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Molecular identification of streptomycetes by exploiting RNA polymerase beta-subunit (*rpoB*) gene in Saudi arabia

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Abstract

This study was designed with the aim of exploring the efficacy of RNA polymerase beta subunit, *rpoB* gene analysis for the identification of streptomycetes. A characteristic marine environment distinguished by their content of streptomycetes were targeted, and two organisms of the genus *Streptomyces* were isolated in Saudi Arabia. The two *Streptomyces* spp., named isolates EH1 and EH2 were isolated and subjected to the regular phenotypic characterization including morphological and physiological studies, analyses of cells hydrolysates, detecting their antibacterial activities and pocks formation ability. These isolates showed inhibitory activity of gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*. The two isolates gave positive results in pocks formation test, which indicated that both must belong to the genus *Streptomyces.* Molecular identification of both isolates was accomplished through analysis of the beta-subunit of the RNA polymerase gene (*rpoB*) to assist in species identification. The target fragments 352 bp were amplified and detected in both isolates using agarose gel electrophoresis, indicating that they belong to the genus *Streptomyces*. The amplified product of the isolate EH1 was sequenced and 296 bp continuous sequence was determined (GenBank accession MK569762.1). By matching the obtained sequence with the *Streptomyces* DNA sequence databases, the isolate EH1 was found very similar to *Streptomyces labedae*. The study concluded with the possibility of using molecular analysis of RNA polymerase beta subunit gene to correctly identify *streptomycetes* to the species level.

Keywords: Molecular identification; Streptomycetes; *rpoB* gene; RNA polymerase; *Streptomyces labedae*

1. Introduction

The genus *Streptomyces* belongs to the filamentous bacteria with the well-known property of producing antibiotics, which can be isolated from many habitats, including marine habitats [1]. Marine environments are rich in bacteria distinguished in their phenotypic and genetic characteristics, which makes the exploration of these habitats for such organisms of great importance. Streptomycetes are unique group of bacteria that the ideal identification of them have raised a lot of controversy through decades. Despite the importance of conducting all the generally accepted identification tests, they always lack proper identification at the species level, which calls for the use of distinctive genetic markers and exploring their ability in a more correct identification. Molecular identification of this genus by detecting (rpoB) gene has proven very constructive compared to the other common targeted genes as 16S ribosomal RNA (16S rRNA) [2]. Also, *Streptomyces* frequently possess linear plasmids, which is a significant feature of this genus [3]. Among bacteria, conjugation in *Streptomyces* is a characteristic phenomenon in its mode of genetic exchange and in the resulted growth morphology. On agar cultures mixing of two streptomycetes, one of which carries a conjugative transferable plasmid, gives a characteristic mode of growth inhibition of the recipient organism. This characteristic delayed growth is called pocks formation and corresponds to the zones of recently attained plasmids [4]. Also, early studies indicated that this process takes place in natural environments [5]. Conjugation in *Streptomyces* usually involves

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the transfer of the intact whole plasmid molecule [6]. Also, many reviews about the mechanisms of gene transfer in *Streptomyces* have been discussed [7]. So, the aim of the study was to isolate streptomycetes from marine habitat and identify them. Through the course of identification all the possible standard methods were applied. Here, a great deal of reliance was placed on the ability of streptomycetes that contain plasmids and can form pocks and detect them to ascertain the genus, as this trait can be detected phenotypically in this genus only. Ultimately, analysis of the beta-subunit of the RNA polymerase gene (*rpoB*) to assist in identification to the genus and species levels were followed.

2. Material and methods

2.1. Sampling and isolation

Sediment samples were collected according to Sivakumar *et al.* [8] from mangrove marine area dominated by *Avicennia marina* vegetation with the coordinates of Latitude, 19.5390 and Longitude, 41.0495, Al-Qunfudhah city in KSA. The agar medium employed for isolation was starch-nitrate by soil dilution plate technique.

2.2. Phenotypic identification of the isolates

To achieve this goal, standard methods mentioned in Kämpfer [9] have been followed, including morphological, cell-hydrolysate, phospholipid analysis, physiological studies, antibacterial activity, and detection of pocks formation ability and plasmid presence.

2.3. Morphological studies

The morphological characteristics of the selected isolates have been studied through an inorganic-salts starch agar, along with the coverslip culture technique. Scanning Electron Microscopy (SEM) preparations were also performed for the selected isolates.

2.4. Analysis of cells hydrolysate and phospholipid type

These analyses were done to detect the cell wall peptidoglycan type and to assign the type of phospholipid.

2.5. Physiological studies

All physiological tests were performed according to the standard methods for streptomycetes.

2.6. Antibacterial activity

The isolates were allowed to grow in liquid starch-nitrate for 10 days at 30 ^{o}C under shaking (200 rpm). The agar diffusion method was employed to detect antibacterial activity according to Abdllha *et al*. [10]. The used test organisms were as follows; gram-positive bacteria, *Staphylococcus aureus* (NCTC7447) and *Bacillus subtilis* (NCTC10400); and gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC10145) and *Escherichia coli* (NCTC10416).

2.7. Pocks formation and plasmid isolation

The selected isolates have been inoculated onto starch-nitrate agar medium plates, to test pocks-forming ability, according to Grohmann *et al*. [4]. The isolates have mixed and incubated for three days at 30^{o}C. Then, the mixed cultures were observed during incubation to detect the formation of the growth-retardation zones. The selected isolates have then inoculated into Tryptone-Yeast-extract broth medium (TY-medium) and incubated at 30^{o}C under shaking conditions for only 24 hours. The growth has been collected in the form of a pellet, which then processed for plasmid isolation using alkaline lysis for the detection of circular plasmids according to Lezin *et al*. [11].

2.8. Molecular identification

2.8.1. DNA extraction and purification

DNA extraction was performed using phenol extraction and isopropanol precipitation according to Kim *et al*. [12]. The isolates have previously grown at 30 °C for 24 hours. The growth medium was PY (Peptone Yeast extract) supplemented with 1% Starch. The extracted DNA was purified with centrifugation (15,000 rpm) and precipitation with ice-cold ethanol (70 %), then, stored at -80 °C.

2.8.2. PCR amplification of rpoB gene

PCR amplification was conducted according to Kim *et al*. [12]. The primers used were SRPOF1 and SRPOR1, being specific for *Streptomyces*, they produce 352-bp PCR product. The reaction contained 2 µL of the extracted DNA suspended in TE buffer (pH 8.0). Twenty picomoles of every primer were used along with the dNTPs and one unit of *Taq*-polymerase. The total volume of the reaction was 20 µL, and the amplification was completed for thirty cycles at the exact temperatures using a thermal cycler (Perkin Elmer Cetus, 9600). Gel electrophoresis was run on a three percent agarose and the amplified products were visualized under ultraviolet light. The DNA marker used was Promega, φx174.

2.8.3. Sequencing of rpoB gene

Sequencing of the gene was conducted according to Kim *et al*. [12] using the same primers mentioned above, SRPOF1 and SRPOR1. Purification of the obtained products was done first. The reaction contained two microliters of the amplified DNA, three picomoles of each primer (forward or reverse), and eight microliters terminator (Applied Biosystems). The total volume of the reaction was twenty microliters, and the sequencing was completed for thirty cycles at the exact temperatures in a sequencer (Applied Biosystems, 377A).

2.8.4. Evolutionary analysis

The obtained sequences with the forward and the reverse primers were aligned to get one superposed partial continuous sequence which was then registered in NCBI. The evolutionary analyses were achieved by the neighbor joining method along with the maximum likelihood method. Evolutionary analyses have conducted by MEGA-X [13].

3. Results

3.1. Isolation of the streptomycetes, isolates EH1 and EH2:

Two isolates have been obtained from the collected mangrove sediment samples. The two isolates named EH1 and EH2 have been purified and stored for subsequent experiments.

3.2. Phenotypic identification of the isolates

The cell wall peptidoglycan belonged to type I (LL-diaminopimelic acid and glycine. Phosphatidylethanolamine was the only characteristic lipid detected, so, the phospholipid type (PII) has assigned for the two isolates. The spore chain of the isolate EH1 is characterized by spirals formation with a spiny spore surface. In contrast, the isolate EH2 is characterized by rectiflexibles formation with a smooth spore surface (Figure 1). Results of the physiological and biochemical studies presented in (Table 1) revealed that the isolate EH1 was aerobic. This isolate has a hydrolytic activity of protein and chitin. Also, the isolate has an efficient degradation of cellulose and NaCl tolerance up to 13 % w/v. Growth was recorded at incubation temperature 45°C. This isolate showed resistance to streptomycin in concentration of 100 ppm. Also, the results of the physiological and biochemical studies presented in (Table 1) revealed that the isolate EH2 was also aerobic. This isolate has a hydrolytic activity of protein and chitin. Also, the isolate has an appropriate cellulose degradative activity and tolerated NaCl concentration up to 10 % w/v. the growth of this isolate was not recorded at incubation temperature 45°C. This isolate was sensitive to streptomycin in concentration of 100 ppm. Based on phenotypic studies, the two isolates have been identified as *Streptomyces*.

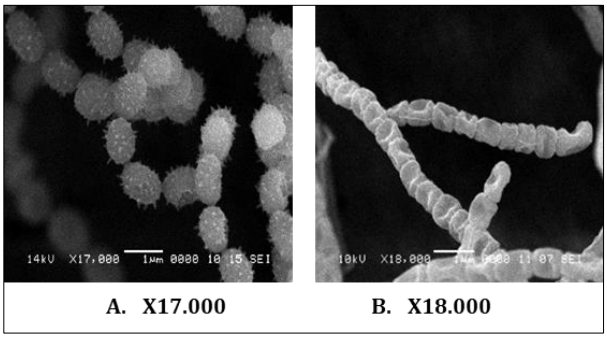

Figure 1 SEM of the isolates (A) EH1 and (B) the isolate EH2

Table 1 Physiological and biochemical characteristics of the isolates EH1 and EH2

Character	**Character State**		**Character**	**Character State**	
	EH1	**EH2**		**EH1**	**EH2**
Aerobic growth	+	+	Growth temperatures and pH:		
Enzyme Activity:			Growth at 10 °C	-	-
Lipase	+	+	Growth at 37 °C	+	+
Protease	+	+	Growth at 45 °C	+	-
Hydrolysis of chitin	+	+	Growth at pH 4.3	-	-
Hydrolysis of pectin	+	-			
Degradation Of:	**EH1**	**EH2**	**Growth in presence of Inhibitory compounds (% w/v):**	**EH1**	**EH2**
DNA	+	-	Crystal violet (0.0001)	+	+
RNA	+	-	Phenol (0.1)	+	+
Starch	+	+	Sodium azide (0.02)	+	+
Gelatin	+	+	Sodium chloride (7)	+	+
Tween 80	+	+	Sodium chloride (10)	+	+
Xylan	+	+	Sodium chloride (13)	+	-
Cellulose	+	+	Streptomycin (100 ppm)	R	S
Nitrogen utilization (0.1 % w/v)			Carbon utilization (1.0 % w/v)		
	EH1	**EH2**		**EH1**	**EH2**
L-arginine	+	+	L-arabinose	+	+
L-cysteine	-	-	Cellobiose	+	+
L-histidine	+	+	Dextran	+	+
L-hydroxyproline	+	+	D-fructose	+	+
L-methionine	+	+	D-galactose	+	+
Potassium nitrate	+	+	*meso*-Inositol	+	-
L-phenylalanine	-	-	lactose	+	+
L-serine	+	+	D-mannitol	+	+
L-threonine	+	-	D-mannose	+	+
L-valine	+	+	D-xylose	+	-
L-tyrosine	+	+	Raffinose	+	-
Urea	+	+	Sucrose	+	-

(+): Positive result; (-): Negative result; (R): Resistant; (S): Sensitive

3.3. The antibacterial activity of the isolates

The two isolates inhibited the gram-positive bacteria *S. aureus* and *B. subtilis* with inhibition zone diameters achieved 23 mm. No activity was recorded in case of the gram-negative bacteria *P. aeruginosa* and *E. coli*.

3.4. Pocks formation and plasmid isolation

Figure (2) shows the growth of the isolates EH1 and EH2 on the starch-nitrate agar plate. It can be observed, macroscopically, the growth of the two isolates together on this medium with characteristic growth-retardation zones of the isolate EH2 forming special structures called pocks. These pocks are characteristic for only the genus *Streptomyces* and give direct evidence for the existence of an auto transportable plasmid within the genetic composition of the isolate EH1, while the isolate EH2 should be devoid of such a plasmid. Detection of conjugative plasmids by pock formation by the transconjugants is considered one of the most important modes of gene exchange in *Streptomyces* commonly employed. The transport of such a plasmid must have accompanied by the over-expression of the specific transfer genes yielding transfer proteins. These proteins are toxic to the cells of the recipient mycelium (the isolate EH2) and led to the visually seen pocks formed. Within these pock structures, the growth of the isolate EH1 (Donor mycelium) can be noticed (Figure 2) in a lawn of the growth of the isolate EH2 (Recipient mycelium). Usually, the process ceases after 12 hours only from the beginning of transfer by the transconjugants. This is observed after 24 hours of incubation of both isolates together (Figure 3). Using the previous mixed culture of the isolate EH1 and EH2 grown on starch-nitrate agar plates, a subculture of both isolates has picked-up (from the edges of the grown mycelia of both isolates within the pock structure) on a new starch-nitrate agar plate. Cultural appearances of the wild type and their subcultures did not show any differences. Also, the two derivatives of both isolates were active against the tested bacterial cultures. Consequently, a preliminary plasmid isolation has carried out for both the wild-type isolates (EH1 and EH2) and their subcultures. The results showed the absence of any circular plasmids in all tested isolates. So, the plasmid isolation experiment gives indirect evidence to the presence of linear plasmid within the mycelium of the isolate EH1. It seemed that isolation using the usual alkaline lysis for the detection of circular plasmids was unable to recover linear DNA.

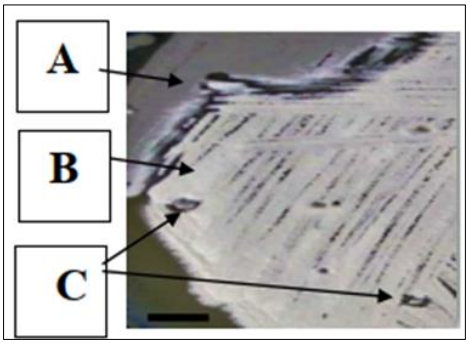

Figure 2 Starch-nitrate agar plate showing the growth of the isolate EH1 (A), the isolate EH2 (B), and the growth retardation zones of the isolate EH2 (Pocks formation) (C). Bar, 1 cm

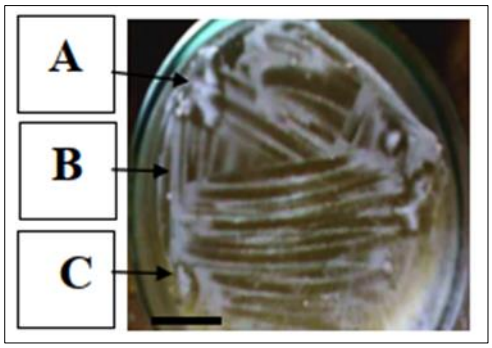

Figure 3 Starch-nitrate agar plate showing the growth of the isolate EH1 (A), the isolate EH2 (B), and the developing growth retardation zones of the isolate EH2 after 24 hours of incubation (C). Bar, 1 cm

3.5. Molecular Identification of the isolates

Molecular identification of the isolates revealed that the two isolates were *Streptomyces* since the target (352 bp) *rpoB* fragments have been generated from both the isolates and detected by agarose gel electrophoresis (Figure 4). The obtained fragment of the isolate EH1 only was successfully sequenced, and (296 bp) continuous sequence has been determined. Figures (5 and 6) show the constructed phylogenetic trees of the partial sequence of the isolate EH1. By matching the obtained sequence with NCBI Genomic Reference for *Streptomyces* DNA nucleotide sequence databases,

the isolate EH1 (GenBank accession MK569762.1) showed a high percent identity to *Streptomyces labedae* (GenBank accession JF424010.1). Figure (7) shows the sequence of the isolate EH1 aligned with the sequence of the closely related *Streptomyces labedae*.

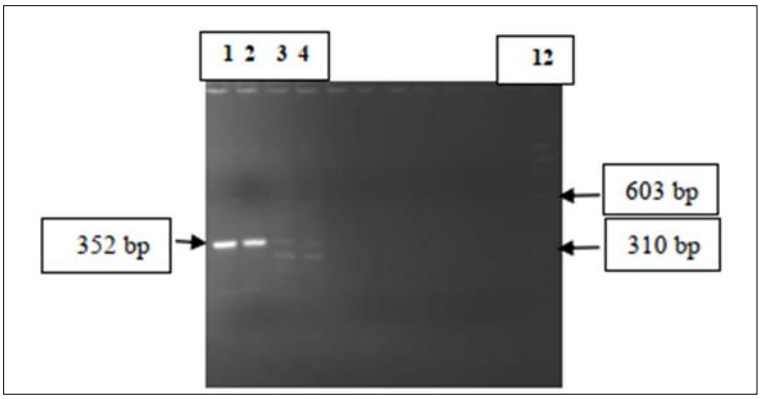

Figure 4 Agarose-gel shows electrophoresed PCR amplicons from isolates EH1 and EH2.Lane 1 & 2: Samples of isolate EH1. Lane 3 & 4: Samples of isolate EH2. Lane 12: Sample of the used marker (φx174)

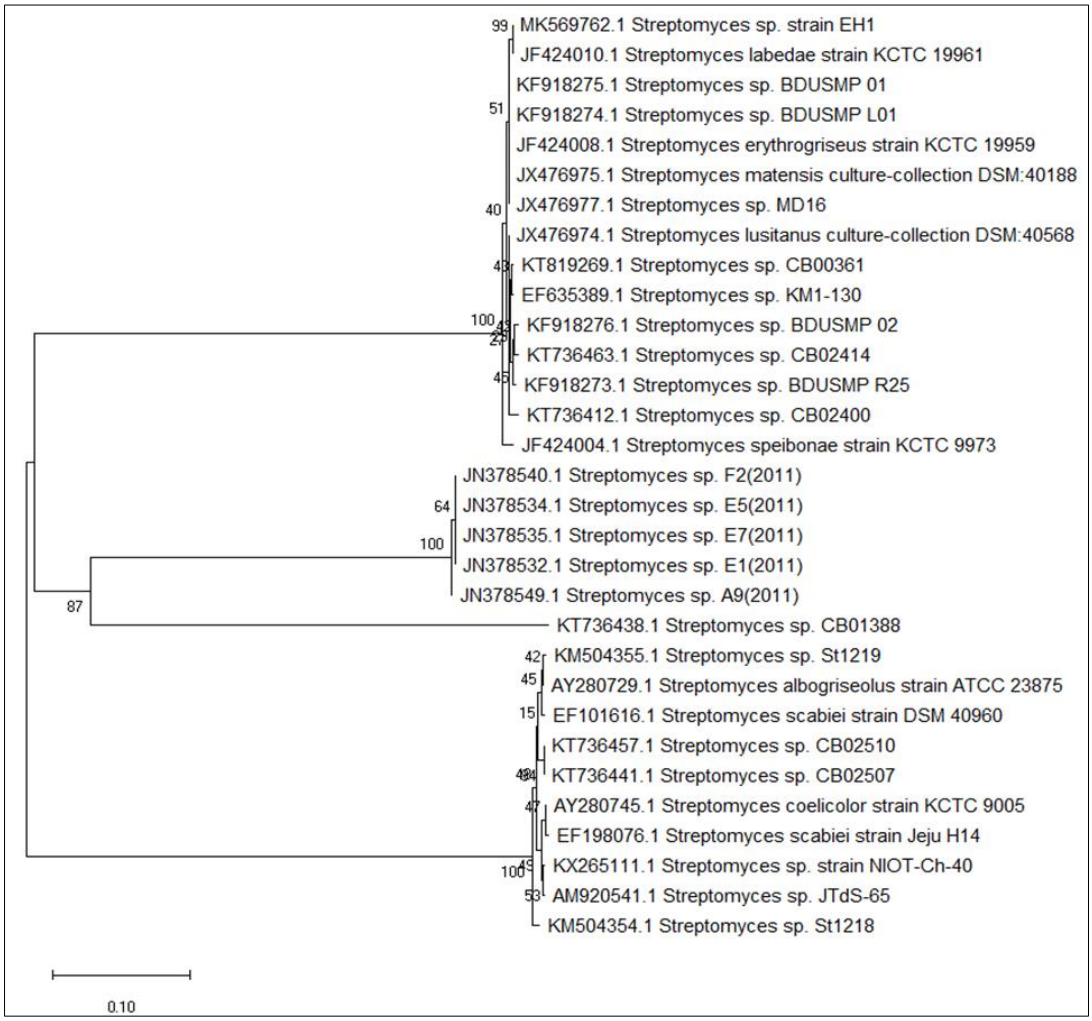

Figure 5 Neighbor-joining tree of the partial *rpoB* gene nucleotide sequence (296 bp) of the isolate EH1 (accession MK569762.1) and nucleotide sequences retrieved from GenBank. Numbers represent bootstrap percentage values based on 1000 replicates

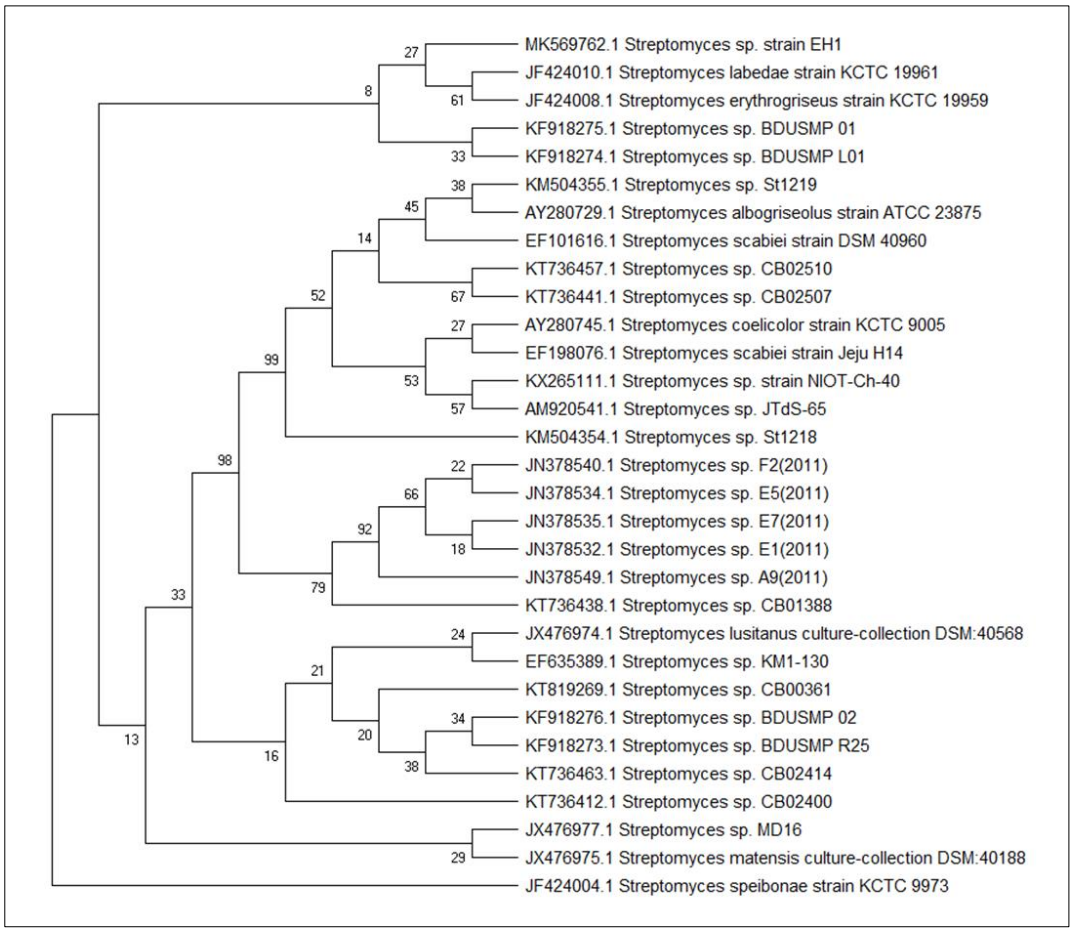

Figure 6 Maximum-likelihood tree of the partial rpoB gene nucleotide sequence (296 bp) of the isolate EH1 (accession MK569762.1) and nucleotide sequences retrieved from GenBank. Numbers represent bootstrap percentage values based on 1000 replicates

Streptomyces labedae strain KCTC 19961 RNA polymerase subunit B (rpoB) gene, partial cds

Sequence ID: **JF424010.1** Length: 993 Number of Matches: 1

Range 1: 4 to 299

Score	Expect	Identities	Gaps	Strand	Frame
547 bits(296)	2e-151()	296/296(100%)	0/296(0%)	Plus/Plus	

```
Query  1    GTCCGTACGGGTCTCGCCCGTATGGAGCGCGTCGTGCGCGAGCGCATGACCACCCAGGAC  60
Sbjct  4    ............................................................  63

Query  61   GTCGAGGCGATCACGCCGCAGACCCTGATCAACATCCGGCCGGTCGTCGCCTCCATCAAG  120
Sbjct  64   ............................................................  123

Query  121  GAGTTCTTCGGCACCAGCCAGCTGTCCCAGTTCATGGACCAGAACAACCCGCTGTCGGGG  180
Sbjct  124  ............................................................  183

Query  181  CTGACGCACAAGCGTCGTCTGAACGCCCTCGGCCCGGGTGGCCTCTCCCGTGAGCGGGCC  240
Sbjct  184  ............................................................  243

Query  241  GGCTTCGAGGTCCGTGACGTGCACCCCTCGCACTACGGCCGCATGTGCCCGATCGA  296
Sbjct  244  ..........................................................  299
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Figure 7 The aligned sequence of the isolate EH1 (Query) and the closest strain in the GenBank *Streptomyces labedae* (Subject)

4. Discussion

Among actinomycetes, members of the genus *Streptomyces* account for a high percentage of secondary metabolites, including antibiotics and bioactive compounds. These compounds are not necessary for the growth of the producers and produced at the end of vegetative growth. Much research has been focused on investigating the activity of

microorganisms from unique marine habitats [14]. *Streptomyces* species with antibacterial activity against many microbes of medical concerns such as *Vibrio* infections have been reported from numerous studies [15]. Here, we report the isolation of streptomycetes from mangrove marine sediments. All the available standard methods were used to identify the isolates at the genus level. Nevertheless, it is always difficult to differentiate the different species from each other. Accordingly, it was confirmed that the two isolates under study belong to the genus *Streptomyces* through the experiments that were conducted to determine the phenotypic characteristics. In addition, relying on the pocks formation test, as this test is distinguished only in the genus *Streptomyces* and all bacterial genera do not give any results in this test except for the genus *Streptomyces*. Crosses between the two isolates EH1 and EH2 on starch-nitrate agar plates revealed the macroscopically seen characteristic growth-retardation zones of the isolate EH2 forming pocks. Plasmid isolation and detection results showed the absence of any circular plasmids in all tested isolates. As the isolation of circular plasmids can be achieved easily when present in *Streptomyces* species and linear plasmids are abundant in *Streptomyces* [16], so, the plasmid isolation experiment gives indirect evidence to the presence of linear plasmid within the mycelium of the isolate EH1. Conjugation systems in *Streptomyces* are under different regulation from conjugation systems in other gram-positive bacteria [17]. Frequently, the plasmid transport occurs upon the physical contact of hyphae [18]. The primary transfer event and the subsequent intramycelial spreading are usually have mediated by the transport protein with the other several spread-proteins [7, 19, 20]. All these studies reveal the importance of *Streptomyces* linear plasmids. Such plasmids in *Streptomyces* are very important when employing cloning and integration vectors [21]. In previous studies, analysis of the *rpoB* gene has been proven functional in the identification and differentiation of streptomycetes species [12, 22, 23]. So, in our study, analysis of this gene was used in the molecular identification of our *Streptomyces* isolates. Through the PCR only this gene can identify the genus since it is a single copy gene. Also, the partially sequenced *rpoB* gene of the isolate EH1 revealed the high sequence similarity of the isolate EH1 to *S. labedae*.

5. Conclusion

This study revealed the importance of molecular identification in addition to the phenotypic studies to describe *Streptomyces* genus. To assign the species, the partial sequencing of the *rpoB* gene was very effective. The study provided a new insight in the potential of using molecular analysis of single copy genes more efficiently in identification of streptomycetes, as this group of filamentous bacteria are ubiquitous and have important roles in nature.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors hereby acknowledge that there is no conflict of interest.

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