Screening of actinomycetes isolated from the soils of the Beni Amir region of Morocco by the 16S sequencing of the rDNA gene

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Abstract— 30 actinomycete strains isolated from the soil of the Beni Amir region were molecularly identified by sequencing the 16S rDNA fragment. After DNA extraction from isolates, amplification of 16S rDNA fragments by PCR (Polymerase Chain Reaction) technique, sequencing of amplified fragments and comparison of the obtained characteristic sequences with the content of a database, phylogenetic studies made it possible to develop the phylogenetic trees of the 30 isolates. The analysis of the obtained results showed the taxonomic affiliation of the isolates to the genus Streptomyces and allowed to assign each of them to the species. On the phylogenetic tree the isolates form seven phylogenetic groups the largest group comprises 33% of the isolates that are placed on the same phylogenetic branch as *Streptomyces griseorubens*. The second phylogenetic group is composed of 30% occupies the same phylogenetic branch with *Streptomyces bellus*. 20% are grouped with *Streptomyces albogriseolus* forming the third phylogenetic group. 6.66% are affiliated with *Streptomyces lilaceus*. 3.33% show similarity to Streptomyces rochei strains, respectively. *Streptomyces labedae*. Streptomyces azureus. This heterogeneous distribution can be explained by the difference in the physicochemical characteristics of the soils of the Beni Amir region.

Index Terms— Molecular identification, Sequencing, rDNA, Phylogenetic trees, Streptomyces.

1 Introduction

The identification of actinomycete genera is mainly based on the study of several morphological characters corresponding to the presence, the abundance and the disposition of the hyphae of the substrate mycelium or aerial mycelium, the presence of spores, their number, their mobility, their shape, their position on the hyphae, the presence of sporangia of sclerotia, or synnemata. These classical methods of bacterial identification based on the determination of cultural, physiological and metabolic characteristics have shown their limits, in particular, for the detection of non-culturable microorganisms [1]. They only led to the description of a very small part of the microbial diversity. These methods of identification have been abandoned by several researchers to replace them with molecular techniques

These molecular approaches are often used for their speed and efficiency [2]-[3]-[4]-[5] Molecular taxonomy has been implemented since the 1980s and involves the application of genetic and molecular analysis methods, including determination of the percentage of GC [6], DNA-DNA hybridization [7] and sequencing of 16S ribosomal RNA [8]-[9]-[10]. These techniques made it possible to trace all the phylogeny of bacteria in particular, that of actinomycetes.

Gupta and Woese (1983) [11] began to study and sequence 16S rDNA genes of different bacteria using DNA sequencing, a state that good technology at present and used sequences for phylogenetic studies.

In different cases, sequencing of the 16S rRNA gene was primarily used to distinguish between Actinomyces and Actinomyces anaerobic Gram-positive bacilli, which is often difficult in clinical microbiology laboratories [12]-[13].

In these early works, the results showed a wide dissemination using PCR and DNA sequencing, 16S rDNA sequencing plays a vital role in the proper identification of bacterial and fungal isolates and also discovery of new strains in the fields of clinical microbiology. By the use of sequencing of 16S rDNA, there are many organisms identified as new bacterial species and belong to genera have been discovered so far [14].

The 16S rDNA gene that encodes 16S rRNA (16S ribosomal RNA) is one of the best preserved genes among prokaryotic organisms (Eubacteria and Archaea). It has been chosen as a phylogenetic marker, providing an effective and reliable basis for comparison to compare and differentiate between bacteria. Indeed, the 16S rDNA:

- Contains well conserved internal sequences that allow the selection of universal primers for the amplification of 16S rDNA of existing bacteria;
- Includes variable internal sequences that, once analyzed, distinguish species of bacteria from one another and classify them according to their phylogeny;
- Is of a sufficiently short size (~ 1500 bp) to be analyzed quickly.

The soils of the region of Beni Amir which are character-

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ized by a high salinity rate were studied for the first time by a work that was done in our laboratory [15].In this study we isolated 80 strains of actinomycetes from these soils [16] .These strains have undergone morphological, physiological and biochemical identification. In this work, of these 80 actinomycete strains, 30 were molecularly identified by sequencing 16S rDNA.

2 Materials and Methods

2.1 Actinomycete strains

The isolation of actinomycetes strains was done in the laboratory of bioprocess and Bio-interfaces of the faculty of sciences and techniques of Beni mellal by the classical methods [15]-[16]. Molecular identification by sequencing of the 16s rDNA was carried out at the Tropical & Mediterranean Symbioses Laboratory. IRD / CIRAD / INRA / SupAgro / UM2 International Campus of Baillarguet Montpellier France. These strains were selected and grouped according to their salinity tolerances (Table 1)

TABLE 1
ISOLATES OF ACTINOMYCETES SELECTED FOR MOLECULAR IDENTIFICATION

Isolates	Origin
A30	Sol 6
A44	Sol 6
A46	Sol 4
A49	Sol 10
A50	Sol 6
A53	Sol 1
A57	Sol 4
A58	Sol 2
A60	Sol 6
A63	Sol 1
A64	Sol 4
A65	Sol 1
A76	Sol 2
A79	Sol 11
A3	Sol 5
A7	Sol 5
A10	Sol 10
A14	Sol 8
A15	Sol 7
A23	Sol 11
A43	Sol 9
A61	Sol 8
A27	Sol 6
A33	Sol 4
A41	Sol 11
A26	Sol 8
A36	Sol 3
A80	Sol 6
A22	Sol 1
A34	Sol 10

2.2 Extraction of the DNA from the strains of actinomycetes

The extraction protocol for genomic DNA from actinomycetes isolates was carried out by thermal shock. From a fresh culture, a small fragment of mycelium of actinomycetes is placed in water called sterile milli-q at 50 µl. The tubes are then put at -20 °C for one hour and then they undergo a bursting protocol according to the cycle shown in Fig. 1. 2 µl of the supernatant (containing DNA) are used for the amplification reaction (PCR) chain polymerization reaction.

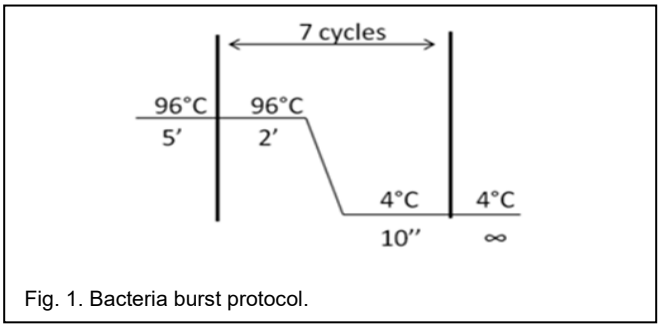

Fig. 1. Bacteria burst protocol.

2.2.1 Amplification of the ribosomal DNA fragment 16s

The amplification was performed by the polymerase chain reaction (PCR). The genes encoding the ribosomal 16S RNA of the strains are amplified by the use of the following primers: FGPS6 (GGAGAGTTAAGATCTTGGCTCA) and FGPS1509 (AAGGAGGGGATCCAGCCGGA) NORMAND et al., 1992 [17]

The amplification was performed in an Applied Biosystems "Verity" thermocycler. Table 2 gives the composition of the PCR reaction medium. The operation of Fig. 2 begins with an initial denaturation at 95 °C for 5 min, then 30 cycles comprising 1 min of denaturation at 95 °C, 1 min of hybridization at 55 °C and 1 min of extension at 72 °C and finally terminated by final elongation at 72 °C for 10 min.

TABLE 2
REACTION MIX AND AMOUNT OF REAGENTS USED FOR A PCR REACTION

Stock	Volume for 1 reaction from volume stock	Concentration of use
Tampon 5X	5	$MgCl_2$ – 1.5 mM
Mix dNTPs 2.5mM	2	200 µM de chaque
Amorce 1 (10 µM)	1.25	0.5 µM
Amorce 2 (10 µM)	1.25	0.5 µM
Taq	0.2	1 U total
H_2O	13.3	

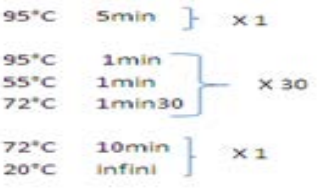

Fig. 2. PCR amplification program of 16S rDNA.

2.3 Electrophoresis on agarose gel

2.3.1 Preparation of the agarose gel

2 g of agarose were mixed with 100 ml of 1 × TAE buffer (Tris base, acetic acid and EDTA). The mixture was heated to boiling, then cooled to about 50 °C. Under water and then a drop of ethanol bromide was added. The preparation was poured into a support provided with an applicator. After gel solidification, the applicator was removed for the wells to appear.

2.3.2 Migration

After amplification, 1 µl of each amplifiât +3 µl of water quality molecular biology +1 µl of bromophenol blue were prepared separately, then placed on the well of the gel for analysis by horizontal electrophoresis for 2 hours at 100 V, in a 1X TAE buffer. In another well, 1 µL of a 100 Ladder Ladder (MW-1700-10) molecular weight marker was deposited as a control DNA.

After migration, the gel was removed from the apparatus for UV observation.

2.3.3 Revelation of DNA bands with UV rays

The agarose gel was then exposed to ultraviolet rays to visualize the DNA bands. The presence of the band that corresponds to the amplified fragment was compared with the (100 base pairs) marker used as a control.

2.4 Purification

The Gel kit SK-GEPU-100 has been used to purify DNA that exhibit parasitic band DNA purification protocol from agarose gel.

2.5 Sequencing

Sequencing was performed according to the automated Sanger [18] technique at the GENOSCREEN Montpellier France Laboratory.

3 Results and Discussion

3.1 Analysis of the 16S rDNA sequence

The results of the sequencing of the actinomycete strains were obtained in the form of crude electrophoregrams under ChromasR software. The alignments of the pair of sense/antisense sequences are performed by Clustal W software to define the consensus sequence.

3.2 Comparison of the sequences with the computer bank

To evaluate the diversity of actinomycetes isolated from the soils of the region of Amir blessed, a PCR method was performed on the 30 isolates using primers FGPS 6 and FGPS1509 [17] which allow the amplification of a 1400 bp fragment which corresponds to the V3 to V5 regions of the 16S rDNA gene [19]. For the sequencing isolates, the sequences obtained are compared with those available in the Gen Bank databases, using the BLAST program (Basic Local Alignment Search Tool) [20] through the National Information Center for Biotechnology (NCBI). Results are expressed as a percentage of similarity of the strain to be identified with the closest species.

The alignment of the nucleotide sequences with those of the database shows that the isolated strains of actinomycetes are classified in the phylum Actinobacteria, class V Actinobacteridae and order I Actinomycetales and all are part of the genus Streptomyces with percentages of similarity variable between 99 and 100% table 3. These results are similar with [21] - [22].

Each isolate is similar to several species in the "Genbank" databank. To affiliate each of the isolates to one of the closest species, a phylogenetic analysis is therefore necessary.

Sequence analysis allowed us to realize a phylogenetic tree using the MEGA 5[23] software by the neighbor-joining method. The reliability of the topology was evaluated by the bootstrap method [24] by making 1000 replicates.

Analysis of this tree shows an assembly of the 16S rDNA gene sequences to the Streptomyces gene species.

The isolates form seven phylogenetic groups on the phylogenetic tree (Fig. 3). The largest group comprises 10 isolates (A63, A23, A15, A80, A3, A14, A79, A64, A22, A33) which are placed on the same phylogenetic branch as *Streptomyces griseorubens*. The second phylogenetic group is consisted of 9 isolates (A44, A46, A49, A7, A10, A43, A61, A41, A34) occupying the same phylogenetic branch with *Streptomyces bellus*. Isolates A 57, A76, A27, A26, A65, A36 are grouped with *Streptomyces albogriseolus* forming the third phylogenetic group. Two isolates (A30, A52) are affiliated with *Streptomyces lilaceus*. The A58, A60 and A50 isolates showed similarity with *Streptomyces rochei strains, Streptomyces labedae and Streptomyces azureus* strains respectively.

The results confirm the abundance of the genus Streptomyces within isolated strains of actinomycetes. These results are in agreement with the literature [25] - [26]. It has been reported that only 0.001-15% of the microbial population present in environmental samples is cultivable [27]. Streptomyces species have the ability to produce a wide variety of extracellular enzymes and antibiotics [28] which may explain their high ability to exist and colonize different environments.

The 16S rDNA gene sequences have played a vital role in microbiology and can be used in a variety of ways in a variety of disciplines, including taxonomy and ecology. This has led to an abundance of information on prokaryotic diversity [29] - [30].

TABLE 3
SPECIES CLOSE TO ACTINOMYCETES ISOLATES

Actionmycetes isolates	close strains	Access number	Percentage of similarity
A30	Streptomyces lilaceus	AB 184457	99%
A44	Streptomyces bellus	Nr_041222	99%
A46	Streptomyces bellus	NR_041222	99%
A49	Streptomyces bellus	NR_041222	99%
A50	Streptomyces azureus	LM644081	100%
A52	Streptomyces lilaceus	AB 184457	99%
A57	Streptomyces albogriseolus	GQ925802	99%
A58	Streptomyces rochei	KC1720301	100%
A60	Streptomyces labedae	KU5003691	100%
A63	Streptomyces griseorubens	EU841556	99%
A64	Streptomyces griseorubens	KY120282	99%
A65	Streptomyces albogriseolus	GQ925802	99%
A76	Streptomyces albogriseolus	GQ925802	99%
A79	Streptomyces griseorubens	KY120282	99%
A3	Streptomyces griseorubens	KY120282	99%
A7	Streptomyces bellus	NR_041222	99%
A10	Streptomyces bellus	NR_041222	99%
A14	Streptomyces griseorubens	KY120282	99%
A15	Streptomyces griseorubens	KY120282	99%
A23	Streptomyces griseorubens	EU841556	99%
A43	Streptomyces bellus	NR_041222	99%
A61	Streptomyces bellus	NR_041222	99%
A27K	Streptomyces albogriseolus	GQ925802	99%
A33	Streptomyces griseorubens	EU841556	99%
A41	Streptomyces bellus	NR_041222	99%
A26	Streptomyces albogriseolus	GQ925802	99%
A36	Streptomyces albogriseolus	KU324449	99%
A80	Streptomyces griseorubens	KY120282	99%
A22	Streptomyces griseorubens	EU841556	99%
A34	Streptomyces bellus	NR_041222	99%

The results showed a heterogeneous distribution of strains in the 11 soils studied. *Streptomyces griseorubens* strains were found in soils 1; 11; 7; 6; 5; 8; 4,*Streptomyces bellus* in soils 6; 4; 10 ; 5; 9; 8; 11,*Streptomyces albogriseolus* in soils 1; 2; 6; 8; 3; Streptomyces lilaceus in soils 6; 1,*Streptomyces rochei in soil 2; and Streptomyces labedae, Streptomyces azureus* in soil 6.

This distribution can be explained by the difference in the physicochemical characteristics of the soils of the region of Beni Amir (Morocco) which have a high salinity level. These results are semilarar to those of Zanane et al., (2018) [16] who showed that the diver- sity of actinomycetes is associated with the physicochemical characteristics of the soil, namely salinity, organic matter, moisture and Ph.

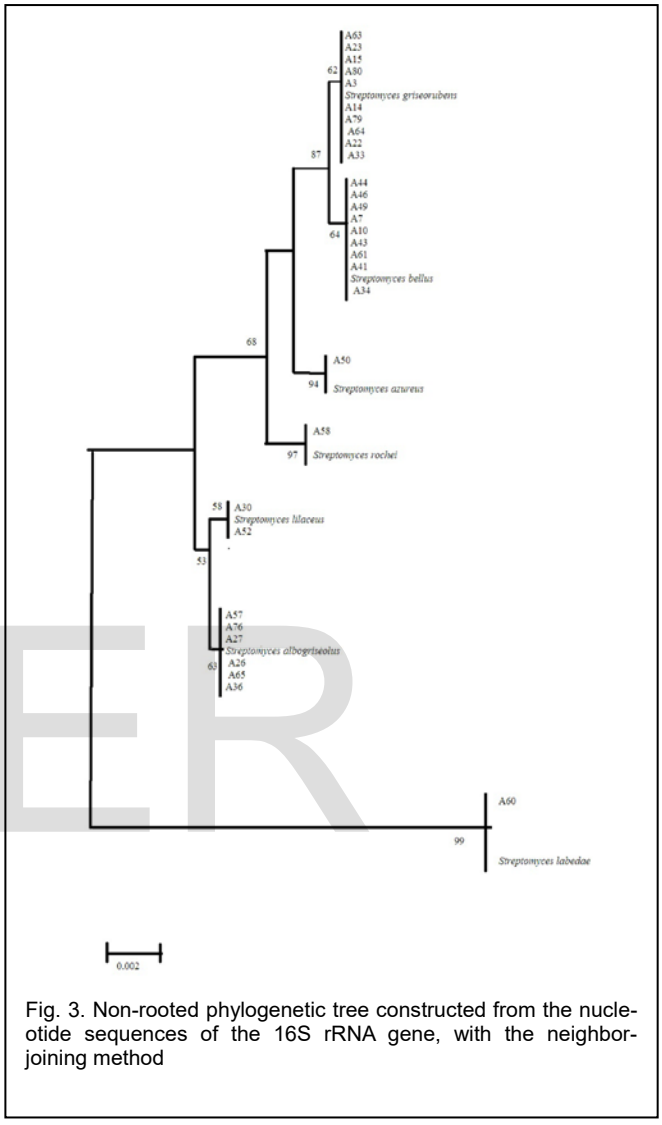

Fig. 3. Non-rooted phylogenetic tree constructed from the nucleotide sequences of the 16S rRNA gene, with the neighbor-joining method

4 CONCLUSION

A comparison of the sequences after amplifications of the 16S rDNA with the actinomycete species available in the database (Genbank) using the blast program showed that the 30 strains studied here were assimilated to the genus Streptomyces with percentages of 99 to 100%. These results confirm the abundance of Streptomyces in telluric ecosystems compared to other genera.

The results showed that the distribution of actinomycetes in soils varies from one soil to another. This diversity can be influenced by the high salinity in the region of Beni Amir.
Molecular techniques based on analysis of 16S rDNA sequences can highlight a great phylogenetic diversity in ecosystems.

Therefore the use of a simple method such as microbial ad-

hesion to solvents (MATS), is necessary to know a physico-chemical characterization of the surface of these bacteria also know The influence of environmental factors on the adhesive behavior using adhesion in the presence of salt

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