Molecular and Phenotypic Characterization of a New *Streptomyces* Strain, SP13', Isolated from an Unusual Ecosystem (Discharge of Boujad Pottery in Morocco)

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Abstract

This work is a part of the search for rare actinomycete strains producing antimicrobial substances. Following a screening of actinomycete strains isolated from different Moroccan ecosystems, the strain called SP13', isolated from an unusual ecosystem and producing interesting biological activity, was selected for molecular and phenotypic characterization. The sequencing results of amplified 16S rRNA fragments were obtained as electropherograms by Sequence Scanner software. The sequences were analyzed by Chromas lite 2.1 software and aligned by DNAMAN software. The obtained consensus sequence was compared with homologous sequences contained in the computerized database "GenBank" using Blast program in http://blast.ncbi.nlm.nih.gov/Blast.cgi. *The actinomycete isolate tested belonged to Streptomyces genus and identified as a specie very similar to Streptomyces crystallinus. The polyphasic approach failed to assimilate the SP13' strain to S. crystallinus. This confirms its originality and the probability that this strain can be a new Streptomyces specie.*

Keywords : *rare actinomycetes, unusual ecosystem, polyphasic identification, 16S rDNA, Streptomyces crystallinus.*

1. Introduction

The antifungal and antibacterial substances have been used for fifty years to treat infectious diseases of humans, animals and plants. The frequent and excessive use of these substances is a key factor in the development and evolution of microbial resistance **[1]**; **[2]**; **[3]**; **[4]**. This phenomenon, known as antibiotic resistance is a concern in agriculture and human and veterinary medicine.

In front of the emergence of antibiotic resistance and in order to combat these pathogens, several studies are currently focusing on research of bioactive substances from microorganisms isolated from ecosystems not yet explored in order to discover the original taxa and subsequently new biologically active molecules. Given their importance in the production of antibiotics and their omnipresence in almost all environments including those where life is extremely hostile **[5]**, actinomycetes are the most promising actors in the production of new antimicrobial metabolites **[6]**; **[7]**; **[8]**.

In order to discover rare original taxa able to produce new bioactive molecules, several Moroccan ecosystems were studied. The strains isolated from an unusual ecosystem (Pottery Discharge of Boujad in Morocco) showed high biological activity. Among these actinomycetale bacteria, SP13' strain showed an interesting activity against all target microorganisms used (Ten multiresistant bacteria and Six pathogenic fungi) **[9]**.

This work aims to identify and characterize the SP13' strain in order to determine its phylogenetic affiliation and to show its originality. The molecular identification is performed by amplification and sequencing of 16S rDNA genes. The SequenceScanner software, chroma lite 2.1 and DNAMAN software were used to analyze the obtained sequences. Phenotypic characterization is performed by determining the cultural, biochemical and physiological characters.

2. Materials and methods

2.1. Molecular identification of actinomycete strain SP13'

The extraction of DNA is performed from 48 hour cultures of the SP13' strain in liquid medium according to the kit "GenElute Bacterial Genomic DNA kit" protocol of SIGMA . The in vitro amplification of 16S ribosomal gene (1500 bp) is carried in a thermocycler type "Verity" of Applied Biosystems. The amplification was achieved by the PCR (Polymerase Chain Reaction) technique using the following primers:

Fd1 (5'-AGAGTTTGATCCTGGCTCAG-3');
Rp2 (5'-AAGGAGGTGATCCAGCC-3').

Purification of PCR products was performed according to the kit protocol "ExoSAP-IT" **[10]**.

After amplification, the samples were analyzed by electrophoresis in a 1% agarose gel in presence of a molecular weight marker 100 bp. After migration, the gel was examined under UV light to detect amplified bands. The photos were visualized by the photo-documentation system "G Box".

The sequencing method used is that automated using an ABI 3130 Genetic Analyser (16 capillary sequencer of Applied Biosystems) by the laboratory "laboratoire de séquencage de l'Unité d'Appui Technique à la Recherche Scientifique (UATRS)" at the "Centre National pour la Recherche Scientifique et Technique (CNRST) Rabat" in Morocco, The method used is that of Sanger based on ddNTPs technology (Society Applied Biosystems). The sequence reactions were performed in 96 well PCR plates according to the sequencing kit (Big Dye Terminator version 3.1 or version 1.1 cycle sequencing-Applied Biosystems).

The results of amplified rDNA fragments sequencing were obtained as gross electropherograms. These latter were visualized by SequenceScanner software (Applied Biosystems) and analyzed by chroma lite 2.1 software. The alignments of the sense/antisense sequences couple are performed by the software DNAMAN to define the consensus sequence. To determine the phylogenetic affiliation of this latter, we compared it with homologous sequences contained in the international data bank called "GenBank" using Blast (Basic Local Alignment Search Tool) **[11]** at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

The results are expressed as a similarity percentage between the strain to identify and the closest species and expressed under phylogenetic trees form.

2.2. Morphological and physiological characterization of actinomycete strain SP13'

After preparation of general inoculum and washed inoculum of actinomycete strain SP13' **[12]**, microscopic observation of the spores chain morphology and study of aerial mycelium and substrate mycelium performed according to the technique recommended by Cross **[13]**.

Then, the determination of morphological and cultural characteristics is performed on specific culture media (ISP2, ISP3, ISP4, ISP5; Bennett, GBA, Olson, GLM; Starch Casein Agar, Glucose asparagine; GLP; Sporulation Agar) **[14]**.

Finally, metabolic and physiological characteristics were determined: production of melanoid pigment and hydrolysis of starch **[15]**, gelatin hydrolysis, hydrolysis of casein and coagulation or peptonization of milk **[16]**, the determination of used carbon sources **[17]**, nitrate reduction **[18]**, research of urease , tolerance to sodium chloride **[19]**, the effect of pH and temperature on the growth and the sensitivity to various antibiotics **[20]**; **[21]**.

3. Results and discussion

3.1. Molecular identification of SP13' strain

After extraction of DNA from the SP13' isolate and dosage of DNA extract by Nanodrop, the purity is particularly analyzed. Figure 1 shows the concentrations of the studied samples. The purity of DNA is considered as correct. Indeed, the optical density 280/260 is greater than 0.8 and the optical density 230/280 is greater than 2.

The 16S rDNA fragments were then amplified by universal PCR. After electrophoresis of PCR products, the agarose gel has been photographed on UV table. The DNA bands were well migrated into the 1500 bp region.

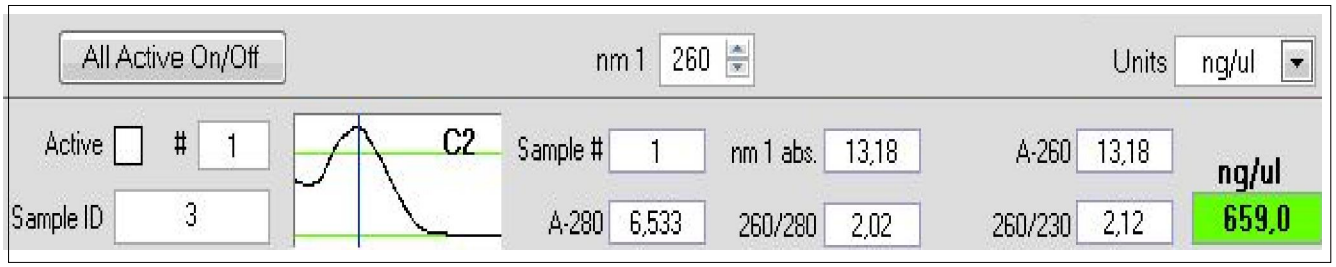

Figure 1: Dosage results of DNA extracted by Nanodrop

The amplified and sequenced fragments are obtained as electrophoregrams by SequenceScanner software (Applied Biosystems) and analyzed by chroma lite 2.1 software. The obtained pairs of sense/antisense sequences were aligned by DNAMAN software to define the consensus sequence (Table 1). This latter is compared with sequences available in the international computer bank (GenBank). The results are expressed as a percentage of similarity between the strain to identify and the closest species

Table 1: Consensus Sequences of the isolate SP13' after aligning the sequence couples.

N°	Isolate	Consensus sequence
3	SP13'	CGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAA.GCGAAAGT GACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT AGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGG.CGGCTTGTCAC GTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCTAGCTAGA GTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG GAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCG TGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGG TGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGG AGTACGGCCGCAAGGCTAAAACTCAAAGGAATTTGACGGGGCCCGCACAAGCAGCGGA GCATGTGGCTTAATTCGACGCAACGCGA.GAACTTTACCGAGGCT.GACATATACTGGAA AGCATCAGAGATGGTGCTCCCCATTGTGGTCGGTATTACAGGTGGGGTGCAATTGAC

The nucleotide sequence alignment with those of data bank shows that this actinomycete strain is classified in phylum of *Actinobacteria*, class V of *Actinobacteridae* and order I of *Actinomycetales* and belongs to genus *Streptomyces*. It has a similarity with several species from database "Genbank". To affiliate the isolates to closely species, a phylogenetic analysis is necessary.

Phylogenetic trees were constructed using the method of Neighbor-Joining distance by the Blast program. Seventeen species belonging to the genus *Streptomyces* have very similar sequences to the SP13' isolate (Figure 2). The percentage of similarity varies between 97% and 98% (Table 2). Phylogenetic trees elaborated under the triangular and radial shape **[10]** showed that *Streptomyces crystallinus* NBRC 15401 is the specie more akin to the SP13' strain.

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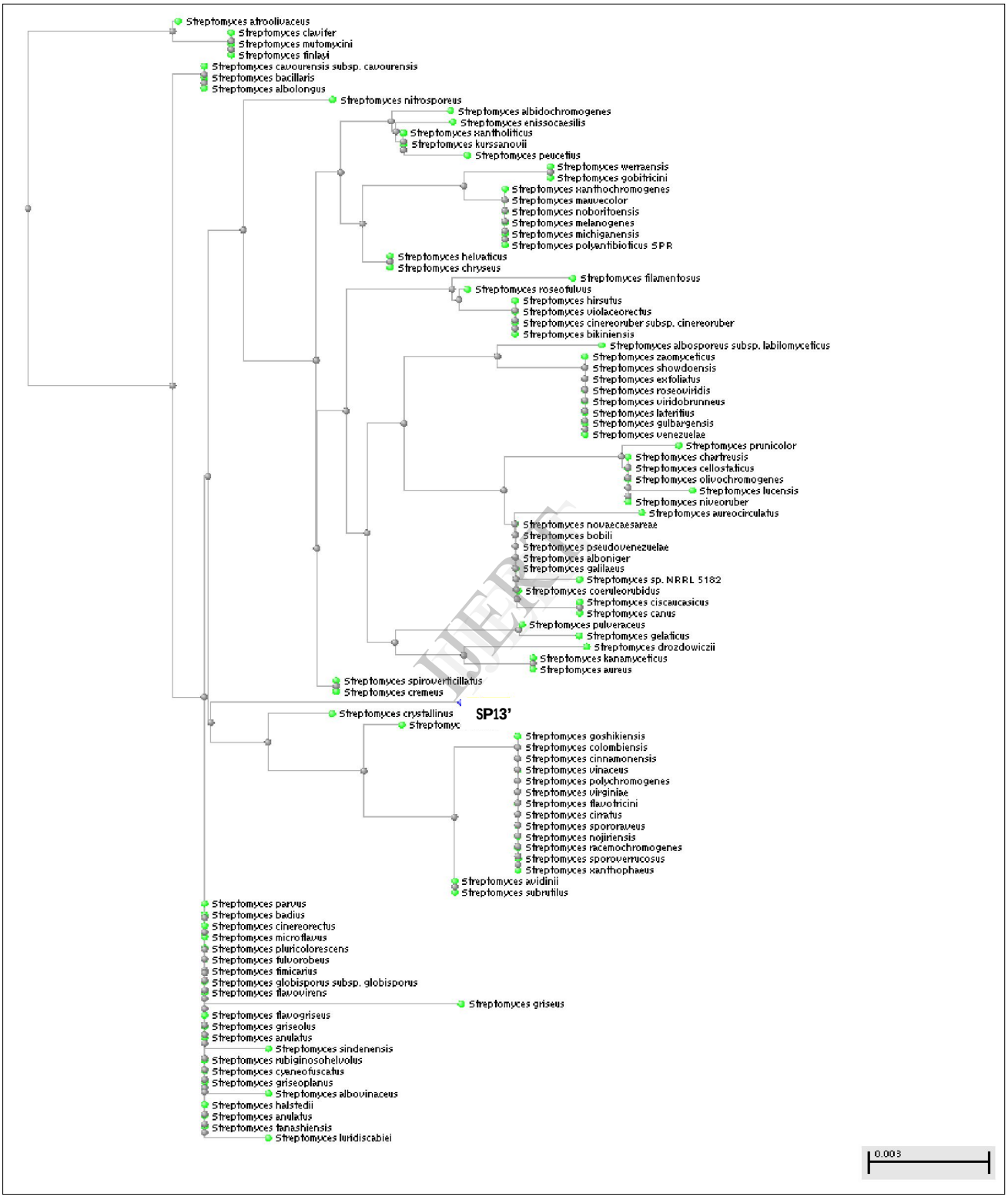

Figure 2: Phylogenetic tree based on 16S rRNA gene showing the position of the isolate SP13'

Table 2: Similar species to SP13' isolate and their degree of similarity

Similar species	Degree of similarity	Accession
Streptomyces crystallinus NBRC 15401	98 %	NR_041177.1
Streptomyces avidinii NBRC 13429	98 %	NR_041132.1
Streptomyces subrutilus DSM 40445	98 %	NR_026203.1
Streptomyces sporoverrucosus NRRL B-16379	98 %	NR_043837.1
Streptomyces goshikiensis NRRL B-5428	98 %	NR_044147.1
Streptomyces colombiensis NRRL B-1990	98 %	NR_043494.1
Streptomyces cinnamonensis NBRC 15873	98 %	NR_041194.1
Streptomyces vinaceus NBRC 13425	98 %	NR_041131.1
Streptomyces polychromogenes NBRC 13072	98 %	NR_041109.1
Streptomyces virginiae NBRC 12827	98 %	NR_041078.1
Streptomyces flavotricini NRRL B-5419	98 %	NR_043380.1
Streptomyces cirratus NRRL B-3250	98 %	NR_043356.1
Streptomyces spororaveus LMG 20313	98 %	NR_042306.1
Streptomyces nojiriensis LMG 20094	98 %	NR_042303.1
Streptomyces racemochromogenes NRRL B-5430	97 %	NR_043499.1
Streptomyces katrae NBRC 13447	97 %	NR_041136.1
Streptomyces xanthophaeus NRRL B-5414	97 %	NR_043848.1

3.2. Phenotypic characterization of actinomycete strain SP13'

The study of cultural characteristics shows that this strain grows on all culture media used except ISP4 and GBA media in which growth was weak. The ISP5, Bennett, GLM and synthetic media gave good growth and promoted the development of an abundant aerial mycelium. This latter is less important on all other used media. Its colouring on all media, tends to a white, beige or light brown. The color of substrate mycelium varies according to the media, but it is generally light brown in most culture media. The presence of a diffusible pigment whose color varies from yellow, beige to light brown, has been highlighted.

The colonies are beige or light brown colored. They come from the accumulation of hyphae with cauliflower aspect. They firmly adhere to the culture medium where they form a slight depression and are difficult to be suspended.

Microscopic observation of the substrate mycelium (zoom x 100) reveals the presence of filaments that are long, thin, branched, not fragmented and with no spores. The aerial mycelium presents thin hyphaes which are branched, non-septate and terminating with long spore-chains. These spore-chains are straight rectiflexible types (RF) and can have between 8 and more than 18 spores per chain. These spores are cylindrical and have no mobility. It should be noted that this strain has neither poranges nor synnemas nor sclerotia.

Concerning the physiological and biochemical characteristics, the SP13' strain assimilates and uses all carbonated substrates tested except rhamnose, inositol, sucrose, arabinose and raffinose. It produces a melanoid pigment on ISP7 medium and shows an important protein metabolism, hydrolyzes casein; it can liquefy gelatin and coagulate skimmed milk. The SP13' strain hydrolyses starch, tolerates up to 9% NaCl, reduced nitrate to nitrite and it can't peptonize milk or produce urease. The suitable temperature for its growth is between 20 °C and 35 °C with an optimum temperature comprised between 28 °C and 30 °C and a pH from 5 to 9 with an optimum value of 7.

According to the sensitivity tests to various antibiotics, this strain has demonstrated antibiotics resistance to the majority of family β-lactam used, while its growth is inhibited by certain antibiotics belonging to cyclins and aminoglycosides.

3.3. Polyphasic identification of SP13' strain

Further to molecular studies based on amplification of the gene coding for 16S rRNA, an actinomycete species (*Streptomyces crystallinus* NBRC 15401) is very close to the SP13' strain. To clarify the belonging of the strain to this species, it is necessary to perform a multiphase identification combining molecular techniques and morphological, biochemical and physiological tests. Table 3 relates the classic characters of the

three strains to compare (two reference strains and SP13' strain). It is clear that the SP13' strain has different phenotypic characteristics of the two reference strains. It differs from it in three points:

- At morphological level, SP13' isolate has a yellow to light brown aerial mycelium bringing only spore chains rectiflexibles, by against the two reference strains produce a red aerial mycelium and bring to maturity only rectiflexibles spore-chains;
- At metabolic level, *S. crystallinus* AZ151 strain uses inositol and raffinose , the *S. crystallinus* reference strain uses inositol, unlike the SP13' strain which does not grow in culture media containing only inositol or raffinose as carbon source;
- At physiological level, SP13' strain could not degrade urea and does not grow at temperatures above 35 °C, but it reduces nitrates and tolerates NaCl concentrations that can go up to 9%. Instead, the *S. crystallinus* AZ151 species produces urease and grows well at 45 °C, but does not reduce nitrates and does not grow in culture media where the NaCl concentration exceeds 5%.

Table 3: Comparison of phenotypic characteristics between SP13' strain and reference strains

phenotypic characteristics		**SP13' isolate**	***S. crystallinus*** AZ151 [**22**]	***S. crystallinus*** (Reference strain)
Morphology	Color of aerial mycelium	Light brown - Beige	Red - Grey reddish	red
	Color of substrate mycelium	Light brown - Light yellow	Light brown - Light yellow	brown
	diffusible pigments	Brown – yellow - Beige	Yellow - Brown	brown
	Melanoid pigments	+	+	+
	Type of spore chain	Rectiflexible	Rectiflexible and Spiral	Rectiflexible and Spiral
	Number of spores per chain	> 18	ND	ND
	Spore's forme	cylindrical	ND	ND
Growth according various carbon sources	Rhamnose	-	-	ND
	Glucose	+	+	ND
	Starch	+	+	ND
	Dulcitol	ND	ND	ND
	Galactose	+	+	ND
	Inositol	-	+++	+
	Fructose	+	+/-	ND
	Glycerol	+	ND	ND
	Maltose	+	-	ND
	Mannitol	+	+++	ND
	Mannose	+	+	ND
	Levulose	ND	ND	ND
	Lactose	+	-	ND
	Sucrose	-	ND	ND
	Melibiose	ND	ND	ND
	Raffinose	-	++	ND
	Dextrin	+	ND	ND
	Ribose	ND	-	ND
	Sorbose	ND	ND	ND
	Xylose	-	-	ND
	Arabinose	-	-	ND
	Citrate	ND	+	ND
Other characters	H_2S production	ND	+	ND
	Nitrate reduction	+	-	ND
	Urease production	-	+	ND
	Gelatin hydrolysis	+	ND	ND
	Casein hydrolysis	+	ND	ND
	Starch hydrolysis	+	+	ND
	coagulate skimmed milk	+	ND	ND
	Peptonization of milk skim	-	ND	ND

ND : Not determined; - : Negative results; + : Positive results

Table 3 : Comparison of phenotypic characteristics between SP13' strain and reference strains (Continued)

phenotypic characteristics		SP13' isolate	*S. crystallinus* AZ151 [22]	*S. crystallinus* (Reference strain)
Growth at different temperatures	10°C	-	-	ND
	20°C	+	ND	ND
	30°C	++	+	ND
	35°C	+	++	ND
	40°C	-	++	ND
	45°C	-	++	ND
	50	-	-	ND
Growth at different pH	3	-	ND	ND
	5	+	ND	ND
	7	++	ND	ND
	9	+	ND	ND
	12	-	ND	ND
Tolerance to NaCl		9 %	5 %	ND
Antibiotics sensitivity	Gentamicin 10 µg	S	ND	ND
	Ceftazidime 30 µg	R	ND	ND
	Pristinamycin 15 µg	R	ND	ND
	fusidic acid 10 µg	R	R	ND
	Doxycicline 30 µg	S	ND	ND
	Amoxicillin 25 µg	R	ND	ND
	Cefotaxime 30 µg	S	ND	ND
	Tetracycline 30 µg	S	ND	ND
	Kanamycin 30 µg	S	ND	ND
	Oxacillin 5 µg	R	ND	ND
	Rifampicin 30 µg	S	ND	ND
	Piperacillin 110 µg	S	ND	ND
	Cefuroxime 30 µg	S	ND	ND
	Ticarcillin 75 µg	R	ND	ND
	Ciprofloxacin 5 µg	R	ND	ND
	Penicillin G 5 µg	R	ND	ND
	Ampicillin 25 µg	ND	R	ND
	Nalidixic acid 30 µg	ND	R	ND
	Cefoperazone 75 µg	ND	R	ND
	Polymyxin 30 µg	ND	S	ND

NT : Not Determined ; R : Resistance ; S: Sensitivity ; - : No growth; + : Low growth; ++ : Moderate growth; +++: Good growth

4. Conclusion

The polyphasic approach which consists in combining molecular, morphological, physiological and biochemical techniques did not permit to assimilate the SP13' isolate to *Streptomyces crystallinus*. this confirms the originality of this isolate which could possibly represent a new specie of the *Streptomyces* genus. Indeed, this strain was isolated from a pottery discharge. This latter is an ecosystem which is not very well or not yet explored. Hence, the species adapted to this environment are specific and particular.

However, to confirm this hypothesis and demonstrate the taxonomic affiliation of the studied isolate, other molecular techniques must be used. Indeed, DNA-DNA hybridization is a technique used in many studies about *Streptomyces* and could be used to estimate the degree of relationship between SP13' strain and *S. crystallinus*.

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