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PLANOMONOSPORA, *SACCHAROTHRIX* AND *ACTINOPHYTOCOLA* GENERA IN SAHARAN SOILS OF ALGERIA: ISOLATION, TAXONOMIC IDENTIFICATION AND ANTAGONISTIC PROPERTIES

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ABSTRACT

With the aim of studying the biodiversity and the antimicrobial potential of three rare actinobacterial genera: *Planomonospora*, *Saccharothrix* and *Actinophytocola* in Algerian Saharan soils, 65 isolates representing the morphological characteristics of *Planomonospora* (17) and *Saccharothrix/Actinophytocola* (48) were isolated from 13 soil samples at 4 different sites in southern Algeria. The isolation was carried out on humic acid-vitamin agar medium using dilution techniques with several soil pretreatment and antibiotics as selective agents. Based on preliminary examination, 21 out of 65 isolates were kept for further investigations: *Planomonospora* (10) and *Saccharothrix/Actinophytocola* (11). 16S rRNA gene sequence analysis and DNA/DNA hybridization (DDH) showed that four strains, isolated from two different sites: Béchar (PM3) and Ghardaïa (MB20 and MB27), were found to represent four novel species. Moreover, they formed a distinct phyletic line within the clade of the most related genus: PM3 (DSM 46752^T) with *Planomonospora*, MB27 (DSM 46885^T) and MB46 (DSM 46886^T) with *Saccharothrix* and MB20 (DSM 46746^T) with *Actinophytocola*. While many novel species belonging to the genera *Saccharothrix* have been isolated from Algerian Saharan soils, this is the first report to describe the isolation of new species of *Planomonospora* and *Actinophytocola* from this extreme habitat. An assessment of the antimicrobial properties of the actinobacterial strains showed that 38 out of 65 have moderate to strong antimicrobial activities against Gram positive bacteria, fungi or yeasts. These results indicated the importance of further exploration of rare Saharan *Actinobacteria* for potentially original antimicrobial agents.

Keywords: *Saccharothrix*, *Planomonospora*, *Actinophytocola*, Biodiversity, Taxonomy, Algerian Saharan soil, Antagonistic properties

INTRODUCTION

Actinobacteria is one of the most common bacterial phyla. This group contains an extremely diverse group of Gram-positive bacteria with high GC-containing genomes. This phylum of bacteria has been extremely useful to the pharmaceutical industry due to their seemingly unlimited capacity of producing secondary metabolites with diverse biological activities and chemical structures **(Watve *et al*., 2001; Tiwari and Gupta, 2013).** Indeed, over 39% of bioactive molecules of microbial origin are produced by *Actinobacteria*, especially the excellent producers belonging to the genus *Streptomyces* **(Berdy, 2012)**. However, the intensive exploitation of terrestrial actinobacteria for many years has resulted in a steadily decreasing discovery rate of novel bioactive compounds, with an estimated 95% rediscovery rate of known compounds **(Fenical *et al*., 1999)**. Even so, the emergence of antibiotic resistance developed in various bacterial pathogens has caused a resurgence of interest in finding new biologically active compounds for drug discovery **(Subramani and Aalbersberg, 2013).** Thus, it is critical that new groups of microbes from unexplored habitats be pursued as sources of novel antibiotics and other therapeutic agents **(Bull *et al*., 2005)**. According to **Berdy (2005)** and **Tiwari and Gupta (2012)** rare actinobacteria produce the most diverse, unique, and occasionally structurally complicated with excellent antibacterial potency and usually low toxicity. Several chemical types such as simple terpenoids or benzenoids are almost completely absent from these compounds. Moreover, more than 50 rare actinobacteria taxa are reported to be the producers of 2,500 bioactive compounds **(Berdy, 2005).** Despite this intrinsic economic importance, little is known about the diversity of *Actinobacteria* in extreme environments, notably desert soils. Indeed, many studies reported that Algerian Saharan soils are harboring a significant amount of diverse actinobacteria, such as *Nocardiopsis*, *Saccharothrix*, *Actinopolyspora*, *Prauserella*, *Saccharomonospora*, *Saccharopolyspora*, *Streptomonospora*, *Actinoalloteichus*, *Planomonospora*, *Streptosporangium* and others **(Sabaou *et al*., 1998; Zitouni *et al*., 2005; Meklat *et al*., 2011; Boudjelal *et al*., 2015; Saker *et al*., 2015, Boubetra *et al*., 2016; Chaabane Chaouch *et al*., 2016a,b)**. One novel family, two novel genera and twenty-three novel rare species belonging to the phylum *Actinobacteria* were reported from Algerian Saharan soils (between 2012 and mid-2016). This is in addition to *Saccharothrix algeriensis* which was published as novel species in 2004. Many of these actinobacterial taxa have been reported to produce several metabolites with antimicrobial activity. Therefore, the isolation of new rare taxa from these extreme environments should provide access to new bioactive products with significant therapeutic potential and contribute to an understanding of their ecological roles. The aims of this study were to investigate the diversity of three rare actinobacterial genera (*Saccharothrix*, *Planomonospora* and *Actinophytocola*) isolated from four arid ecosystems (Ghardaïa, Béchar, Tamanrasset and Adrar) in the Algerian Sahara, and to evaluate their potential to produce different antimicrobial components.

MATERIALS AND METHODS

Soil samples and bacterial strain isolation

Thirteen non-rhizospheric soil samples (5–20 cm of depth) were collected aseptically from four different Saharan regions in the south of Algeria: Mzab

(province of Ghardaïa), Saoura (province of Béchar), Hogggar (province of Tamanrasset) and Touat (province of Adrar). The samples were placed in sterile polyethylene bags, closed tightly and stored at 4°C until analysis. The soil samples were suspended in sterile distilled water, serially diluted and spread-plated on humic acid-vitamin agar medium **(Hayakawa and Nonomura, 1987)** some of which was supplemented with penicillin (25 mg l^{-1}) and/or polymyxin (25 mg l^{-1}). If thermal pretreatment was performed as a selective method, the soil was baked at 120°C for 1 h **(Hayakawa *et al*., 1991).** The antifungal cycloheximide (50 µg/ml) was used to inhibit development of invasive fungi. These selective agents were chosen on the basis of good results obtained previously in our laboratory during work on the selective isolation of rare *Actinobacteria* from Saharan soils. The plates were incubated at 30°C for 28 days, and all colonies were examined directly by light microscopy (Model B1, Motic) to identify isolates that appeared to be *Planomonospora*, *Saccharothrix* and *Actinophytocola*.

Cultural and morphological characteristics of the isolates

Cultural and morphological features of each strain were determined by naked-eye examination of 1 to 4 week-old cultures grown at 30°C on various International *Streptomyces* Project (ISP) media: yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3) and inorganic salt-starch agar (ISP4) **(Shirling and Gottlieb, 1966),** and also on Bennett's medium**.** The colors of the substrate and aerial mycelia and of any soluble pigment secreted were determined with ISCC-NBS color charts **(Kelly and Judd, 1976).** The micromorphology of the strains was observed by light microscopy (Model B1, Motic).

Physiological and biochemical studies

Sixty-three physiological tests were performed to characterize the isolated strains. Physiological characteristics were determined according to the several methods **(Marchal and Bourdon, Marchal *et al*., 1978)**. The aims of these tests were to assess the assimilation of carbohydrates and derivatives as sole carbon sources; the decomposition of adenine, aesculin, arbutin, casein, cellulose, gelatin, guanine, hypoxanthine, starch, Tween 80, tyrosine, and xanthine, organic acids, reduction of nitrate; peptonization and coagulation milk, the growth at different values of pH and temperature, tolerance of several NaCl concentrations and production melanoid pigments.

Chemical studies of cell constituents

For the chemotaxonomic study, analysis of diaminopimelic acid and whole-cell sugars was carried out using the methods of **Becker *et al*. (1964)** and **Lechevalier and Lechevalier (1970)**. Phospholipids were analyzed according to the procedures developed by **Minnikin *et al*. (1984).** Menaquinones were isolated according to **Minnikin *et al*. (1984)** and were analyzed by HPLC **(Kroppenstedt, 1982, 1985).**

16S rRNA gene sequence and phylogenetic analysis

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR product were carried out following the protocols of **Rainey *et al*. (1996)**. The almost-complete 16S rRNA gene sequences were compared with those in the GenBank database using the EzTaxon-e server **(Kim *et al*., 2012)**. Phylogenetic analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) software version 6 **(Tamura *et al*., 2013)**. The 16S rRNA gene sequences were aligned against neighboring nucleotide sequences using the CLUSTAL W (with default parameters) in MEGA version 6. Phylogenetic trees were reconstructed with the neighbor-joining method **(Saitou and Nei, 1987)**. Evolutionary distances were calculated using the model of **Jukes and Cantor (1969)**. Topologies of the resultant trees were evaluated by bootstrap analyses **(Felsenstein, 1985)** based on 1000 resamplings.

DNA–DNA Hybridization

DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by **Cashion *et al*. (1977).** DNA–DNA hybridization was carried out as described by **De Ley *et al*. (1970)**, incorporating the modifications described by **Huss *et al*. (1983)**, using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). DNA-DNA hybridization experiment was done as duplicates in 2 × SSC in the presence of 10% formamide at 71°C.

Evaluation of antimicrobial activity

Seventy-two isolates were assessed by preliminary screening of antimicrobial activity using the cross streak method **(Lemos *et al*., 1985)**. The isolates were cross streaked on ISP2 medium and incubated at 30°C for 10 days. After observing a good growth of actinobacterial cultures, overnight cultures of ten different pathogens were used for the screening; namely, *Bacillus subtilis* ATCC 6633, Methicillin-resistant *Staphylococcus aureus* 639c, *Escherichia coli* (E52), *Klebsiella pneumonia* (E40), *Saccharomyces cerevisiae* ATCC 4226, *Candida albicans* (M3), *Fusarium culmorum* (Fc), *Aspergillus carbonarius* (M333), *Umbelopsis ramanniana* NRRL 1829 and *Penicillium expansum* (Pe) were streaked perpendicularly to the Actinobacterial cultures. Plates were incubated at 30°C for 48 h and the zone of inhibition was recorded. ISP2 plates without *Actinobacteria* isolates but streaked with the same stock of pathogens were used as controls.

RESULTS AND DISCUSSION

Isolation of strains from Saharan soil samples

Individual colonies were picked from the selective medium plates according to different types of morphology and pigment formation, and further isolated on ISP 2 medium as pure cultures. A total of 65 strains were isolated from Saharan soil samples, 48 of which were isolated from Ghardaïa, 14 from Béchar, 2 from Adrar and 1 isolate was recovered from Tamanrasset. Among the 65 isolates collected, 42 were obtained with the addition of polymyxin in the medium as a selective agent. Whilst 13 isolates were collected from soil samples which were baked at 120°C for 1 h and using polymixin as selective agents in the medium. No colony was obtained on medium supplemented with penicillin, while 10 isolates were collected from plates with no selective agents (table 1).

Morphological properties, chemotaxonomic characters and bacterial strain grouping

All isolates were identified to the genus level on the basis of cultural, morphological, physiological and chemotaxonomic tests. The 65 isolates were classified into 2 groups:

Group I included 17 strains (such as PM1, PM2, PM3). These isolates were found to have morphological and chemotaxonomic properties associated with members of the genus *Planomonospora*. They developed cylindrical sporangia arranged in double parallel row on aerial mycelium; each one containing a single motile sporangiospore. They produced orange or cream substrate mycelium and white scanty aerial hyphae. Strains in this group were characterized by a type III cell wall composition (*meso*-diaminopimelic acid) and a type B whole-cell sugar pattern (madurose). The predominant menaquinones were MK-9(H2, H4), and the phospholipid type was type PIV (phosphatidylethanolamine, and glucosamine-containing phospholipids).

Group II contained 48 strains (such as MB1, MB5, MB15) which were characterized by excessive fragmentation of both substrate and aerial mycelia into rods and ovoid elements, type III cell wall *meso*-diaminopimelic acid without glycine, the presence of galactose, rhamnose and small amounts of mannose as diagnostic whole-cell sugars, a phospholipid type PII (phosphatidylethanolamine) or PIV (phosphatidylethanolamine and glucosamine-containing phospholipids) pattern **(Labeda and Lechevalier, 1989)**, the presence of MK-9(H_4) as the predominant menaquinone and the absence of mycolic acids **(Labeda and Kroppenstedt, 2000)**. These characteristics are those of the genera *Saccharothrix* and/or *Actinophytocola*.

Phylogenetic analysis and DNA-DNA hybridization

The almost-complete 16S rRNA gene sequences of the 21 representative actinobacterial strains were submitted to the EzTaxon-e database for BLAST search to obtain the closest match for preliminary taxonomic assignment. It is evident from the BLAST results that the taxonomic placement of these actinobacterial strains is in agreement with phenotypic studies.

According to blast results, strains belonging to group I were most closely related to *Planomonospora parontospora* and *P. sphaerica*. Seven strains (PM1, PM2, PM3, PM4, PM12, PM18 and PM20) were affiliated to *P. sphaerica* JCM 9374^T (with 99.3 to 100% sequence similarity). Nevertheless strains PM9 and PM15 were most closely related to *P. parontospora* subsp. *antibiotica* JCM 3094^T (99.15 and 100% of similarity, respectively). However strain PM11 shared 99.72% sequence similarity with both *P. sphaerica* JCM 9374^T and *P. parontospora* subsp. *antibiotica* JCM 3094^T. The phylogenetic relationship between *Planomonospora* strains and their closely related type strains is shown in figure 1.

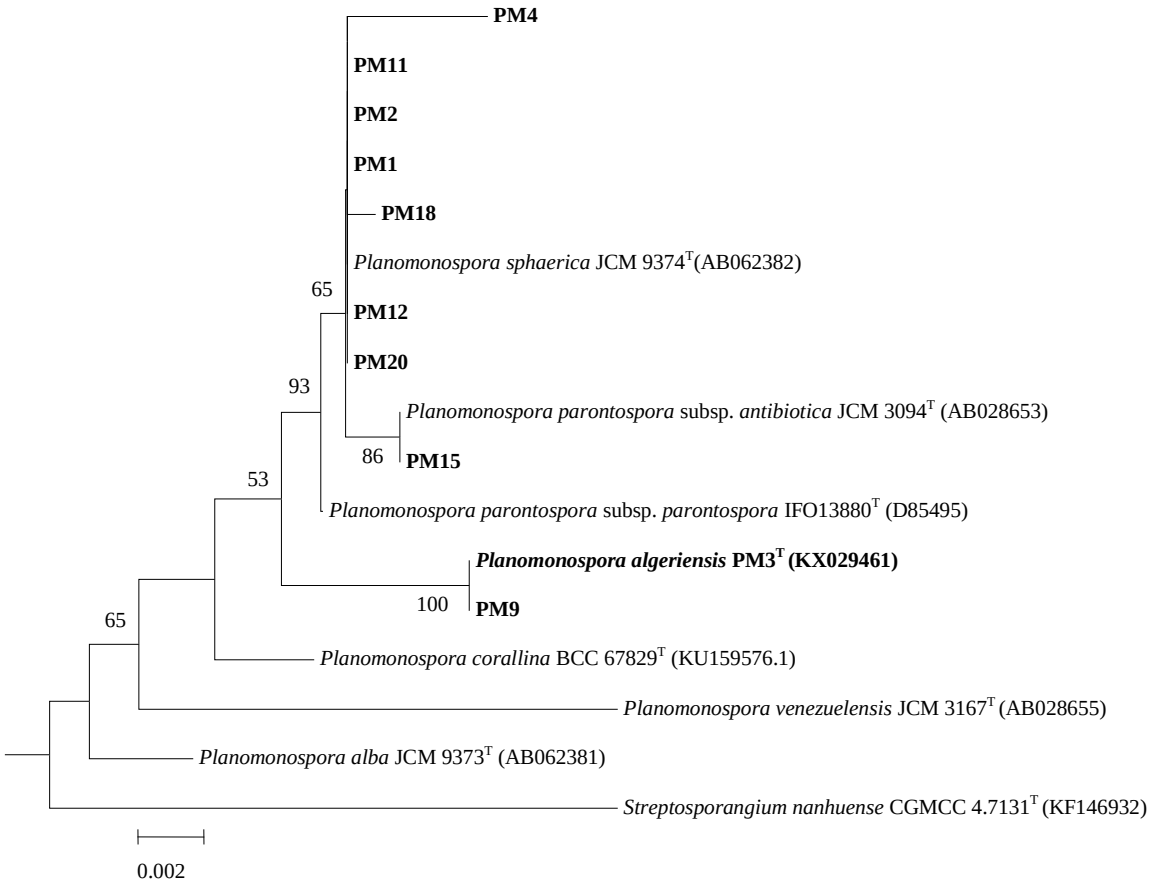

Figure 1 Neighbor-joining (NJ) tree based on 16S rRNA gene sequences showing relationships between *Planomonospora* isolates, and between these and their nearest phylogenetic neighbors. *Streptosporangium nanhuense* CGMCC 4.7131^T was used as an outgroup. Bootstrap values of >50% are indicated at nodes. GenBank accession numbers are given in parentheses. Bar: 0.002 substitution per nucleotide position.

For the strains belonging to group II, the 16S rRNA gene sequence analysis showed that strains MB1, MB5, MB15, and MB40 were most closely related to *Saccharothrix texasensis* NRRL B-16107^T (99.7, 99.03, 99.52 and 99.65% of similarity, respectively), MB28 and MB30 to *Saccharothrix hoggarensis* DSM 45457^T (99.86% for both strains), MB29 to *Saccharothrix longispora* NRRL B-116116^T (99.79%), MB43 to *Saccharothrix yanglingensis* KCTC 19722^T (99.31%), MB27 to *Saccharothrix ecbatanensis* DSM 45486^T (99.85%) and MB46 to *Saccharothrix espanaensis* DSM 44229^T (99.17%). The phylogenetic representation of *Saccharothrix* strains and their neighboring type strains belonging to this genus is illustrated in figure 2.

Table 1 Summary of the number of strains belonging to *Saccharothrix*, *Planomonospora* and *Streptosporangium* genera that were obtained by using the selective isolation methods

Province	**Regions**	**Genus**		**Total number of strains**
		Saccharothrix/Actinophytocola	***Planomonospora***	
Ghardaïa (Mzab)	Metlili (MT)	8 (C) + 8 (poly)	-	48 *Saccharothrix/Actinophytocola*
	Berriane (BR)	2 (C) + 3 (poly)	-	
	Tafilalt (TF1)	4 (Poly)	-	
	Tafilalt (TF4)	1 (Poly)	-	
	Tinemmirine (TM2)	7 (Poly)	-	
	El-Ateuff (AT)	10 (Poly)	-	
	Ntissa (NT3)	4 (Poly)	-	
	Ahbas (AB7)	1 (Poly)	-	
Béchar (Saoura)	Béni-Abbès (BA1)	-	2 (poly + p)	14 *Planomonospora*
	Béni-Abbès (BA5)	-	7 (poly + p) + 4 (poly)	
	Béni-Abbès (BA8)	-	1 (poly)	
Adrar (Touat)	Bouda (TC)	-	2 (poly)	2 *Planomonospora*
Tamanrasset (Hoggar)	Hoggar (HG1)	-	1 (poly)	1 *Planomonospora*
Total number of strains by genus		48 strains	17 strains	**Total: 65 strains**

-: no one strain; C: control (without soil pretreatment or antibiotic addition); poly: polymixin; peni: penicillin; p: thermal pretreatment.

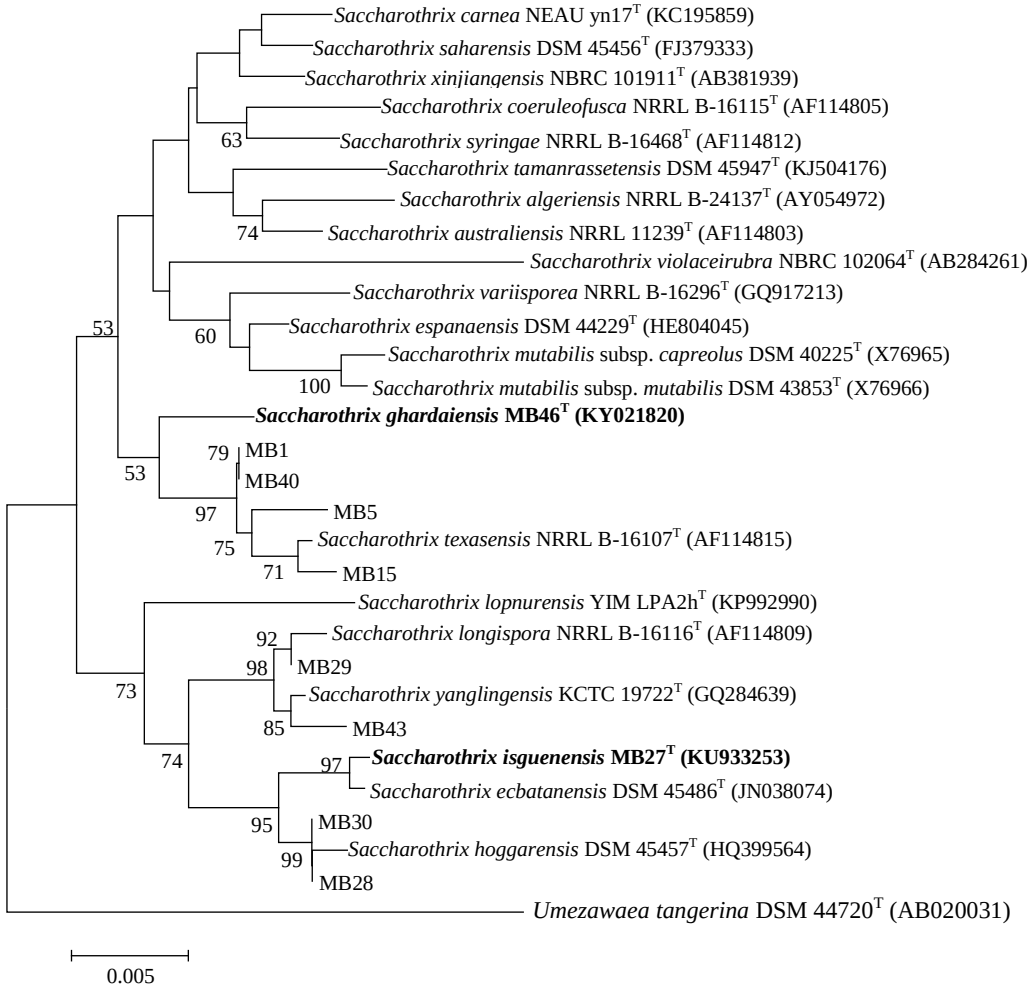

Figure 2 Neighbor-joining (NJ) tree based on 16S rRNA gene sequences showing relationships between *Saccharothrix* isolates, and between these and their nearest phylogenetic neighbors *Umezawaea tangerina* DSM 44720^T was used as an outgroup. Bootstrap values of >50% are indicated at nodes. GenBank accession numbers are given in parentheses. Bar: 0.005 substitution per nucleotide position

Although, strain MB20 which had the same morphological characteristics as *Saccharothrix*, was found to be closely related to members of the genus *Actinophytocola* (figure 3). Furthermore, the similarity of the 16S rRNA sequence of strain MB20 to those of the other species of the genus *Actinophytocola* ranged from 97.5 to 98.5%, with *A. gilvus* DSM 45828^T having the closest match.

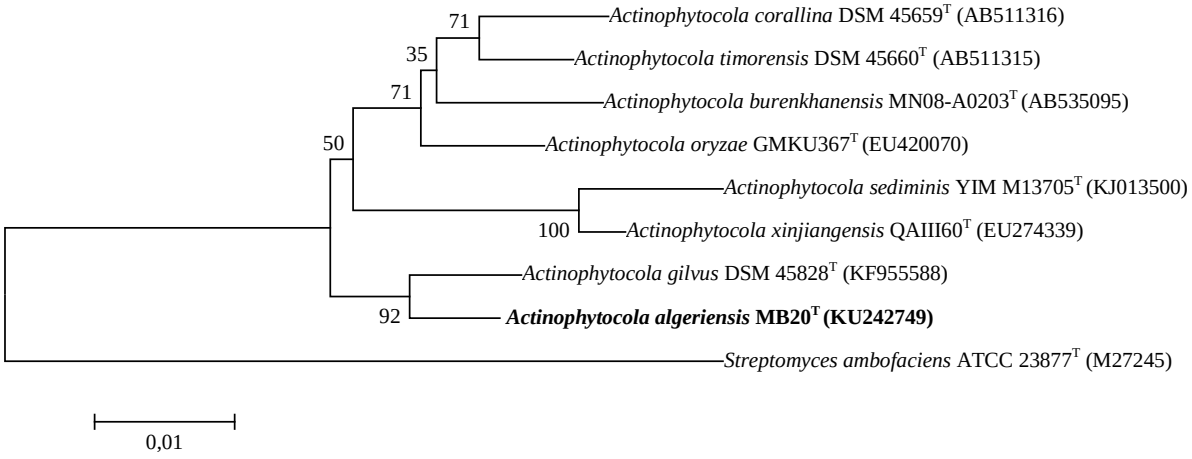

Figure 3 Neighbor-joining tree (Saitou and Nei, 1987) based on almost-complete 16 rRNA gene sequences showing the position of strain *Actinophytocola algeriensis* sp. nov. (MB20^T) amongst its phylogenetic neighbors. *Streptomyces ambofaciens* ATCC 23877^T was used as an outgroup. Numbers at nodes indicate levels of bootstrap support (%); only values ≥50% are shown. GenBank accession numbers are given in parentheses. Bar, 0.01 substitutions per site

For instance, complete polyphasic taxonomic analysis for strains PM3, MB27, MB46 and MB20 showed that strain PM3 (DSM 46752^T) was found to represent a novel species of the genus *Planomonospora*, named *Planomonospora algeriensis* **(Chaabane Chaouch *et al.*, 2016c)**, strain MB27 (DSM 46885^T) and MB46 (DSM 46886^T) to be represent two other novel species of the genus *Saccharothrix*, named respectively *Saccharothrix isguenensis* **(Bouznada *et al.*, 2016b)** and *Saccharothrix ghardaiensis* (accepted paper), while strain MB20^T DSM 46746^T is a novel species of the genus *Actinophytocola*, named

Actinophytocola algeriensis **(Bouznada *et al*., 2016a)**. It is worth mentioning that many other strains (PM4, PM18, MB5 and MB43) may represent also novel species belonging to the *Planomonospora* and *Saccharothrix* genera. More complete taxonomic studies on these promising novel organisms are underway. In the last years, four novel species of the genus *Saccharothrix* **(Zitouni *et al*., 2004; Boubetra *et al*., 2013a,b; Boubetra *et al*., 2015)** have been isolated from Saharan soils in Algeria. However, this is the first time that new species of *Planomonospora* and *Actinophytocola* have been isolated from these soils.

Actinobacterial diversity in Saharan soils

All stains of *Saccharothrix/Actinophytocola* (48) were harvested from the soils of Mzab region, whilst nearly all *Planomonospora* strains were collected from soils of Saoura region, except for PM9 and PM10 which collected from Touat region and PM11 from Hoggar province (table 1).

The use of polymyxin appeared to be important for the selection of *Planomonospora* and *Saccharothrix* genera. In addition, the thermal pretreatment of the soil was found to enhance the selection of *Planomonospora*. No colony was obtained on medium supplemented with penicillin. These results are in agreement with previous works carried out on *Actinobacteria* collected from several soil samples of the Sahara (unpublished data).

The obtained results revealed the effectiveness of selective methods of isolation as well as the biodiversity and richness of Algerian Saharan soils with respect to the genera *Planomonospora* and *Saccharothrix*, from which several novel members (novel species) are described.

Preliminary screening for antibacterial activity

The preliminary antimicrobial analysis showed that the Saharan strains may prove to be a potential source of antimicrobial compounds. Indeed, 58.5% of these strains were active against at least one test microorganism (table 2), while some of them inhibited the growth of more than one target microorganism (data not shown). This indicates the large diversity of these bioactive compounds and the high potential of these strains to produce unique antimicrobial agents.

Table 2 Number of actinobacterial strains belonging to *Saccharothrix*, *Planomonospora* and *Streptosporangium* genera producing antimicrobial metabolites

Genus	Total number of the active strains	Number of the Active strains against each target microorganism									
		Bs	Sa	E52	E40	Sc	M3	Fc	Ac	Ur	Pe
Saccharothrix/Actinophytocola	25	17	15	2	2	18	10	11	25	20	19
Planomonospora	13	13	13	0	0	0	0	0	08	06	0
Total number of the active strains	**38**	**30**	**28**	**2**	**2**	**18**	**10**	**11**	**33**	**26**	**19**

Bs: *Bacillus subtilis* ATCC 6633; **Sa:** *Staphylococcus aureus* Methicillin-resistant *Staphylococcus aureus* 639c; **E52:** *Escherichia coli*; **E40:** *Klebsiella pneumoniae*; **Sc:** *Saccharomyces cerevisiae* ATCC 4226; **M3:** *Candida albicans*; **Fc:** *Fusarium culmorum*; **Ac:** *Aspergillus carbonarius* (M333); **Ur:** *Umbelopsis ramanniana* NRRL 1829; **Pe:** *Penicillium expansum*.

CONCLUSION

In this study, 65 isolates representing the morphological characteristics of *Planomonospora* and *Saccharothrix/Actinophytocola* were isolated from Algerian Saharan soil. Four of which were found to be novel species *Planomonospora*, *Saccharothrix* and *Actinophytocola*. The results obtained also show the evidence of the high potential of the majority isolated strains to produce antimicrobial compounds. Hence, it provides further evidence that the Saharan soils of Algeria are a rich source for new rare actinobacterial taxa and, potentially, original bioactive compounds.

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