different annealing temperature was carried out next (Fig. 2B and C). Annealing temperature of 59°C gave a pair of clear bands at about 700 bp (Fig. 2B, lane 2). Raising annealing temperature to above 59°C gave either extra bands or no bands at all (Fig. 2C). DNA from the 10 colonies was screened by PCR for the presence of aphE gene, however, none of these samples showed the expected bands.

PCR amplification of strA gene in control bacteria was carried out in present of 1 to 4 mM $MgCl_2$ and annealing temperature of 57°C (data not shown). Under the optimal condition of the 2 mM $MgCl_2$, a band of 924 bp was observed in control bacteria (PTCC 1125). This band was present in some soil samples (Fig. 3B, W1, W5: Fig. 3C, F5, F26) and was not present in others (Fig. 3B, W2, W3 and W8, Fig. 3C, F3 and F4).

DISCUSSION

The aim of this study was to identify the active strains of *Streptomyces griseus* from various samples of Iranian soils using biochemical and molecular techniques. In addition to many modern sensitive molecular technologies, traditional methods based on morphology and biochemical properties seem to keep their importance in *Streptomyces* detection. Distinguishable morphological factors such as the size of colony, pigmentation and the colony status on the plate were used to separate the *Streptomyces* species from the others in first step. Using the above mentioned criteria, together with the microscopic observations, the total of 50 colonies was screened and 19 were found to be of *Streptomyces* origin. Large spores, mode of branching and non-segmented mycelia were among the most prominent factors for discriminating *Streptomyces* spp. from other genera within *Actinomycetes* family. Although macroscopic and microscopic detections are fast and easy ways for discrimination, differentiation between *Streptomyces* and some species such as *Nocardiodes* could be difficult for an inexperienced eye. This fact reduces the reliability of microscopic-based method.

Using 41-criteria matrix of biochemical test, 16 out of 19 primarily-detected *Streptomyces* colonies matched the major clusters' criteria. However, the biochemical tests could not be fully relied on, as so many natural soil habitats do not form clusters with recognized reference strains (Huddleston, 1997), as it happened in our study either. Morphological and biochemical results showed a close resemblance between *S. griseus* ATCC 11429 and *S. anulatus* cluster group which confirms finding of Kampfer *et al.* (1991) about synonymy of *S. anulatus* and *S. griseus* cluster (Kampfer *et al.*, 1991).

PCR technique was used to further identification of colonies that were found to be a member of *S. anulatus* cluster group and colonies with positive antimicrobial activity. In 10 isolates which were screened for the presence of aphE gene, we found no obvious band related to this gene. The results of strA amplification showed that some soil isolates possessed strA gene and therefore, they could be considered as potential producer of streptomycin or related genera. The majority of strains possessing strA gene, have streptomycin biosynthesis genes as well (Tolba *et al.*, 2002). Genes for resistance and biosynthesis have been shown to coexist in one gene cluster (Piepersberg, 1995). This gene cluster comprises of many resistance genes including strA gene (Huddleston, 1997). Although AphE and strA genes were present in *S. griseus* ATCC 11429 as our standard strain and producer of streptomycin, the possibility of horizontal gene transfer should not be forgotten as resistance genes can be transferred even to non-producing strains (Anderson and Wellington, 2004). So it is not unusual that in some strains which were not related to *S. anuluatus* cluster group, strA band was seen. However, S. *griseus* is the dominant soil bacteria which possessed resistance genes. Other genera may receive only a part of biosynthesis gene cassette (resistance genes).

In spite of PCR limitations, this method is more sensitive than culture method in detection of Streptomyces. As Amann *et al.* (1995) reported only 0.001-15% of microbial population in environmental samples is detectable by culture method. Great variability in *Streptomyces* characteristics and differences between taxonomic criteria exacerbates this problem. Although culture method seems to keep its importance, the accuracy and specifity of PCR amplification suggest more dominant role than culture method in future screening projects and a more proper tool for solving the dilemma of *Streptomyces* classification. For such a screening, amplification of 16s rRNA, DNA-DNA hybridization, randomly amplified polymorphic DNA (RAPD) PCR assay and other genotypic approaches are taking over traditional ways.

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