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Streptomyces brollosae sp. nov., NEAE-115, A Novel L-Asparaginase Producing Actinomycete Isolated from Brollos Lake at the Mediterranean Coast of Egypt

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An actinomycetes strain producing L-asparaginase, strain NEAE-115, was isolated from a soil sample collected from Brollos Lake at the Mediterranean coast of Egypt. The identification of this strain was established using the morphological, cultural, physiological, biochemical properties and 16S rRNA sequence. The organism was found to have morphological and chemotaxonomic characteristics typical of streptomycetes. Phylogenetic analysis based on the almost complete 16S rRNA gene sequence indicated that the strain NEAE-115 belongs to the genus *Streptomyces* and consistently falls into a clade together with *Streptomyces niveoruber* strain NRRL B-2724, *Streptomyces polychromogenes* strain EAAG77, *Streptomyces nashvillensis* strain 1534, *Streptomyces roseoviridis* strain JS-9 and *Streptomyces venezuelae* strain JS-11. 16S rRNA gene sequence similarities between NEAE-115 and above mentioned type strains were between 80 and 81%. The comparative study on the basis of morphological, physiological and biochemical characteristics of the strain NEAE-115 in relation to its closest phylogenetic neighbours of the genus *Streptomyces* revealed that the strain NEAE-115 represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces brollosae* NEAE-115 is proposed and its sequencing product was deposited in the GenBank database under accession number KJ410229.

Key words: *Streptomyces brollosae* NEAE-115, 16S rRNA sequences analysis, scanning electron microscope, phenotypic characteristics.

L-asparaginases are a cornerstone of treatment protocols for acute lymphoblastic leukemia (ALL), it has been an integral part of combination chemotherapy protocols of pediatric acute lymphoblastic leukemia and in the majority of adult treatment protocols[1]. L-asparaginase administration has been limited by a high rate of hypersensitivity in the long-term use and development of anti-asparaginase antibodies, which cause an anaphylactic shock or neutralization of the drug effect. To overcome these limitations, L-asparaginase from other new sources, pegylated formulations, and L-asparaginase loaded into erythrocytes have been recently proposed [2]. The demand for L-asparaginase will be increase several fold in the coming years due to its potential industrial applications as food processing aid in addition to its clinical applications [3,4]. Hence, studies are continued to screen soil samples from various sources for isolation of potential microbes, which have the ability to produce the enzyme.

Amongst prokaryotes, members of actinomycetes are the richest source of natural products, especially clinically useful antibiotics,

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antimetabolites and antitumor agents[5]. In addition to antibiotics, actinomycetes also produce other economically important compounds including vitamins, enzymes and immunomodulators[6]. Actinomycetes taxonomy was formerly thought to be associated with morphology. Morphological characteristics alone are not adequate in differentiating between different species of many genera. The fast development of the molecular biology, the use of phylogenetic and molecular evolutionary approaches has been of great importance to the classification methods[7]. Recently, the identification of the actinomycetes species and phylogenies are commonly derived from 16S rRNA and the use of polymerase chain reaction (PCR) for sequence analyses[8].

In the course of our screening programme for L-asparaginase activity, one actinomycete, strain NEAE 115 which isolated from a soil sample collected from Brollos Lake at the Mediterranean coast of Egypt showed high L-asparaginase activity.

The present study reports a taxonomic analysis of the strain NEAE-115 by using a combination of chemotaxonomic, morphological, physiological criteria and 16S rRNA gene nucleotides.

MATERIALS AND METHODS

Microorganisms and cultural conditions

Actinomycetes from the soil had been isolated using standard dilution plate method procedure on Petri plates containing starch nitrate agar medium of the following composition (g/L): Starch, 20; KNO_3, 2; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; $CaCO_3$, 3; $FeSO_4.7H_2O$, 0.01; agar, 20 and distilled water up to 1 L; then plates were incubated for a period of 7 days at 30°C. Actinomycete isolates were purified and maintained as spore suspensions in 20 % (v/v) glycerol at -20 °C for subsequent investigation.

Morphology and cultural characteristics

The morphology of the spore chain and the spore surface ornamentation of strain NEAE-115 were examined on starch nitrate agar medium after 14 days at 30°C. The gold-coated dehydrated specimen was examined at different magnifications[9] with Analytical Scanning Electron Microscope Jeol JSM-6360 LA operating at 20 Kv at the Central Laboratory, City of Scientific Research and Technological Applications, Alexandria, Egypt. Aerial spore-mass color, substrate mycelial pigmentation and the production of diffusible pigments were observed on yeast extract-malt extract agar (ISP medium 2), oatmeal agar (ISP medium 3), inorganic salt starch agar (ISP medium 4), glycerol- asparagine agar (ISP medium 5) peptone-yeast extract iron agar (ISP medium 6) and tyrosine agar (ISP medium 7) as described by Shirling and Gottlieb[10]; all plates were incubated at 30°C for 14 days.

Chemotaxonomy

Sugars were identified by the method described by Staneck and Roberts[11].

Physiological characteristics

Carbon source utilization was tested on plates containing ISP basal medium 9[10] supplemented with a final concentration of 1% of the tested carbon sources. The plates were incubated at 30°C and examined after 14 days. Melanoid pigment production was examined on peptone-yeast extract-iron agar (ISP medium 6), on tyrosine agar (ISP medium 7), and in tryptone-yeast extract broth (ISP medium 1)[10]. Growth in the presence of sodium chloride was determined according to Tresner *et al*. [12] (1968). Degradation of casein was tested following the method of Gordon *et al*. [13] and reduction of nitrates to nitrites [14] was examined. Liquefaction of gelatin was evaluated by using the method of Waksman[15]. The ability to coagulate or to peptonize milk and hydrogen sulphide production was determined as described by Cowan and Steel[16]. Lecithinase activity was conducted on egg–yolk medium according to the method of Nitsch and Kützner[17] and the capacity to decompose cellulose was tested following the method of Ariffin *et al*. [18]. The ability of strain to produce α-amylase was determined; the isolate was streaked onto starch nitrate medium plates containing 2% soluble starch and incubated at 30°C for 7 days. After incubation, the plate is flooded with Gram's iodine solution and zone of clearance was observed[19]. The ability of the organism to inhibit the growth of five bacterial strains (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*), five fungal strains (*Rhizoctonia solani*, *Fusarium oxysporum*, *Alternaria solani*, *Bipolaris oryzae*, *Aspergillus*

niger) and two yeast (*Sacchromyces cerevisiae, Candida albicans*) was determined. Some additional tests can be considered to be useful in completing the description of a strain or species, even if they are not very significant or indicative on their own, The ability of strain NEAE-115 to produce uricase[20]; asparaginase[21] and chitosanase activity [22] were tested.

16S rRNA sequencing

The preparation of genomic DNA of the strain was conducted in accordance with the methods described by Sambrook *et al*.[23]. The PCR amplification reaction was performed according to the method of El-Naggar [9] in a total volume of 100 µl, which contained 1 µl DNA, 10 µl of 250 mM deoxyribonucleotide 5'-triphosphate (dNTP's); 10 µl PCR buffer, 3.5 µl 25 mM $MgCl_2$ and 0.5 µl Taq polymerase, 4 µl of 10 pmol (each) forward 16s rRNA primer 27f (5'-AGAGTTTGATCMTGC CTCAG-3') and reverse 16s rRNA primer 1492 r (5'-TACGGYTACCTTGTTACGACTT-3') and water was added up to 100 µl. The PCR-apparatus was programmed as follows: 5 min denaturation at 94°C, followed by 35 amplification cycles of 1 min at 94°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C, followed by a 10 min final extension at 72°C. The PCR reaction mixture was then analyzed via agarose gel electrophoresis, and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA). The purified PCR product was sequenced by using two primers, 518F; 5'-CCA GCA GCC GCG GTA ATA CG-3' and 800R; 5'-TAC CAG GGT ATC TAA TCC-3'. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing product was resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). The complete 16S rRNA gene sequence (1565 bp) was deposited in the GenBank database under accession number KJ410229.

Sequence alignment and phylogenetic analysis

The 16S rRNA gene sequence of strain NEAE-115 was aligned with the corresponding 16S rRNA sequences of the type strains of representative members of the genus *Streptomyces* retrieved from the GenBank, EMBL, DDBJ and PDB databases by using BLAST program (www.ncbi.nlm.nih. gov/blst) [24] and the software package MEGA4 version 2.1[25] was used for multiple alignment and phylogenetic analysis. The phylogenetic tree was constructed via the bootstrap test of neighbor-joining algorithm[26] based on the 16S rRNA gene sequences of strain NEAE-115 and related organisms.

RESULTS AND DISCUSSION

Morphology and cultural characteristics of the strain no. NEAE-115

Strain NEAE-115 grew well on all tested media (yeast extract -malt extract agar, oatmeal agar, inorganic salt-starch agar, glycerol–asparagine agar, peptone-yeast extract iron agar and on tyrosine agar) (Table 1). Aerial mass color falls in the gray color series on yeast extract -malt extract agar, inorganic salts-starch agar (Fig.1), peptone-yeast extract iron agar and tyrosine agar while is in grayish beige on oatmeal agar and glycerol-asparagine agar. Reverse side of colony is yellowish gray on yeast extract -malt extract agar; faint yellowish gray on oatmeal agar and glycerol

Fig. 1. Color of the aerial mycelium of *Streptomyces* sp. NEAE-115 grown on A) inorganic salt-starch agar, B) yeast extract -malt extract agar

asparagines agar and olive on inorganic inorganic salts-starch agar, peptone-yeast extract iron agar and tyrosine agar; substrate pigment is not pH indicator. No pigment produced in all tested media. The organism is aerobic, mesophilic. The mycelium does not fragment. Spore chains in section spirales (Fig. 2), with open spirals intergrading through flexuous spore chains suggestive of section rectiflexibiles. Mature spore chains are generally long. Spore surface is smooth. This morphology is

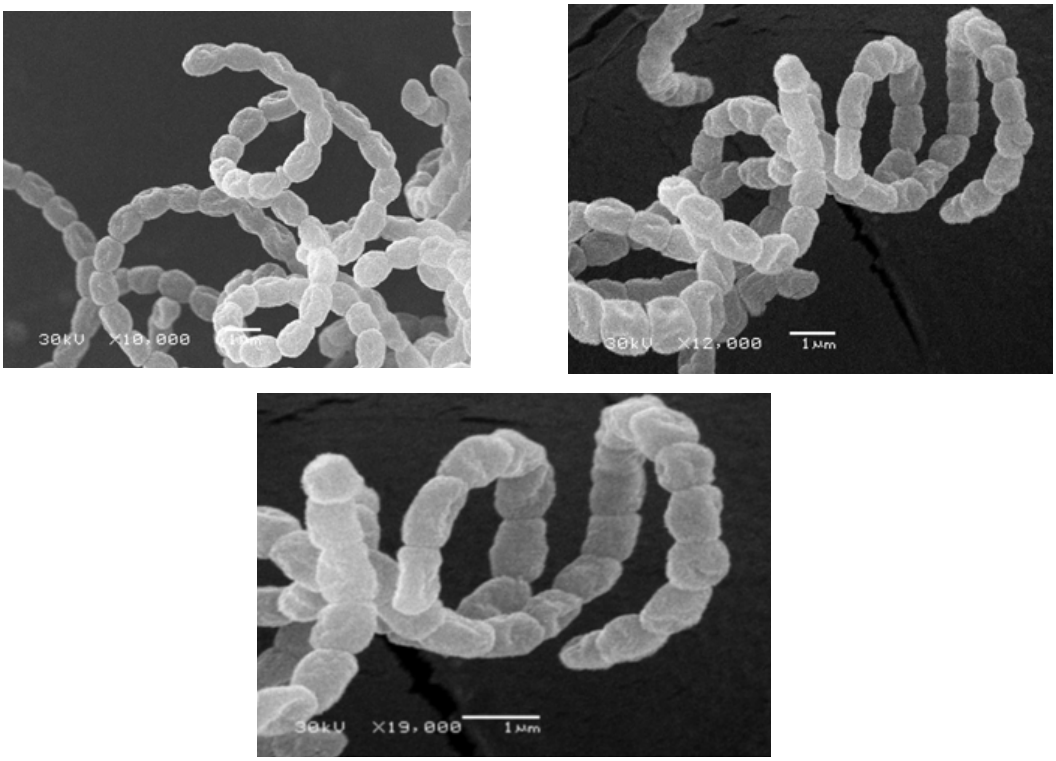

Fig. 2. Scanning electron micrograph showing the spore-chain morphology and spore-surface ornamentation of strain NEAE -115 grown on starch nitrate agar medium for 14 days at 30 °C at magnification of 10000X(A),12000X (B)and 19000X (C)

Table 1. Culture characteristics of the *Streptomyces* sp. strain NEAE-115

Medium	Color of		Diffusible pigment	Growth
	Aerial mycelium	Substrate mycelium		
ISP medium 2 (Yeast extract -malt extract agar)	Gray	Yellowish gray	Non-pigmented	Excellent
ISP medium 3 (Oatmeal agar)	Grayish beige	Faint yellowish brown	Non-pigmented	Excellent
ISP medium 4 (Inorganic salt-starch agar)	Gray	Faint olive	Non-pigmented	Excellent
ISP medium 5 (Glycerol asparagines agar)	Grayish beige	Faint yellowish brown	Non-pigmented	Very good
ISP medium 6 (Peptone-yeast extract iron agar)	Gray	Olive	Non-pigmented	Very good
ISP medium 7 (Tyrosine agar)	Gray	Olive	Non-pigmented	Excellent

The substrate mycelium pigment was not pH sensitive when tested with 0.05 N NaOH or 0.05 N HCl.
The diffusible pigment was not pH sensitive when tested with 0.05 N NaOH or 0.05 N HCl.

seen on starch nitrate agar medium. Spore surface is smooth (0.679-0.775 x 0.744- 1.325 µm in diameter) (Fig. 2).

Physiological and chemotaxonomic characteristics

The physiological characteristics of strain NEAE-115 are shown in Table 2. D-fructose, D-xylose, D-galactose, D-glucose, L-arabinose, ribose, D-mannose, maltose, rhamnose, raffinose, cellulose and trehalose are utilized for growth. Only traces of growth were produced on sucrose. α–amylase (starch hydrolysis), protease (degradation of casein), cellulase (growth on cellulose) and asparaginase of strain NEAE-115 were produced while uricase, lecithinase, chitosanase and gelatinase (gelatin liquification) were not produced. Coagulation and peptonization of milk were positive while hydrogen sulphide production and reduction of nitrate to nitrite were negative. The optimal growth temperature was 30

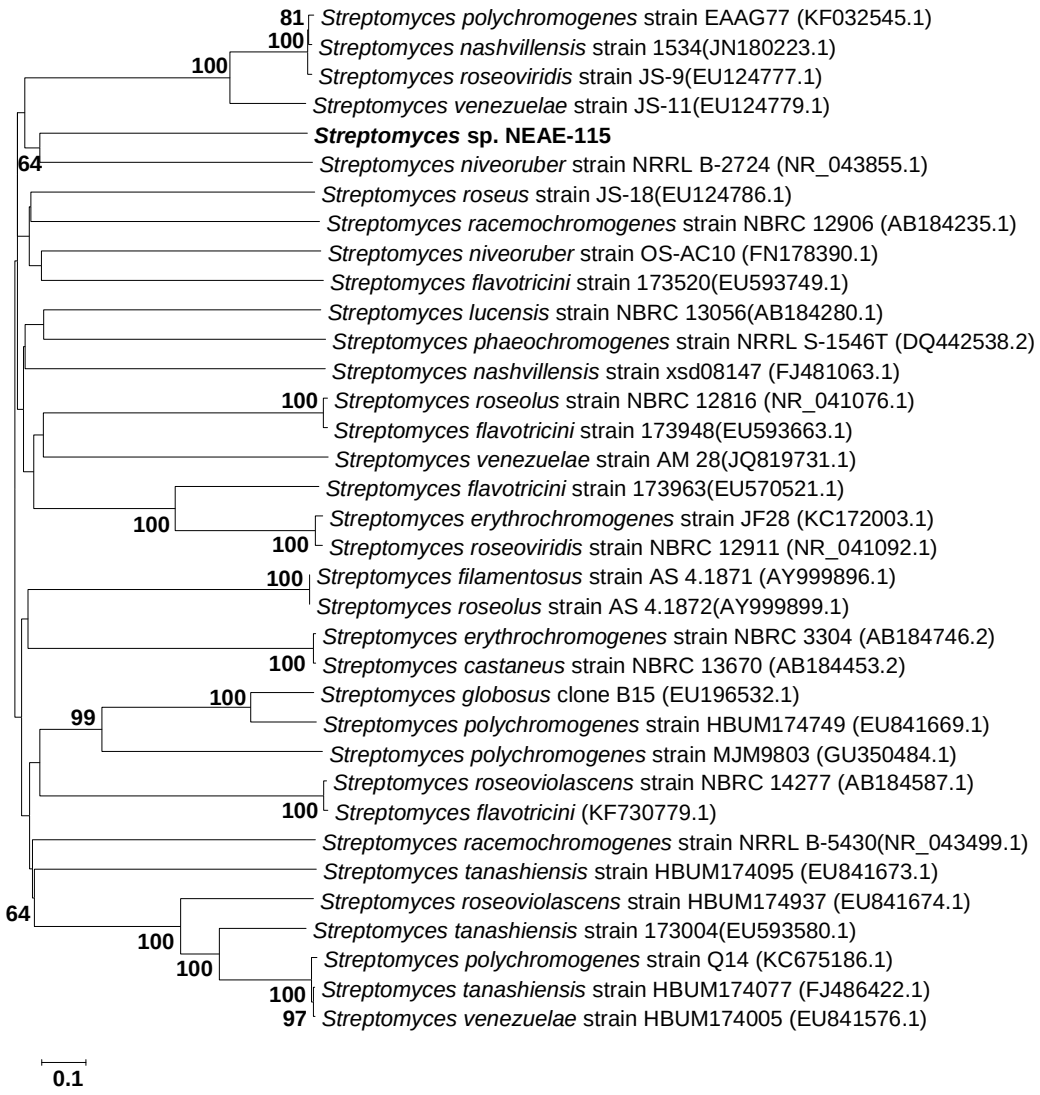

Fig. 3. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain NEAE-115 and related species of the genus *Streptomyces*. Only bootstrap values above 50 %, expressed as percentages of 1000 replications, are shown at the branch points. GenBank sequence accession numbers are indicated in parentheses after the strain names. Phylogenetic analyses were conducted in the software package MEGA4. Bar, 0.1 substitution per nucleotide position.

Table 2. Phenotypic properties that separate strain *Streptomyces* NEAE-115 from related *Streptomyces* species.
Data for reference species were taken from Bergey's Manual® of Systematic Bacteriology -volume five the actinobacteria [27].

Characteristic	*Streptomyces* sp. strain NEAE-115	*Streptomyces niveoruber*	*Streptomyces venezuelae*	*Streptomyces roseoviridis*	*Streptomyces polychromogenes*	*Streptomyces nashvillensis*
Aerial mass color on ISP mdeium 2	Gray	White-red	Gray	Red or yellow color	Red (grayish yellowish pink)	Gray
Reverse side of colony on ISP medium 2	Yellowish grey	Yellow/orange	No distinctive pigments (grayish yellow)	No distinctive pigments	No distinctive pigments	Strong brown to light grayish brown
Production of diffusible pigment	None	Yellow	No pigment	Green diffusible pigment	No pigment, or only a trace of yellow	Yellow to brown
Spore chain morphology	Spirals	Spirals	Rectiflexibilies	Rectiflexibiles	Rectiflexibiles, A few spore chains may terminate in spirals	Rectiflexibiles, some spore chains spirals
Spore surface	Smooth	Smooth	Smooth	Smooth	Smooth(1)	Smooth
Spore shape	Rod-shaped to cylindrical		Oval to oblong			
Sensitivity of diffusible pigment to pH	–					+(2)
Melanin production on						
Peptone-yeast extract iron agar	–	–	+	+	+	+
Tyrosine agar	–	–	+	+	+	+
Tryptone-yeast extract broth	–	–	+	+	+	+
Maximum NaCl tolerance (%, w/v)	7	2.5	2.5			
Degradation of						
Lecithin	–		+			
Casein	+		+			
Starch	+		+			
Coagulation of milk	+		-			
Peptonization of milk	+		+ (slow)			
Nitrate reduction	–		+			

Table 2. Continued

Characteristic	*Streptomyces* sp. strain NEAE-115	*Streptomyces niveoruber*	*Streptomyces venezuelae*	*Streptomyces roseoviridis*	*Streptomyces polychromogenes*	*Streptomyces nashvillensis*
H_2S production	–	+				
Gelatin liquification	–	+	+			
Utilization of carbon sources(1%,w/v)						
D(-) Fructose	+	+	+	±	+	±
D(+) Xylose	+	–	+	+	+	+
D(+) Galactose	+					
D(+) Glucose	+	+	+	+	+	+
L-arabinose	+	+	+	+	+	+
Ribose	+					
D(+) Mannose	+	+				
Sucrose	±	+	±	–	+	–
Maltose	+					
Rhamnose	+	–	+	–	±	–
Raffinose	+	–	±	–	±	+
Cellulose	+	–				
Trehalose	+					

Abbreviations : +, Positive; –, Negative; ±, Doubtful; Blank cells, no data available.The optimal growth temperature was 30°C and optimal pH was 7.0. It exhibited no antimicrobial activities against *Staphylococcus aureus, Alternaria solani and Bipolaris oryzae. Sacchromyces cerevisiae, Candida albicans, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Rhizoctonia solani, Fusarium oxysporum, Aspergillus niger* and *Klebsiella pneumoniae.* β–amylase (starch hydrolysis), protease (degradation of casein), cellulase (growth on cellulose) and asparaginase of strain NEAE-115 were produced while uricase, lecithinase, chitosanase and gelatinase were not produced.

(1) Knots and nest-like tangles may be seen in the aerial mycelium. Some of these tangles fragment into spore-like bodies and one observer reports that spores may also be seen on the substrate mycelium.

(2) Reverse mycelium pigment is somewhat pH-sensitive changing from yellowish brown to reddish brown with addition of 0.05 M NaOH or from yellowish brown to yellow with addition of 0.05 M HCl.

°C and optimal pH was 7.0. Strain NEAE 115 grew well in the presence of 7 % (w/v) NaCl. It exhibited no antimicrobial activities against *Staphylococcus aureus, Alternaria solani and Bipolaris oryzae. Sacchromyces cerevisiae, Candida albicans, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Rhizoctonia solani, Fusarium oxysporum, Aspergillus niger* and *Klebsiella pneumoniae.* Chemotaxonomic tests showed that the whole-cell hydrolysates contained mainly mannose and arabinose. Melanoid pigments were not formed in peptone-yeast-iron agar, tryptone-yeast extract broth or tyrosine agar.

16S rRNA gene sequence comparisons and phylogenetic analysis

The complete 16S rRNA gene sequence (1565 bp) was determined for strain NEAE-115. A BLAST search[24] of the GenBank database using this sequence showed its similarity to that of many species of the genus *Streptomyces*. A phylogenetic tree (Fig. 3) based on 16S rRNA gene sequences of members of the genus *Streptomyces* was constructed according to the bootstrap test of neighbor-joining algorithm method of Saitou and Nei [26] with MEGA4 [25]. This tree shows the close phylogenetic association of strain NEAE-115 with certain other *Streptomyces* species. Phylogenetic analysis indicated that the strain NEAE-115 consistently falls into a clade together with *Streptomyces polychromogenes* strain EAAG77 (GenBank/EMBL/ DDBJ accession No. KF032545.1 with a similarity of 80%), *Streptomyces nashvillensis* strain 1534 (GenBank/EMBL/ DDBJ accession No. JN180223.1 with a similarity of 80%), *Streptomyces roseoviridis* strain JS-9 (GenBank/ EMBL/ DDBJ accession No. EU124777.1 with a similarity of 81%), *Streptomyces venezuelae* strain JS-11 (GenBank/EMBL/ DDBJ accession No. EU124779.1 with a similarity of 81%) and *Streptomyces niveoruber* strain NRRL B-2724 (GenBank/EMBL/ DDBJ accession No. NR_043855.1 with a similarity of 80%).

Taxonomic conclusions

It is evident from Fig. 3 that strain NEAE-115 formed a distinct phyletic line with *Streptomyces niveoruber* strain NRRL B-2724. This phyletic line was consistently in the same clade along with *Streptomyces polychromogenes* strain EAAG77, *Streptomyces nashvillensis* strain 1534, *Streptomyces roseoviridis* strain JS-9 and *Streptomyces venezuelae* strain JS-11. The similarities of 16S rRNA gene sequence between the strain NEAE-115 and the forementioned type strains were between 80 and 81%. Comparison of the morphological, cultural and physiological characteristics of strain NEAE-115 and its closest phylogenetic neighbours revealed significant differences (Table 2). Data for reference species were taken from Bergey's Manual of Systematic Bacteriology -volume five the actinobacteria[27]. Strain NEAE 115 mainly differed from *Streptomyces polychromogenes, Streptomyces nashvillensis, Streptomyces roseoviridis* and *Streptomyces venezuelae* in that it produced spiral spore chains and did not produce melanin pigments. On the contrary, these strains produce rectiflexibiles spore chains and melanoid pigments formed on peptone-yeast-iron agar; tyrosine agar and tryptone-yeast extract broth. In addition, strain NEAE 115 mainly differed from *Streptomyces niveoruber* in that it produced gray aerial mycelium, yellowish gray substrate mycelial pigment and did not produce a diffusible pigment on ISP medium 2 while *Streptomyces niveoruber* produced white-red aerial mycelium, yellow/orange substrate mycelium pigment and yellow diffusible pigment. Strain NEAE 115 also differed from *Streptomyces niveoruber* in pattern of utilization of carbon sources. D-fructose, D-xylose, D-galactose, D-glucose, L-arabinose, ribose, D-mannose, maltose, rhamnose, raffinose, cellulose and trehalose are utilized for growth of strain NEAE 115, only traces of growth on sucrose. D-fructose, D-glucose, L-arabinose, D-mannose and sucrose are utilized for growth of *Streptomyces niveoruber*, whereas D-xylose, rhamnose, raffinose and cellulose are not utilized by *Streptomyces niveoruber.*

In conclusion, the strain NEAE 115, isolated from soil sample collected from Brollos Lake at the Mediterranean coast of Egypt, is clearly distinct from its closest phylogenetic neighbours described in the literature, and thus it can classified as a novel status in the genus *Streptomyces*. Therefore, we propose this organism as a new species, for which we suggest the name *Streptomyces brollosae* NEAE 115. Sequencing product was deposited in the GenBank database under accession number KJ410229.

Description of *Streptomyces brollosae* NEAE 115 sp. nov.

***Streptomyces brollosae* (*brollosae* pertaining to Brollos Lake, the geographical location of this type strain).**

The strain is aerobic, mesophilic, Gram-positive actinomycete that develops abundant and well-developed substrate and aerial mycelium. It develops gray aerial mycelium, yellowish gray substrate mycelium on yeast extract -malt extract agar. Verticils are not present and the mycelium does not fragment. The color of the substrate mycelium is not sensitive to changes in pH. Strain NEAE 115 grows well on yeast extract -malt extract agar, oatmeal agar, inorganic salt-starch agar, glycerol–asparagine agar, peptone-yeast extract iron agar and tyrosine agar. Melanoid pigments are not formed in peptone-yeast-iron agar, tryptone-yeast extract broth or tyrosine agar. No pigment found in medium in yeast extract -malt extract agar, oatmeal agar, inorganic salts-starch agar, glycerol-asparagine agar, peptone-yeast extract iron agar or tyrosine agar. The organism forms aerial hyphae which carries smooth-surfaced spores in spiral spore chains with open spirals intergrading through flexuous spore chains suggestive of section Rectiflexibiles (Fig. 2). The strain produces α–amylase (starch hydrolysis), protease (degradation of casein), cellulase (growth on cellulose) and asparaginase, whereas uricase, lecithinase, chitosanase and gelatinase (gelatin liquification) were not produced. Coagulation and peptonization of milk by this strain are positive while hydrogen sulphide production and reduction of nitrate to nitrite are negative (Table 2). Strain NEAE 115 grows well in the presence of 7 % (w/v) NaCl. D-fructose, D-xylose, D-galactose, D-glucose, L-arabinose, ribose, D-mannose, maltose, rhamnose, raffinose, cellulose and trehalose are utilized for growth. Only traces of growth were formed on sucrose. It exhibited no antimicrobial activities against *Staphylococcus aureus, Alternaria solani and Bipolaris oryzae. Sacchromyces cerevisiae, Candida albicans, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Rhizoctonia solani, Fusarium oxysporum, Aspergillus niger* and *Klebsiella pneumoniae.* Chemotaxonomic tests showed that the whole-cell hydrolysates contain mainly mannose and arabinose.

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