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Melghirimyces thermohalophilus sp. nov., a thermoactinomycete isolated from an Algerian salt lake

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A novel filamentous bacterium, designated Nari11A^T, was isolated from soil collected from a salt lake named Chott Melghir, located in north-eastern Algeria. The strain is an aerobic, halophilic, thermotolerant, Gram-stain-positive bacterium, growing at NaCl concentrations between 5 and 20 % (w/v) and at 43–60 °C and pH 5.0–10.0. The major fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{17:0}$. The DNA G+C content was 53.4 mol%. LL-Diaminopimelic acid was the diamino acid of the peptidoglycan. The major menaquinone was MK-7, but MK-6 and MK-8 were also present in trace amounts. The polar lipid profile consisted of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine and three unidentified phospholipids. Results of molecular and phenotypic analyses led to the description of the strain as a novel member to the genus *Melghirimyces*, family *Thermoactinomycetaceae*. Strain Nari11A^T shows 96.7 % 16S rRNA gene sequence similarity to the type strain of *Melghirimyces algeriensis.* On the basis of phenotypic, physiological and phylogenetic data, strain Nari11A^T (=DSM 45514^T =CCUG 60050^T) represents the type strain of a novel species, for which the name *Melghirimyces thermohalophilus* sp. nov. is proposed.

The genus *Melghirimyces* was described by Addou *et al.* (2012) with one species, *Melghirimyces algeriensis*, a thermotolerant and halotolerant filamentous actinomycete first isolated from an Algerian chott. The genus belongs to the family *Thermoactinomycetaceae* and shares the characteristics of members of this family except for the presence of LL-diaminopimelic acid in the cell wall, which is rather unusual in the family *Thermoactinomycetaceae*, which is characterized by the presence of *meso*-diaminopimelic acid (Matsuo *et al.*, 2006). This particular characteristic was also found for the recently isolated species *Kroppenstedtia eburnea* (Von Jan *et al.*, 2011) and *Marininema mesophilum* (Li *et al.*, 2012) and has led to the emendation of the description of the family.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Nari11A^T is JX861508.

Two supplementary figures are available with the online version of this paper.

Here, we characterize a new member of the family *Thermoactinomycetaceae* isolated from the soil of an Algerian salt lake. The strain was isolated during our investigation of extremophilic actinomycetes with the aim of exploring their potential to produce original bioactive metabolites.

Samples were collected from soil taken from an Algerian salt lake named Chott Melghir located in north-eastern Algeria (located between 34° 00′ 00″ N 6° 07′ 30″ E and 34° 30′ 01″ N 6° 30′ 02″ E). The soil is characterized by an alkaline pH (8.49) and a salinity of 112 g l^{-1}. One gram of the collected soil sample was transferred to 100 ml liquid ISP2 medium (Shirling & Gottlieb, 1966) supplemented with 10 % NaCl in a 500 ml flask and incubated in a rotary shaker incubator at 55 °C with shaking at 160 r.p.m. for 4 days. From this culture, an aliquot of 0.1 ml was inoculated each day onto the surface of solid ISP2 medium with 10 % NaCl; plates were incubated at 55 °C and monitored after 48, 72 and 96 h. Strain Nari11A^T was

isolated by this procedure and maintained as a glycerol suspension (20 % w/v) at −80 °C.

Determination of phenotypic and cultural characteristics was achieved by cultivating the strain on ISP media (ISP1, ISP2, ISP4, ISP5, ISP6 and ISP7) as recommended by Shirling & Gottlieb (1966), and on nutrient agar. All media were supplemented with 10 % NaCl and incubated at 55 °C. On these media, the colours of aerial and vegetative mycelia and the development of growth were observed. Additionally, the production of melanoid pigments was examined on ISP6 and ISP7.

Cells taken at the exponential phase of growth on nutrient agar for 3 days were used for the study of morphological characteristics under an Optiphot phase-contrast microscope (Nikon). Transmission electron microscopy studies were carried out by using methods described by Fardeau *et al.* (1997).

Utilization of carbohydrates and organic acids as sole carbon and energy sources was examined on ISP9 medium (Shirling & Gottlieb, 1966). The same basal medium without NH_4SO_4 was used for determination of the utilization of amino acids as sole carbon and nitrogen sources; 10 % (w/v) NaCl was added to the medium. Each substrate tested (Table 1) was added to the medium to a final concentration of 20 mM. Mineral medium without carbon source was used as a control. Acid production from carbohydrate utilization was determined by addition of 0.2 g yeast extract l^{-1} and 20 ml 0.04 % (w/v) bromocresol blue solution l^{-1} as pH indicator to the basal medium. Acidification of the medium was demonstrated by a shift of the colour of the pH indicator from purple to yellow.

Strain Nari11A^T was tested for its ability to grow on meat extract, yeast extract, peptone and Casamino acids (supplied individually at 1 % w/v) as sole carbon and energy sources. Detection of hydrolysis of casein and tyrosine was carried out by the method of Staneck & Roberts (1974). Hydrolysis of starch was examined as described by Cowan (1974). Aesculin and arbutin agar were used to test the ability of the strain to degrade aesculin and arbutin, respectively, according to the method of Gordon *et al.* (1974). Hydrolysis of xylan (0.4 %, w/v) was detected on the basal medium of Tsukamura (1966). The method recommended by Sierra (1957) was used to determine the hydrolysis of Tweens 20 and 80. Degradation of xanthine, hypoxanthine, adenine and gelatin was determined according to the method of Gordon (1966) and Gordon & Mihm (1957). Urease activity was determined according to the method of Lányí (1987). Indole production, the Voges–Proskauer reaction and nitrate reduction/denitrification were investigated by using the methods of Gordon & Mihm (1957), Guérin-Faublée *et al.* (1992) and Joffin & Leyral (2006). The temperature range for growth was determined by incubating the culture on ISP2 medium at temperatures between 30 and 60 °C. The strain was tested at 55 °C for its ability to grow in the range pH 5–10 on ISP2 medium buffered with the following solutions: citrate buffer for pH 4–6, Tris/HCl for pH 7–9, tetraborate buffer for pH 9.5–10 and Na_2HPO_4 buffer for pH 11. For these two tests, the medium was supplemented with 10 % (w/v) NaCl. NaCl requirement for growth of the strain was determined by increasing the amount of NaCl (0–20 % w/v) in the ISP2 medium at pH 7 and at 55 °C.

The phylogenetic position of the strain was established by 16S rRNA gene sequencing. The methods of Thabet *et al.* (2004) were used for DNA purification, PCR amplification and sequencing of the 16S rRNA gene. The partial sequences generated were assembled using BioEdit version 5.0.9 (Hall, 1999) and the consensus sequence of 1532 nt was corrected manually for errors. The most closely related sequences in GenBank (version 178) and the Ribosomal Database Project (release 10) were identified using BLAST (Altschul *et al.*, 1997) and the Sequence Match program (Cole *et al.*, 2009). These sequences were extracted and aligned and the alignment was adjusted manually according to the 16S rRNA secondary structure using BioEdit. Evolutionary distances were calculated by using the Jukes and Cantor option (Jukes & Cantor, 1969). Dendrograms were reconstructed with the TREECON program using the neighbour-joining method (Saitou & Nei, 1987). The tree topology was re-examined by the bootstrap method (1000 replications) of resampling (Felsenstein, 1985); its topology was also supported by using the maximum-parsimony and maximum-likelihood algorithms (results not shown).

Biomass for chemotaxonomic and phylogenetic studies was obtained from 72- to 96-h-old cultures of strain Nari11A^T in ISP6 medium supplemented with 10 % (w/v) NaCl and incubated at 55 °C under aerobic conditions. Fatty acid methyl esters were obtained following the method of Stead *et al.* (1992) and were analysed by gas chromatography (model 6890N; Agilent Technologies) using the Microbial Identification System (MIDI; Sherlock version 6.1, TSBA40 database). Polar lipids were determined according to the method described by Minnikin *et al.* (1979) and separated by two-dimensional TLC. To identify spots, ninhydrin, Zinzadze reagent and molybdophosphoric acid were used (Embley & Wait, 1994). Isoprenoid quinones were extracted as described by Collins *et al.* (1977) and analysed by HPLC (Groth *et al.*, 1996).

DNA for the determination of the G+C content was obtained after disruption of cells by a French pressure cell (Thermo Spectronic) and purification on hydroxyapatite as described by Cashion *et al.* (1977). The method of Mesbah *et al.* (1989) was used for analysis of the DNA base composition and the G+C content was determined by reversed-phase HPLC according to the method described by Tamaoka & Komagata (1984). The G+C content of the DNA was calculated from the ratio of deoxyguanosine to thymidine.

The isomer of diaminopimelic acid was identified in whole-cell hydrolysates (4 M HCl, 100 °C, 16 h) by TLC on a cellulose sheet (cat. no. 1.05577.0001; Merck) and sugars were analysed by TLC on cellulose plates after

Table 1. Characteristics of strain Nari11A^T and *M. algeriensis* NariEX^T

Data for *M. algeriensis* NariEX^T were taken from Addou *et al.* (2012). ND, Not determined.

Characteristic	Nari11A^T	*M. algeriensis* NariEX^T
Colour of aerial mycelium	Light brown	Cream yellow
Colour of substrate mycelium	Brown	Yellow
Utilization of carbohydrates as sole carbon sources		
Glycerol	−	+
Maltose	−	+
Cellobiose	−	+
Rhamnose	−	+
Sucrose	−	+
myo-Inositol	−	+
Fructose	−	+
Ribose	−	+
Mannose	−	+
Glucose	−	+
Raffinose	−	+
Utilization of amino acids as sole carbon and nitrogen sources		
Alanine	−	+
Threonine	−	+
Proline	−	+
Glycine	−	+
Asparagine	−	+
Glutamic acid	−	+
Arginine	−	+/−
Aspartic acid	−	+
Utilization of organic acids as sole carbon sources		
Acetate	−	+
Oxalate	−	+
Succinate	−	+
Malate	−	+
Degradation of:		
Aesculin	+	−
Arbutin	+	ND
Ranges for growth		
Temperature (°C)	43–60	37–60
pH	5–10	5–9.5
NaCl (%, w/v)	5–20	0–21
DNA G+C content (mol%)	53.4	47.3
Major fatty acids*	i-$C_{15:0}$, ai-$C_{15:0}$, i-$C_{17:0}$	i-$C_{15:0}$, ai-$C_{15:0}$

*ai, Anteiso-branched; i, iso-branched.

hydrolysis of whole cells (0.5 M H_2SO_4, 100 °C, 2 h) according to published protocols (Schumann, 2011).

The almost-complete 16S rRNA gene sequence (1514 bp) of strain Nari11A^T was obtained and compared with sequences available in GenBank, indicating that the strain belongs to the genus *Melghirimyces*. The strain is closely related to *M. algeriensis*, with 96.7 % sequence similarity to the type strain, as shown in the phylogenetic tree (Fig. 1).

Strain Nari11A^T formed light-brown aerial mycelium and brown substrate mycelium on ISP2 medium after 72 h of incubation at 55 °C. Good growth occurred on ISP1, ISP6 and nutrient agar after 48 h of incubation at 55 °C. Moderate growth was observed on ISP7, and no growth was observed on ISP4 or ISP5. Colonies formed on ISP2 medium were dull with irregular margins and radial wrinkles, dark in the centre, clearer at the edges and surrounded by a halo. The diameter was 1.3 cm, and could reach 4 cm when the culture was incubated for long period. On ISP1 and nutrient agar, the strain formed small, beige colonies, 2–3 mm in diameter. The best growth was obtained on ISP6, and abundant aerial mycelium was produced as a layer. The strain did not produce any diffusible pigment. Long, straight to flexuous, moderately branched hyphae that developed single endospores were observed by means of phase-contrast microscopy (Figs S1

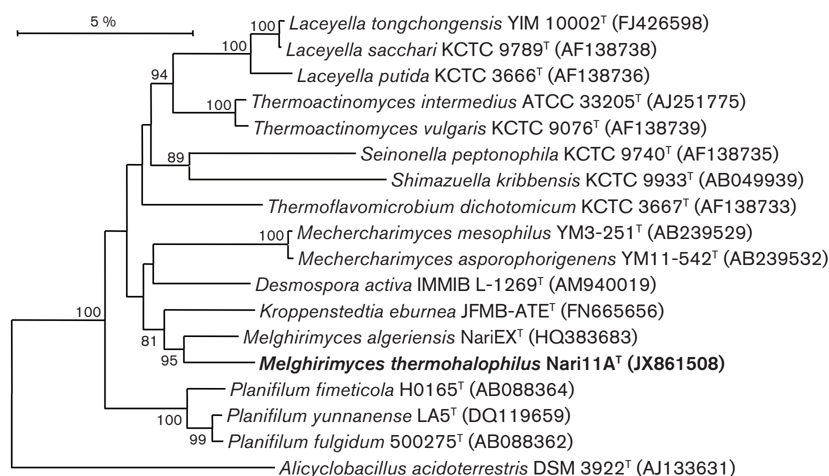

Fig. 1. Neighbour-joining phylogenetic dendrogram based on 1342 unambiguous nucleotides showing the relationship between strain Nari11A^{T} and organisms belonging to the family *Thermoactinomycetaceae*. The dendrogram was reconstructed from 16S rRNA gene sequences. GenBank accession numbers are shown in parentheses. Numbers at branch points specify the reliability of the branching order determined for 1000 resamplings; only bootstrap values above 70 % are shown. Bar, 0.05 substitutions per nucleotide position.

and S2, available in IJSEM Online). The nearest relative *M. algeriensis* showed a similar micromorphology.

Strain Nari11A^{T} was aerobic, Gram-stain-positive, thermophilic and strictly halophilic. Growth occurred at 43–60 °C, with very weak growth at 37 °C. The strain required NaCl at 5–20 % (w/v). Very weak growth was observed in 2 % (w/v) NaCl after 15 days. Unlike the nearest species *M. algeriensis*, which is halotolerant and able to develop on media free of NaCl, the strain is considered as moderately halophilic according to Larsen (1986). This character also distinguishes the new isolate from all other members of the family *Thermoactinomycetaceae*, for which no halophilic strain has been described so far. Strain Nari11A^{T} grew at pH 5–10, with good growth observed at pH 6–8.

Strain Nari11A^{T} did not use any of the carbohydrates, amino acids or organic acids tested as sole carbon sources. No growth was observed on ISP9 supplemented with each of the organic compounds used for this test. A similar case has already been reported for another member of the family *Thermoactinomycetaceae*, namely members of the genus *Planifilum*, which grow weakly on media containing carbohydrates as sole carbon sources (Hatayama *et al.*, 2005). The species *Kroppenstedtia eburnea* was also unable to use amino acids as nitrogen sources for growth (Von Jan *et al.*, 2011). This was explained by the fact that the basal media used were not rich enough for examining species of the *Thermoactinomycetaceae* (Von Jan *et al.*, 2011). This characteristic discriminates strain Nari11A^{T} from the nearest species *M. algeriensis*, which uses many organic compounds as sole carbon sources (Table 1).

Good growth was obtained on yeast extract, peptone and beef extract, while no growth was obtained on Casamino acids. Gelatin, aesculin, arbutin and casein were degraded, while xanthine, hypoxanthine, cellulose, starch, tyrosine, adenine, xylan and Tweens 20 and 80 were not hydrolysed. Urease activity was negative. Nitrate reduction, indole production and the Voges–Proskauer reaction were negative. Results of physiological and biochemical tests are summarized in Table 1.

The DNA G + C content of strain Nari11A^{T} was 53.4 mol%, which is much higher than the G + C content reported for *M. algeriensis* (47.3 mol%). The fatty acid profile of strain Nari11A^{T} consisted of iso- and anteiso-$C_{15:0}$ and iso-$C_{17:0}$ as main components (34.77, 19.38 and 17.36 %, respectively); analysis of cellular fatty acids also revealed the presence of iso-$C_{11:0}$ (0.12 %), anteiso-$C_{13:0}$ (0.38 %), iso-$C_{14:0}$ (1.50 %), $C_{14:0}$ (0.81 %), $C_{15:0}$ (1.22 %), iso-$C_{16:0}$ (8.98 %), $C_{16:0}$ (5.16 %), anteiso-$C_{17:0}$ (8.66 %) and $C_{17:0}$ (0.55 %). The profile of fatty acids is an effective differentiating character between strain Nari11A^{T} and *M. algeriensis* NariEX^{T}, which contains iso- and anteiso-$C_{15:0}$ as major components, like strain Nari11A^{T}, but not iso-$C_{17:0}$ (Table 2).

Whole-cell sugars consisted of galactose and ribose, but no other diagnostic sugars according to Lechevalier & Lechevalier (1970) were detected.

Strain Nari11A^{T} corresponded to the type strain of *M. algeriensis* in displaying LL-diaminopimelic acid as the diamino acid of the peptidoglycan and in its menaquinone profile, which contained MK-7, MK-6 and MK-8 in an approximate molar ratio of 94 : 1 : 1. Its polar lipid pattern differed from that of *M. algeriensis* by the absence of the unidentified minor lipid components PL1, PL2, PL5 and L (see Fig. S4 of Addou *et al.*, 2012). The polar lipids PL1 and PL2 of strain Nari11A^{T} (Fig. 2, this paper) may correspond to PL3 and PL4, respectively, of *M. algeriensis* NariEX^{T} (Addou *et al.*, 2012).

Several phenotypic and chemotaxonomic characteristics differentiate the new isolate from *M. algeriensis*, such as colony morphology, inability to utilize organic compounds as carbon sources, to hydrolyse urea and to reduce nitrate, G + C content, polar lipid pattern and cellular fatty acids. These differences, together with the strictly halophilic nature of strain Nari11A^{T}, confirmed the distinct species status of this strain, as shown by phylogenetic data based on the 16S rRNA gene sequence.

On the basis of significant phenotypic, phylogenetic and chemotaxonomic differences between strain Nari11A^{T} and

Table 2. Cellular fatty acid profiles of strain Nari11A^T and *M. algeriensis* NariEX^T

Values are percentages of total fatty acids. −, Not detected/not reported. Data for *M. algeriensis* NariEX^T were taken from Addou *et al.* (2012).

Fatty acid	Strain Nari11A^T	*M. algeriensis* NariEX^T
$C_{10:0}$	−	0.39
$C_{12:0}$	−	0.21
iso-$C_{11:0}$	0.12	−
iso-$C_{13:0}$	0.85	1.25
anteiso-$C_{13:0}$	0.38	0.20
iso-$C_{14:0}$	1.50	1.59
$C_{14:0}$	0.81	3.12
iso-$C_{15:0}$	34.77	59.13
anteiso-$C_{15:0}$	19.38	18.18
$C_{15:0}$	1.22	0.73
iso-$C_{16:0}$	8.98	1.84
$C_{16:0}$	5.16	4.62
iso-$C_{17:0}$	17.63	6.66
anteiso-$C_{17:0}$	8.66	1.54
$C_{17:0}$	0.55	−
$C_{18:1}\omega9c$	−	0.26

M. algeriensis, strain Nari11A^T represents a novel species of the genus *Melghirimyces*, for which the name *Melghirimyces thermohalophilus* sp. nov. is proposed.

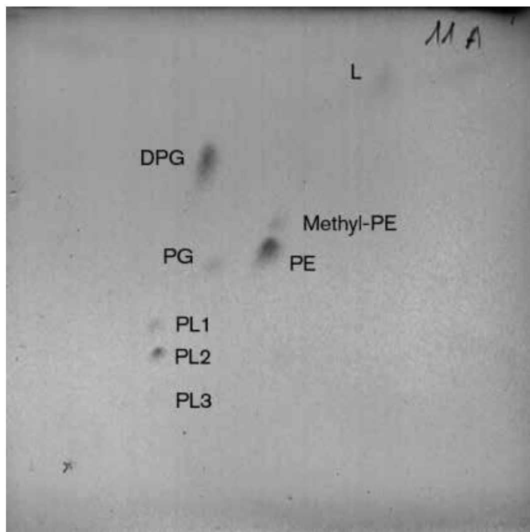

Fig. 2. Polar lipids of strain Nari11A^T after two-dimensional TLC and detection with molybdatophosphoric acid and heating at 200 °C for 10 min. DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; methyl-PE, phosphatidylmonomethylethanolamine; PL1–3, unknown phospholipids; L, unknown lipid.

Description of *Melghirimyces thermohalophilus* sp. nov.

Melghirimyces thermohalophilus (ther.mo.ha.lo′phi.lus. Gr. n. *thermê* heat; Gr. n. *hals*, *halos* salt; Gr. masc. adj. *philos* loving; N.L. masc. adj. *thermohalophilus* heat- and salt-loving).

Aerobic and Gram-positive-staining. Forms brown substrate mycelium and light-brown aerial mycelium on ISP2 medium. Beige aerial and substrate mycelium are formed on ISP1, ISP6 and nutrient agar. Growth occurs at 43–60 °C, 5–20 % (w/v) NaCl and pH 5–10. Does not use carbohydrates, amino acids or organic acids as sole carbon sources. Able to degrade gelatin, aesculin, arbutin and casein, but not xanthine, hypoxanthine, tyrosine, starch, xylan or cellulose. Major fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{17:0}$. MK-7 is the major menaquinone; MK-6 and MK-8 are minor menaquinones (approximate molar ratio 94 : 1 : 1). The polar lipid pattern consists of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine and three unidentified phospholipids. Whole-cell sugars consist of galactose and ribose, but no diagnostic sugars are detected. The DNA G+C content of the type strain is 53.4 mol%.

The type strain is Nari11A^T (=DSM 45514^T =CCUG 60050^T), isolated from soil of an Algerian salt lake.

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