

Bounagaea algeriensis gen. nov., sp. nov., an extremely halophilic actinobacterium isolated from a Saharan soil of Algeria

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Abstract A novel halophilic actinobacterium strain, designated H8^T, was isolated from a Saharan soil sample collected in El-Goléa, South Algeria. Strain H8^T was identified as representing a new genus using a polyphasic taxonomic approach. Phylogenetic analysis revealed that strain H8^T shared the highest degree of 16S rRNA gene sequence similarity with '*Mz-abimyces algeriensis*' DSM 46680^T (93.0 %), *Saccharopolyspora ghardaiensis* DSM 45606^T (91.2 %), *Halopolyspora alba* DSM 45976^T (90.8 %) and *Actinopolyspora mortivallis* DSM 44261^T (90.0 %). The strain was found to grow optimally at 28–35 °C, at pH 6.0–7.0, and in the presence of 15–25 % (w/v)

NaCl. The substrate mycelium was observed to be well developed and fragmented in liquid medium and on solid medium. The aerial mycelium was observed to be moderately abundant and to form long chains with non-motile, smooth-surfaced and ovoid or spherical spores at maturity. The cell wall of strain H8^T was found to contain *meso*-diaminopimelic acid. The whole-cell hydrolysates were found to mainly contain arabinose and galactose. The diagnostic phospholipid detected was phosphatidylcholine, and MK-9(H₄), MK-9(H₂) and MK-10(H₂) were found to be the predominant menaquinones. The major cellular fatty acids were determined to be anteiso-C_{17:0} and iso-C_{15:0}. The genomic DNA G+C content of strain H8^T was determined to be 71.3 mol%. The genotypic and phenotypic data showed that the strain represents a novel genus and species, for which the name

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Bounagaea algeriensis gen. nov., sp. nov. is proposed, with the type strain H8^T (=DSM 45966^T = CECT 8470^T).

Keywords *Bounagaea algeriensis* gen. nov., sp. nov. · Halophilic actinobacterium · Algerian Sahara · Polyphasic taxonomy

Introduction

Halophilic actinobacteria are a valuable resource for discovery of novel products of industrial interest, including antimicrobial, cytotoxic, neurotoxic, antimutagenic, antiviral and antineoplastic activities (Zhao et al. 2011; Hamedi et al. 2013; Jang et al. 2013; Tian et al. 2013, 2014). Despite this finding, halophilic actinobacteria are under-explored for the discovery of novel bioactive secondary metabolites.

In recent years, research has intensified to discover new halophilic taxa, some of which could have a biotechnological interest. Most known halophilic actinobacteria belong to the family *Actinopolysporaceae*, and certain species to the family *Pseudonocardiaceae* (*Amycolatopsis*, *Prauserella*, *Saccharomonospora*, *Saccharopolyspora*, etc.) and to the family *Nocardiopsaceae* (*Nocardiopsis*, *Streptomonospora* and related genera).

In 2014, two halophilic actinobacterial genera were successively discovered: *Halopolyspora* (type species *Halopolyspora alba*) isolated from Dead Sea sediments and *Mzabimyces* (type species *Mzabimyces algeriensis*) isolated from a Algerian Saharan soil. The name of the latter taxon has not yet been validated.

Saharan soils, many of which are salted, have proven to be an interesting source of halophilic actinobacteria and many new taxa belonging to *Actinopolyspora*, *Nocardiopsis*, *Mzabimyces*, *Saccharopolyspora* and *Streptomonospora* have been described (Meklat et al. 2012, 2013a, b, c, 2014a, b; Saker et al. 2014, 2015; Bouras et al. 2015). In this context, a halophilic strain, named H8^T, was isolated from the Saharan desert of Algeria. The aim of the present study was to determine the taxonomic position of this strain using a polyphasic taxonomic approach.

Materials and methods

Isolation and maintenance of strain

Strain H8^T was isolated from a Saharan saline soil (electrical conductivity = 10.5 mS cm⁻¹) collected from El-Goléa (30°58'N, 2°87'E), South of Algeria. A serially diluted sample was plated on complex medium (CM) agar (Chun et al. 2000) supplemented with 20 % (w/v) NaCl and incubated for 3 weeks at 30 °C.

After isolation and purification, strain H8^T was conserved both on slants of CM agar containing 15 % (w/v) NaCl at 4 °C, and as 20 % (v/v) glycerol suspensions at -20 °C. The strain has been deposited in the German Collection of Microorganisms and Cell Cultures as strain DSM 45966^T and in Spanish Type Culture Collection as strain CECT 8470^T.

Phenotypic characterization

Cultural characteristics of the newly isolated strain were determined after 3 weeks of incubation at 30 °C on media of the International *Streptomyces* Project, ISP 2 and ISP 4 (Shirling and Gottlieb 1966), CM agar (Chun et al. 2000) and nutrient agar (Waksman 1961) media. The colours of the substrate and aerial mycelia and any soluble pigments produced were determined by comparison with ISCC-NBS colour name charts (Kelly and Judd 1976). The morphological characteristics of strain H8^T, including spore size, spore-chain morphology and surface ornamentation, were examined by light microscopy (Motic, B1 Series) and scanning electron microscopy (model S450, Hitachi, Japan) after 3 weeks growth on CM agar at 30 °C. All media used for morphological characteristics contained 15 % (w/v) NaCl. Growth at different temperatures (15, 20, 25, 28, 30, 32, 35, 40 and 45), various values of pH (4, 5, 6, 7, 8 and 9) and NaCl concentrations (0, 7, 10, 15, 20, 25, 28, 30, 32 and 35 %; w/v), and also in the presence of antibiotics, were determined by using nutrient agar medium, after incubating for 21 days at 30 °C. All media used for physiological tests contained 15 % (w/v) NaCl (except for the NaCl concentration test). Utilisation of carbohydrates and decarboxylation of organic acids were evaluated using the method of Gordon et al. (1974).

Degradation of other organic compounds was studied as described by Goodfellow (1971). Production of nitrate reductase and lysozyme sensitivity was determined according to the methods of Gordon and Barnett (1977) and Marchal et al. (1987), respectively. Production of melanoid pigments was evaluated on ISP 6 and ISP 7 media as recommended by Shirling and Gottlieb (1966).

Chemotaxonomical analyses

Biomass for chemotaxonomic analyses was obtained by cultivating the cell in shake flasks at 30 °C for 10 days on a rotary shaker (250 rpm) using CM broth containing 15 % (w/v) NaCl. Biomass was harvested by centrifugation at 3500 rpm and washed several times with distilled water. The isomeric form of diaminopimelic acid and the presence (or not) of glycine in the cell wall were determined according to the method described by Becker et al. (1964). The composition of whole-cell sugars was determined as described by Lechevalier and Lechevalier (1970). Polar lipids were extracted, examined and identified by using the procedure developed by Minnikin et al. (1977), with separation by two dimensional TLC. Cellular menaquinones were extracted and purified according to the procedure of Minnikin et al. (1984) and were analysed by HPLC (Kroppenstedt 1982, 1985). Cellular fatty acid composition was analysed using the microbial identification system (MIDI) Sherlock software version 6.1 (method TSBA40, TSBA6 database) as described by Sasser (1990). The analysis of mycolic acids was performed using the method of Minnikin et al. (1980).

Phylogenetic analyses

For extraction of chromosomal DNA, strain H8^T was grown on CM broth supplemented with 15 % (w/v) NaCl. The genomic DNA was extracted according to the method of Liu et al. (2000). PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out as described by Rainey et al. (1996). PCR products were purified with a PCR product purification kit (Qiagen, Germany). The primers used for sequencing are listed in Coenye et al. (1999). The 16S rRNA gene sequence has been deposited in the GenBank data library and assigned the accession number KF981441. The

resulting 16S rRNA gene sequence was compared with sequences present in the public sequence databases as well as with the EzTaxon-e server (Kim et al. 2012). Phylogenetic analyses were conducted using MEGA version 5 (Tamura et al. 2011). The 16S rRNA gene sequence of strain H8^T was aligned using the CLUSTAL W program (Larkin et al. 2007) against corresponding nucleotide sequences retrieved from GenBank.

Phylogenetic trees were constructed using the neighbour-joining (Saitou and Nei 1987) with Jukes and Cantor (1969) model, maximum-likelihood (Felsenstein 1981) with Kimura 2-parameter (Kimura 1980) model and maximum-parsimony (Fitch 1977) methods. Bootstrap analysis was performed with 1000 replicates (Felsenstein 1985) to validate the tree topology of the neighbour-joining method.

The G+C content of the chromosomal DNA was determined by HPLC according to the method described by Mesbah et al. (1989).

Results and discussion

Strain H8^T was found to show good growth on ISP 2, nutrient agar and CM agar media, and the aerial mycelium was observed to be moderately abundant with white colour on these media. However, poor growth was observed on ISP 4 medium. The substrate mycelium was observed to be a dark reddish orange colour on ISP 2 and nutrient agar media, and light yellow on CM agar and ISP 4 media. Melanoid pigments and other diffusible pigments were not produced on ISP 6, ISP 7 and other media. The substrate mycelium was found to be well developed and fragmented into non-motile cocci. The mycelium exhibited extensive fragmentation on solid and liquid media. The aerial mycelium was observed to form long chains of non-motile, and oval or spherical spores (0.8–0.9 × 0.8–1.3 μm) with smooth surfaces (Fig. 1). No morphological forms or structures resembling sporangia, sclerotia or synnemata were observed.

The strain was found to grow at 20–40 °C (optimum, 28–35 °C), pH 5.0–8.0 (optimum, 6.0–7.0), and 10–35 % NaCl (optimum, 15–25 %). The strain was found to be able to use the majority of the tested sugars and other organic compounds for its growth. The strain was found to be resistant to kanamycin (5 μg ml⁻¹), erythromycin (10 μg ml⁻¹), streptomycin (10 μg ml⁻¹),

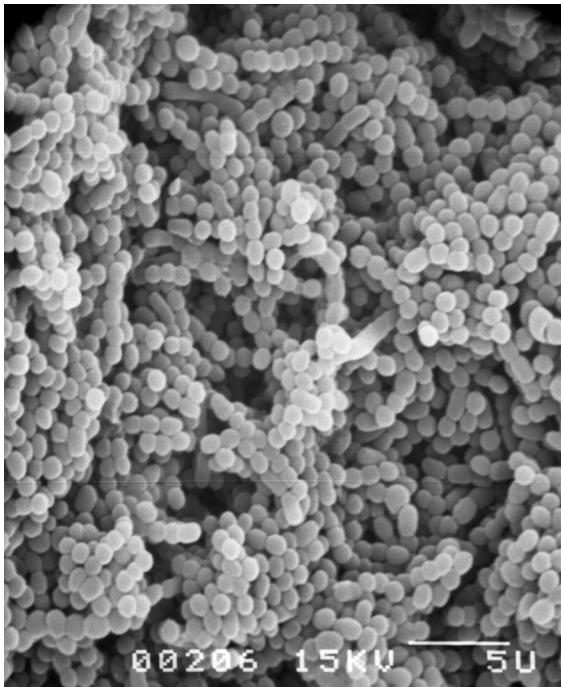


Fig. 1 Scanning electron micrograph of aerial mycelium of strain H8^T grown on complex medium agar containing 15 % (w/v) NaCl for 3 weeks at 30 °C. Bar 5 μm

penicillin G (25 μg ml⁻¹) and lysozyme (0.005 % w/v), but sensitive to chloramphenicol (25 μg ml⁻¹). The detailed physiological and biochemical characteristics of the type strain H8^T are given in the species description and in Table 1.

The strain H8^T was found to contain *meso*-diaminopimelic acid (but not glycine) in its cell wall, and the whole-cell hydrolysate was found to contain arabinose and galactose (chemotype pattern IVA of Lechevalier and Lechevalier 1970). Furthermore, an amount of glucose was also detected. Mycolic acids were not detected. The diagnostic phospholipid detected was phosphatidylcholine, corresponding to phospholipid type PIII (Lechevalier et al. 1977). Phosphatidylinositol, diphosphatidylglycerol, phosphatidylglycerol, two unidentified glycolipids and two unidentified glycopospholipids were also detected (Fig. S1). The predominant menaquinones were determined to be MK-9(H₄) (26.3 %), MK-9(H₂) (23.0 %) and MK-10(H₂) (11.9 %). MK-10(H₄) (6.8 %) and unidentifiable peaks (32 % in total) were also detected. The fatty acids profile was found to contain the following: anteiso-C_{17:0} (36.5 %), iso-

C_{15:0} (18.0 %), iso-C_{17:0} (10.5 %), iso-C_{16:0} (9.5 %), *cis*9 iso-C_{17:1} (7.8 %), anteiso-C_{15:0} (6.9 %) and *cis*8 C_{17:1} (4.3 %). The percentages of each of the other fatty acids were found to be lower than 1.6 % each.

Phylogenetic analysis of an almost complete 16S rRNA gene sequence (1497 bp, GenBank accession number KF981441) revealed that strain H8^T formed a distinct monophyletic clade in the phylogenetic trees obtained by the neighbour-joining (Fig. 2), maximum-parsimony (Fig. S2) and maximum-likelihood methods (Fig. S3). BLAST results for comparison between the 16S rRNA gene sequence of the strain H8^T and those of the closely related representatives of the families *Pseudonocardiaceae* and *Actinopolysporaceae*, and also of the genera *Halopolyspora* and ‘*Mzabimycetes*’, indicated very low percentages of similarity to sequences from the members of these families and genera. These percentages are 93.0 % with ‘*M. algeriensis*’ DSM 46680^T, 91.2 % with *Saccharopolyspora ghardaiensis* DSM 45606^T (*Pseudonocardiaceae*), and 90.8 % with *H. alba* DSM 45976^T and 90.0 % with *Actinopolyspora mortivallis* DSM 44261^T (*Actinopolysporaceae*). The G+C content of DNA of strain H8^T was determined to be 71.3 mol%.

In addition to the above, we noticed within the phylogenetic tree (Fig. 2) that ‘*M. algeriensis*’ is very closely related to *H. alba*. The percentage of similarity between the 16S rRNA gene sequences of these two taxa is 99.1 %. This suggests that ‘*M. algeriensis*’ may belong to the genus *Halopolyspora* and thus should be reclassified. Furthermore, while Lai et al. (2014) classified the genus *Halopolyspora* in the family *Actinopolysporaceae*, Saker et al. (2014) classified the genus ‘*Mzabimycetes*’ in the new family ‘*Mzabimycetaceae*’, a name that has not yet been validated.

The patterns of 16S rRNA gene signature nucleotides detected in the strain H8^T and the members of the related genera (‘*Mzabimycetes*’ and *Halopolyspora*) and families (*Pseudonocardiaceae* and *Actinopolysporaceae*) are shown in Table 2. Strain H8^T appears more closely related to ‘*Mzabimycetes*’ and *Halopolyspora* (only 3 differences) in comparison to the representatives of *Actinopolysporaceae* (7 differences) or *Pseudonocardiaceae* (14 differences). In addition, no differences were obtained between the ‘*Mzabimycetes*’ and *Halopolyspora* sequences, which share the same signatures between them, suggesting that they belong to the same genus. These results

Table 1 Differential phenotypic and chemotaxonomic characteristics of strain H8^T and its closest relative recognized species of the genera *Saccharopolyspora*, *Halopolyspora*, ‘*Mzabimyces*’ and *Actinopolyspora*

Characteristics	1	2	3	4	5
Spore surface	Smooth	Smooth	Rugose	Rugose	Smooth
Utilization as sole carbon source					
Adonitol	–	+	ND	+	ND
L-Arabinose	–	+	–	–	–
D-Cellobiose	–	+	+	+	ND
D-Galactose	+	+	–	–	+
meso-Inositol	+	+	+	–	–
D-Lactose	–	+	+	+	ND
D-Maltose	+	+	–	+	ND
D-Mannitol	–	–	+	+	–
D-Mannose	+	+	+	–	ND
D-Raffinose	–	–	+	+	+
L-Rhamnose	–	–	+	–	–
D-Ribose	+	–	+	–	ND
Salicin	–	–	+	–	ND
D-Sorbitol	–	–	+	+	ND
D-Xylose	+	+	+	–	+
Decomposition of					
Gelatin	+	–	–	–	+
Starch	–	+	+	–	ND
Tween 80	+	+	+	+	+
Xanthine	+	–	ND	+	+
Butyrate	–	–	ND	+	ND
Propionate	–	–	ND	–	+
Serine	–	+	+	–	ND
NaCl range (%, w/v)	10–35	7–32	10–35	7–30	10–32
Temperature range (°C)	20–40	25–45	20–50	20–45	10–50
Predominant menaquinones (%) (more than 10 %)	MK-9(H ₄) (26.3), MK-9(H ₂) (23.0) and MK-10(H ₂) (11.9)	MK-9(H ₄) (88.9)	MK-9(H ₄) (93.0)	MK-9(H ₄) (72.9)	MK-10(H ₄) (47.0) and MK-9(H ₄) (32.0)
Predominant fatty acids (%) (more than 10 %)	Anteiso-C _{17:0} (36.5), iso-C _{15:0} (18.0), iso-C _{17:0} (10.5)	C _{17:0} (27.8), iso-C _{15:0} (26.2), <i>cis</i> 9 iso- C _{17:1} (14.7) and iso- C _{17:0} (14.0)	Iso-C _{16:0} (20.5), iso- C _{17:0} (20.5), iso-C _{15:0} (16.9) and anteiso- C _{17:0} (14.5)	Iso-C _{15:0} (27.3), iso- C _{16:0} (18.4), iso- C _{17:0} (15.8) and anteiso-C _{17:0} (15.7)	Iso-C _{17:0} (28.0), anteiso-C _{17:0} (29.0), C _{16:0} (18.0) and iso- C _{15:0} (16.0)
DNA G+C content (mol%)	71.3	72.6	66.7	68.2	68.0

Data for reference strains were taken from this study except those of *Halopolyspora alba* DSM 45976^T (taken from Lai et al. 2014) and *Actinopolyspora mortivallis* JCM 7550^T (taken from Yoshida et al. 1991)

Strains: 1, *Boungaea algeriensis* H8^T; 2, *Saccharopolyspora ghardaiensis* DSM 45606^T; 3, *Halopolyspora alba* DSM 45976^T; 4, *Mzabimyces algeriensis* DSM 46680^T; 5, *Actinopolyspora mortivallis* JCM 7550^T

All strains producing non-motile spores, have a chemotype pattern IV A and phospholipid type PIII (phosphatidylcholine as phospholipid diagnosis)

+ positive, – negative, ND not determined

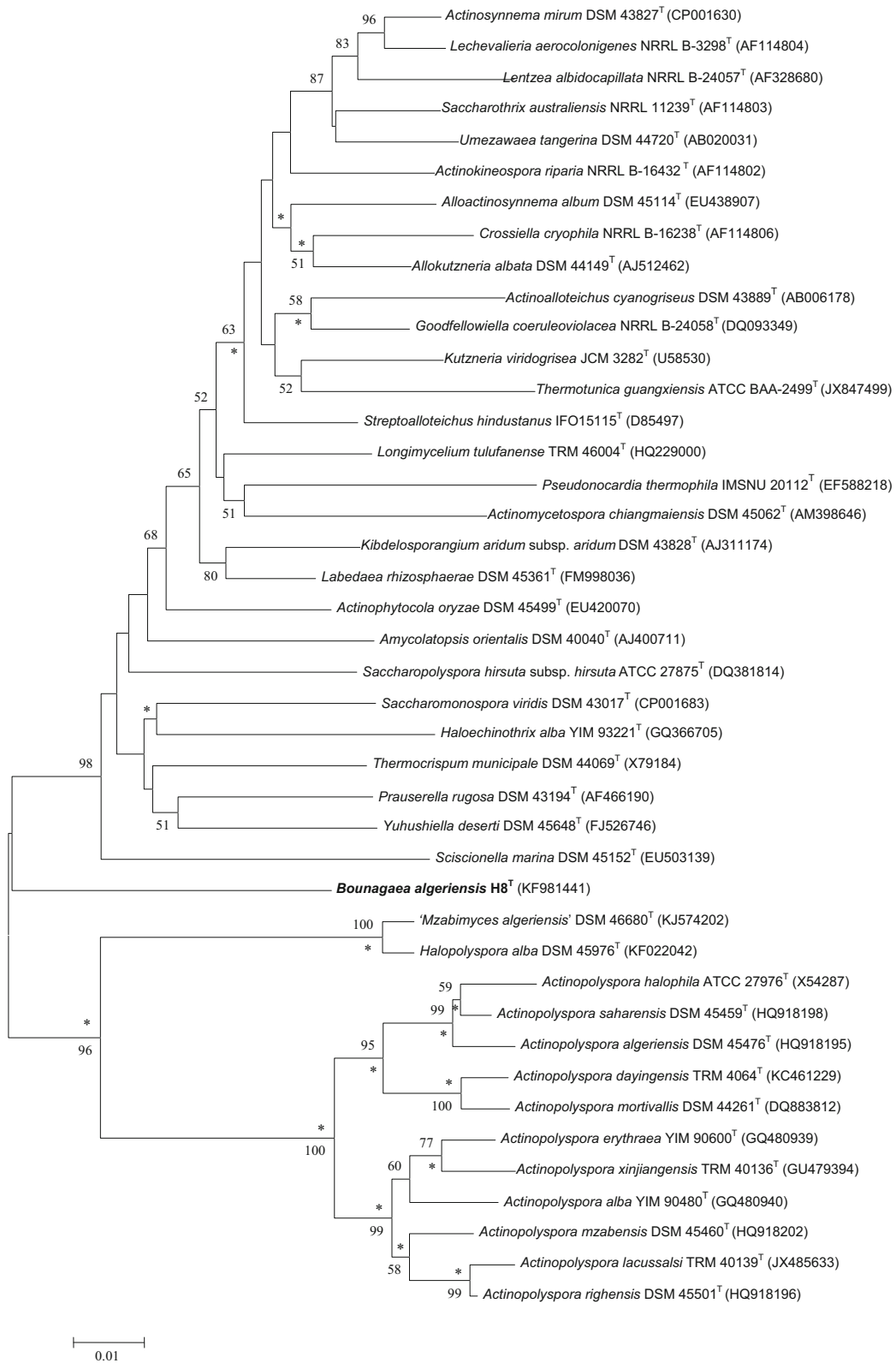


Fig. 2 Phylogenetic tree calculated from almost complete 16S rRNA gene sequences (1497 bp) using Kimura 2 parameter (Kimura 1980) evolutionary distance methods and the neighbour-joining method of Saitou and Nei (1987). This illustrates the taxonomic position of strain H8^T (1497 bp), and members of the family *Pseudonocardiaceae* and *Actinopolysporaceae*, and also the genera *Halopolyspora* and ‘*Mzabimyces*’. Asterisks indicate branches of the tree that were also found using the maximum-likelihood (Felsenstein 1981) or maximum-parsimony (Fitch 1977) tree-making algorithms. Numbers on branch nodes are bootstrap values (1000 resamplings; only values over 50 % are given). Bar 0.01 nucleotide substitution per site

suggest that the strain H8^T belongs to a different family compared to *Actinopolysporaceae*, *Pseudonocardiaceae* and the family ‘*Mzabimyces*’ proposed previously by Saker et al. (2014). Additional studies are needed to clarify the taxonomic position of the strain H8^T, and also the genera ‘*Mzabimyces*’ and *Halopolyspora*, at the suprageneric level.

Phylogenetically, strain H8^T differs very clearly from all known genera. On the basis of the phenotypic,

chemotaxonomic and phylogenetic data presented, strain H8^T should be placed as representing a new species of a new genus, for which we propose the name *Bounagaea* gen. nov. The type species of the genus is *Bounagaea algeriensis* sp. nov. with strain H8^T as the type strain.

Description of *Bounagaea* gen. nov

Bounagaea (bou.na.ga’ea, N.L. gen. masc. n. *bounagaea* of Bounaga, named in honour of the memory and untimely death of our late Professor Djilali Bounaga (1931–1980) of the Centre National de Recherche sur les Zones Arides (CNRZA), and also in honour of our Professor Nicole Bounaga-Riveill (CNRZA and URZA) for their broad contributions in teaching and training of researchers in the field of biology in Algeria).

Gram-stain positive, extremely halophilic, filamentous, aerobic actinobacteria. Diffusible pigments are not produced. Substrate hyphae are well developed

Table 2 Patterns of 16S rRNA gene signature nucleotides detected in the strain H8^T and the closely related genera and families

Position	Strain H8 ^T ^a	‘ <i>Mzabimyces</i> ’ ^a	<i>Halopolyspora</i> ^a	<i>Pseudonocardiaceae</i> ^b	<i>Actinopolysporaceae</i> ^c
127:234	U–U	G–A	G–A	G–C	A–U
242:284	C–G	G–G	G–G	G–A	C–G
480	G	G	G	U	U
564	C	C	C	U	C
657:749	G–C	G–C	G–C	U–A	G–C
672:734	C–G	C–G	C–G	U–G	C–G
828	A	A	A	A	A
829:857	G–C	G–C	G–C	G–C	G–C
831:855	U–G	U–G	U–G	U–G	U–G
832:854	G–U	G–U	G–U	G–U	G–U
833:853	U–G	U–G	U–G	U–G	U–G
840:846	C–G	C–G	C–G	C–A	C–G
952:1229	U–A	U–A	U–A	U–A	U–A
986:1219	U–G	U–G	U–G	U–A	U–A
1100	A	A	A	A	U
1117:1183	C–G	C–G	C–G	U–U	G–C
1309:1328	G–G1	G–G	G–G	G–C	G–U

Nucleotides that differentiate the strain H8^T and the closely related genera ‘*Mzabimyces*’ and *Halopolyspora*, and the families *Pseudonocardiaceae* and *Actinopolysporaceae*, are shown in bold

^a Data from this study

^b Data from Zhi et al. (2009), Mao et al. (2011) and Labeda and Goodfellow (2012)

^c Data from Zhi et al. (2009) and Goodfellow and Trujillo (2012)

and fragment with age into cocci-shaped elements. Forms aerial mycelium with long chains of non-motile, smooth-surfaced and oval or spherical spores. The cell wall contains *meso*-diaminopimelic acid as the diamino acid, and arabinose and galactose are present as major whole-cell sugars. The diagnostic phospholipid is phosphatidylcholine. The major fatty acids are anteiso-C_{17:0}, iso-C_{15:0}, iso-C_{17:0} and *cis*9 iso-C_{17:1}. The predominant menaquinones are MK-9(H₄), MK-9(H₂) and MK-10(H₂). Mycolic acids are not present. The G+C content of the genomic DNA of the type strain of the type species is 71.3 mol%. The type species is *Bounagaea algeriensis*.

Description of *Bounagaea algeriensis* sp. nov

Bounagaea algeriensis (al.ge.ri.en'sis. N.L. fem. adj. *algeriensis*, pertaining to Algeria, the source of the soil from which the type strain was isolated).

The species exhibits the following properties in addition to those given in the genus description. Aerial mycelium is moderately abundant with white colour on ISP 2, nutrient agar and CM agar media. Forms long chains of non-motile, smooth-surfaced, and oval or spherical spores (0.8–0.9 × 0.8–1.3 μm). Substrate mycelium is dark reddish orange colour on ISP 2 and nutrient agar media, and light yellow on CM agar and ISP 4 media. The substrate mycelium is well developed on all media tested and fragments into non-motile cocci. Optimal culture conditions are 28–35 °C and pH 6–7, in the presence of 15–25 % (w/v) NaCl. Growth is not observed in the absence of NaCl or in the presence of 7 % (w/v) NaCl. Not able to reduce nitrate to nitrite. Degrades adenine, gelatin, hypoxanthine, testosterone, Tween 80, L-tyrosine and xanthine, but not casein, guanine and starch. The following compounds are utilised as sole carbon sources: erythritol, D-fructose, D-galactose, D-glucose, glycerol, *meso*-inositol, maltose, D-mannose, D-ribose, sucrose, D-trehalose, D-xylose and L-proline. The following substances are not utilised: adonitol, L-arabinose, D-cellobiose, D-lactose, D-mannitol, D-melezitose, D-melibiose, D-raffinose, L-rhamnose, salicin, D-sorbitol, L-alanine and L-serine. Acetate, citrate and pyruvate are decarboxylated, but not benzoate, butyrate, oxalate, propionate, succinate and L-tartrate. The G+C content of the genomic DNA of the type strain is 71.3 mol%.

The type strain H8^T (=DSM 45966^T = CECT 8470^T) was isolated from a Saharan soil sample collected from El-Goléa (Ghardaïa, South Algeria). The 16S rRNA gene sequence of strain H8^T has been deposited in GenBank under the accession number KF981441.

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Conflict of interest The authors declare that there are no conflicts of interest to declare.

Ethical statement This article does not contain any studies with human participants and/or animals performed by any of the authors. The formal consent is not required in this study.

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