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Research Article

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Molecular identification and characterization of active actinomycete strain isolated from El Mellah Lake of El Kala

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ABSTRACT

The aim of this study was to establish the taxonomic position of the EG76 strain isolated from the water of "El Mellah"lake in "El Kala", North East of Algeria that showed antifungal activity against Candida albicans resistant to 5 fluorocytosine. The EG76 was isolated from water samples from Gauss agar medium, screened for its antifungal activity on ISP2 agar using double layer and cylinder disc agar methods, the time course of antifungal metabolites production was evaluated against C. albicans IPA549 on three culture broth, ISP1, ISP2 and GYEA, the taxonomic position was determined regarding phenotypic and 16S rDNA sequences features. the EG76 showed a high in vitro activity against S. aureus and C. albicans even against clinical strain resistant to 5-fluorocytosine, its 16S rRNA sequence shared 98% similarity with the Streptomyces malaysiensisATB-11, their phenotypic, biochemical and physiological characters were also compared, the organism appeared to produce high antifungal compounds at 6 days of incubation period of production medium culture. Glucose and peptone were the best carbon sources, the double layer method was found better for antifungal assay than the cylinder and disc diffusion method, EG76 had a broad spectrum activity which represents a potential source of antibiotics with a pharmaceutical interest.

Keywords: Antifungal activity, salt water, Mellah Lake, *Streptomyces*, *Candida albicans*.

INTRODUCTION

The epidemiology of invasive fungal infections has changed over the last twenty years, their impact overall has increased, as well as the population of patients at risk, many studies reports the increasing prevalence of resistance of fungi to various antifungal agents**[1]**, *C. albicans* reigning as the most prevalent invasive fungal pathogen of humans **[2]**there are a limited number of drug targets that can be exploited to selectively kill the pathogen with minimal host toxicity, resistance to the available classes of antifungals has emerged as a severe problem, therefore, the search for new sources of natural antifungal producers makes a challenge **[3, 4, 5].**

In this context, members of the genus *Streptomyces* remain a unique source of natural products, including antifungal agents **[6, 7, 8, 9]**, saline lakes and marine ecosystems are interesting models for microbiologists that work on microbial diversity and secondary metabolites produced by novel microorganisms isolated from extreme environments, many studies have shown their novel metabolic pathways and their adaptations**[10, 11, 12],** another unique feature of this genus is the large number of species it contains, at this time, the genus *Streptomyces* contained over than 600 species**[13]**, Our study aims to establish the taxonomic position of a *Streptomyces* strain designated *Streptomyces malaysiensis* that were isolated from salty waters of El Mellah lake in Algeria. A polyphasic taxonomic study based on a combination of genotypic and phenotypic procedures showed that the isolate EG76 shared 98% of similarity with *Streptomyces malaysiensis* ATB-11 (= DSM 41697^T)**[14]**, the isolate EG76 showed activity against *S.aureus* and clinical *C.albicans* sensitive and resistant to 5-fluorocytosine.

EXPERIMENTAL SECTION

Organisms and cultural conditions

Strain EG76 was isolated after 14 days of incubation at 28°C from water samples on Gauss agar (KNO_3, 1g ; KH_2PO_4, 0.5g; $MgSO_4,7H_2O$, 0.5g ; NaCl 0.5g ; $FeSO_4,7H_2O$, 0.01g ; starch 20g ; H_2O 1000ml ; agar 30g ; pH 7.2 **[15]** medium supplemented by lake water**[16]**. Water samples were collected from El Mellah Lake, Park National of El Kala, Algeria, kept at 4°C and transferred immediately to the laboratory.

Antimicrobial activity screening

The selected isolate was further tested for its antibacterial activity against *S. aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and for its antifungal activity against *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258 and two clinical *Candida albicans* strains, CS1 and CS2, provided by the Parasitology and Mycology laboratory at "La Timone" Hospital, Marseille, France.

The antimicrobial activity was evaluated on ISP2 agar using the agar cylinder method with 5 mm diameter discs for bacteria **[17]** and the double layer method for yeast**[18].**

EG76 strain characterization

The morphological characteristics were described on ISP1, ISP2, ISP3, ISP4 and ISP5 media at 7, 14 and 21 days of culture at 28°C, as recommended by Shirling and Gottlieb **[19]**. The production of melanoïd pigments was observed at ISP6 and ISP7 medium, observed after 2 and 4 days at 28° C **[19]**. The growth in the presence of NaCl (0.5-4%) concentration gradient was studied on ISP2 agar **[20]**. The resistance to phenol 0.1% (w/v) was tested according to Goodfellow**[21]**. The proteolytic activity was determined as described by Larpent and Larpent-Gourgaud**[15]**. The capacity to use various carbon sources was observed on ISP basal medium 9 as preconized by Shirling and Gottlieb **[19]**, sterile carbon source was added to a final concentration of 1% (w/v); the capacity of using different nitrogen sources (1% w/v) was tested using a basal medium as preconized by Williams et al., **[22]**. Negative control was the culture medium without any carbon or nitrogen sources. The hydrolysis activity on starch, gelatin, casein and tween 80 was tested as previously described by Gordon et al., **[23]**; Gordon and Smith **[24]**; Pickett et al., **[25]**; and Sierra **[26]**. All these physiological and biochemical tests were performed at 28° C and read after 7, 14 and 21days.

Sequencing of the 16S rDNA

Genomic DNA of the test strain was extracted in accordance with the methods described by Edwards et al.,**[27].** The PCR amplification of the 16S rRNA gene was conducted using two primers (fD1 and RP2) as reported by Weisburg et al.,**[28]**. The sequences of the 16S rRNA gene obtained were compared to those in the NCBI nucleotide database using the BLAST algorithm. The sequences with the highest percentage of similar nucleotides were aligned using Clustal X2 program, and the phylogenetic tree was constructed via the neighbor-joining algorithm **[29]** based on the 16S rRNA gene sequences of the strain EG76 and related organisms.

Time course of antifungal metabolites production

The time course of the antifungal active metabolite of the strain was carriedout using the *C. albicans* IPA 549 strain, three fermentation broth media: ISP1, ISP2 and GYEA (glucose yeast extract agar) were used in this study **[30]**. Each 500 ml Erlenmeyer flask containing 150 ml of broth were incubated at 28°C with continuous shaking at 150 rpm for 14 days. Each day, a 10 ml sample of the broth culture was sterilely collected and centrifuged at 5000g for 10 min, then, 50 µl of supernatant was absorbed onto 6 mm diameter Whatman No.1 filter paper disks to be finally deposited onto Muller Hinton agar (Sigma Aldrich) which was previously inoculated with the organism to test,diameter of inhibition zones and pH variations of the media were daily measured **[8].**

RESULTS AND DISCUSSION

Strain EG76 grew on a range of agar media (table 1), the aerial mycelium is white on Gauss and white to light grey on ISP media, aerial hyphae differentiated into long spiral chains of cylindrical spores; the color of substrate mycelium tented to be white, light grey or yellow without soluble pigmentation, the isolate do not produce melanin pigments on ISP6 and ISP7 agar.

According to investigated results on the morphological, physiological, and biochemical characteristics of the strain compared with the Bergey's Manual of Systematic Bacteriology**[31]**, EG76 was preliminary classified to be *Streptomyces* genus. The rRNA 16S sequences analysis (1241 nucleotides) has given 98% similarity with *Streptomyces malaysiensis* type strain ATB- 11^T (= DSM 41697^T), the neighbor joining tree (figure 1) represents the relationship between EG76, the type strain ATB- 11 and other representatives *Streptomyces* available on Genbank.

Table 1. Phenotypic properties of the EG76 and *S.malaysiensis*from Al-Tai et al., [14]

Agar medium	Aerial mycelium		Substrate mycelium		Soluble pigment	
	EG76	***S.malaysiensis***	**EG76**	***S.malaysiensis***	**EG76**	***S.malaysiensis***
ISP2	Grey	Dark grey	Brown/ grey	Brown/ grey	None	Brown
ISP3	White	Smoky black	Pale yellow	Yellow/ brown	None	None
ISP4	White/ grey	Smoky black	Light grey	Grey	None	None
ISP5	White	White/ grey	white	Pale yellow/ grey	None	None
ISP7	White	Grey	Pale yellow/ white	Brown/ grey	None	Dark brown

Table 2. Physiological and biochemical characteristics of the Eg76 comparing with *S.malaysiensis* from Al-Tai et al., [14]

Test	Results	
	EG76 strain	***Streptomyces malaysiensis***
Degradation of:		
Casein	+	+
Starch	+	+
L-tyrosine	ND	+
Xanthine	ND	-
Growth at:		
10 °C	+	-
25 °C	+	+
45 °C	-	-
Growth on sole carbon sources (1 % w/v)		
L-Arabinose	+	+
D-Fructose	+	+
D-Galactose	+	+
D-Glucose	+	+
meso-Inositol	ND	+
D-Maltose	ND	+
D-Mannitol	+	+
D-Mannose	+	+
D-Raffinose	+/-	+
α-L-Rhamnose	+	+
D-Sorbitol	-	-
D-Sucrose	-	-
D-Xylose	+/-	+
D-melibiose	+/-	ND

ND: not defined

Table 3. Other characteristics of *Streptomyces* EG76

Character	Results
Melanoïd pigments on ISP6/ISP7	None
Tolerance to NaCl	> 4%
Biochemical characters	
Gelatin degradation	+
Urea	-
Protease	+
caseinase	+
Tween 80 degradation	+
Simmons citrate	+
Resistance to phenol 0.1% (w/v)	-

The phenotypic characteristics of the EG76 compared with the *S.malaysiensis* from Al Tai et al. **[14]** are detailed on table 1, on ISP media, the mycelium morphology of the two strains showed big differences, and in addition, *S.malaysiensis* formed brownish diffusible pigments on ISP2 and ISP7 plates while EG76 did not. They were also different in physiological and biochemical characteristics (table 2), *S.malaysiensis* was able to assimilate D-Raffinose and D-Xylose as sole carbon source while EG76 was not; our strain grew well at 10 °C while *S.malaysiensis* does not. Finally results reported that our strain produced a caseinase, gelatinase, protease, and an esterase, EG76 was able to grow in presence of NaCl up to 4% (table 3).

The identification of the *Streptomyces* is actually a very complex process, early depending on morphological and physiological characteristics and classified them into clusters **[22, 32].** Now, with the development of molecular biology, method of the 16S rRNA gene sequence analysis is widely used for the classification because of the high conservation of the 16S rDNA genes in prokaryote **[33, 34, 35].**

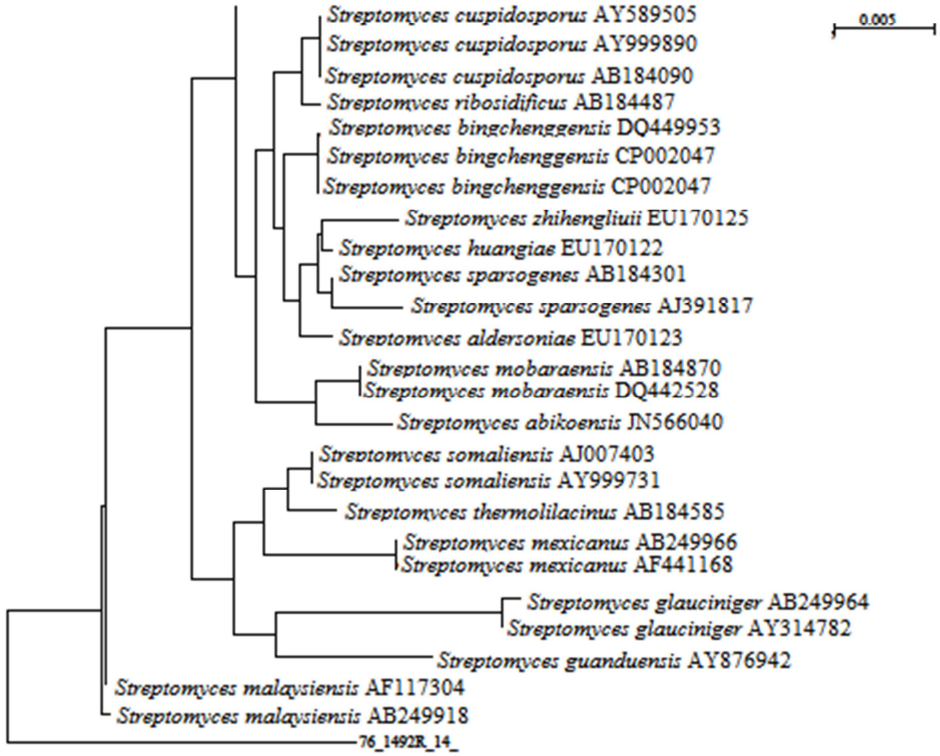

Fig. 1. Neighbor joining tree based on 16S rRNA sequences showing relationship between *Streptomyces* EG76 and related sequences obtained from "NCBI Blast". Bootstrap = 500. The scale bar indicates 0.005 substitutions

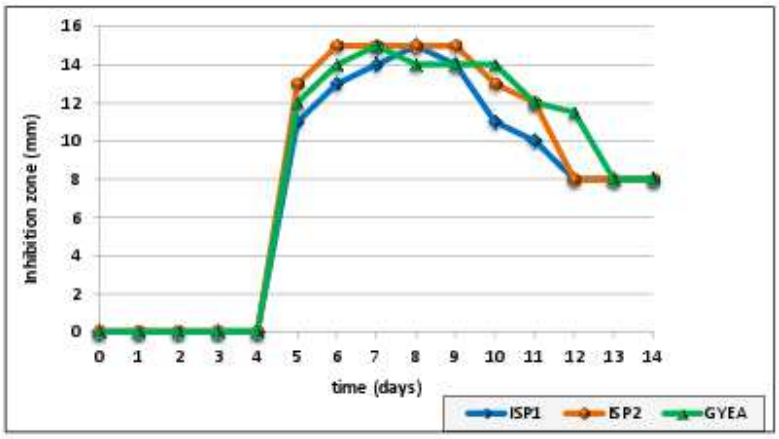

Fig. 2. Time course of antifungal metabolites production by *Streptomyces* EG76 and in three culture media (ISP1, ISP2, GYEA)

However, with the increasing number of species over the two last decades especially in *Streptomyces*, with at the same time, the gene mutated in different degrees, a combined classification has improved the taxonomy of actinomycetes with the study of the phenotypic, physiological and biochemical properties **[36, 37, 38].**

In our study, EG76 is an actinomycete isolated from water sample from Mellah lake with a broad spectrum antimicrobial activity, according to its morphological and cultural characteristics, as well as the physiological and biochemical properties and evidence from the 16S rRNA gene sequence analysis, our strain shared 98% similarity with *Streptomyces malaysiensis***[14].** However, changes recorded in its characteristics can be explained by the uncommon combination of selective pressures that have led to the evolution of new morphological and biochemical adaptations and the possibility of the presence of indigenous species **[39, 40].** Salty waters of "El Mellah" lake represent a stratified brackish water body that confers to this ecosystem all its originality, halotolerant and halophilic microorganisms grow in environments with moderate to high salinity concentration and the majorities studied so far

produce compounds with great potential in industrial processes and are also appreciated for their physiological properties which facilitate their use with commercial aims **[41, 42, 43].**

The time course of the production of antifungal active metabolites of the EG76 strain on ISP2, ISP1 and GYEA (glucose yeast extract agar) broth was detected after 5 days of culture, reached a maximum after 9 days as illustrated in Figure 2. The pH varied between 6.8 and 9 during the incubation (figure 3), initial screening revealed that ISP2 medium was found to be the best base for antifungal metabolite production with a maximum of production after 6 days of culture.

The production of antifungal metabolites by EG76 was more efficient in solid culture media, compared with submerged media, previous studies reported that many active isolates may decrease of even cease completely their activity in liquid media **[44, 45, 46]**, the detection of bioactive compounds in liquid media requires high concentration of them **[47].** Our strain exhibited a broad spectrum antimicrobial activity against the two clinical *C.albicans* SC1 sensitive and SC2 resistant to 5-fluorocytosine, *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258 and *S.aureus* ATCC 25923 but not against *E.coli*.

Flucytosine (or 5-fluorocytosine) is a pyrimidine analogue, which inhibits the fungal DNA/RNA synthesis, in *C.albicans*, primary resistance remains low (about less than 2%), secondary resistance results from the inactivation of different enzymes of the pyrimidine pathway, mutations in FCY1, FCY2 and FUR1 genes are known to be implicated in these resistance mechanisms **[48, 49].** So our isolate can be considered an efficient target for use in the production of antifungal compounds against *Candida* sp. with clinical resistance to 5-fluorocytosine, and further studies should be conducted to isolate and characterize its bioactive molecule.

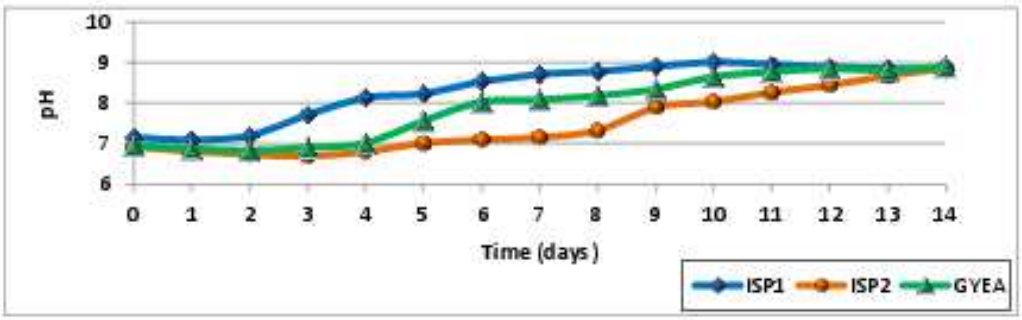

Fig. 3. Time course of pH variation of the *Streptomyces* EG76 in three culture media (ISP1, ISP2, GYEA)

CONCLUSION

This study is the first to introduce the finding of actinomycetes in original environment like “El Mellah” lake producing antifungal molecules that may give others therapeutic approach. Marine actinomycetes have evolved the greatest genomic and metabolic diversity; efforts should be directed toward exploring actinomycetes from this unique ecosystem as a source for the discovery of novel secondary metabolites.

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