TAXONOMY AND ANTIFUNGAL ACTIVITY OF THERMOPHILIC STRAIN NT-T1 OF *Streptomyces* sp. ISOLATED FROM SAHARAN SOIL OF ALGERIA

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Abstract. A thermophilic actinobacterium strain designated NT-T1, was isolated from a Saharan soil sample by the agar plate dilution method using chitin-vitamin agar supplemented with cycloheximide and nalidixic acid. The taxonomic status of this strain was determined using a polyphasic approach. Optimal growth was found to occur at 40-65°C, pH 6-7, and in the presence of 0-5% (w/v) NaCl. Strain NT-T1 shows a greyish aerial mycelium with chains of spores of the *spiralis* and *rectiflexible* types. Melanin pigments were not produced. The strain NT-T1 contains LL-diaminopimelic acid and glycine. The whole-cell hydrolysate shows the strain does not contain taxonomically important sugars in its cell wall. The diagnostic phospholipid detected was phosphatidylethanolamine. The dominant fatty acid was iso-$C_{15:0}$ (65.93%). Strain NT-T1 was able to use the majority of the tested sugars and other organic compounds for its growth. The morphological and chemotaxonomic properties of strain NT-T1 are consistent with those shared by members of the genus *Streptomyces*. Furthermore, phylogenetic analysis based on the 16S rRNA sequence revealed similarities between NT-T1 and other members of the genus *Streptomyces*. The most closely related species, the strain *Streptomyces thermovulgaris* NRRL B-12375^T and *S. thermogriseus* NBRC 100772^T, showed a similarity level of 100% with NT-T1. NT-T1 exhibited good antifungal activity against *Fusarium culmorum*.

Key words: Saharan soil; Thermophilic actinobacteria; *Streptomyces*; Taxonomy; antimicrobial activities.

INTRODUCTION

The phylum *Actinobacteria* is one of the most dominant phyla in the bacteria domain, they are Gram-positive bacteria with a genomic guanine-cytosine content higher than 55%, and are the most attractive source of several types of bioactive secondary metabolites, especially members of the genus *Streptomyces*, which biosynthesize over 70% of the clinically useful antibiotics of natural origin [24, 43, 49]. The Algerian Saharan soils are rich and diversified in *Actinobacteria* with interesting antimicrobial properties [7, 33, 34, 42, 50]. More than 20 new species of *Actinobacteria* were described from the Algerian Sahara such as *Actinokineospora mzabensis* [2], *Saccharothrix ghardaiensis* [8], *Actinophytocola algeriensis* [9], *Streptosporangium becharense* [10] and *Prauserella isguenensis* [41]. *Actinobacteria* from extreme habitats are considered not only to have extensive taxonomic diversity, but also have an interesting potential to synthesize important natural molecules [3]. *Actinobacteria* from such environments are known to be a source of novel molecules. An earlier study has targeted *Actinobacteria* that can survive in extremely high temperatures [22]. These thermophilic *Actinobacteria* are well documented to produce novel antibiotics and other valuable bioactive metabolites [54], gaining the attention of microbiologists. The diverse and natural metabolites, especially antimicrobial agents from these strains, can be safely applied to synthetic antibiotics as there are fewer chances of adverse side effects [52].

In the last decades, thermophilic *Actinobacteria* were recognized as valuable sources of important secondary metabolites [45]. It is surprising that thermophilic Streptomycetes have received so little attention given their possible importance in microbial technology. Algerian ecosystems are a promising source for *Actinobacteria* with diverse biological activities, presupposing that it may be necessary to characterize such bacteria for novel and active molecules from Saharan soils. Therefore, the main objective of the current work is the isolation and identification of the *Streptomyces* strain NT-T1 from Saharan soils, and to describe this actinobacterial strain using a polyphasic approach based on morphological, physiological, chemotaxonomic, and molecular investigations as well as the screening of antifungal activities.

MATERIALS AND METHODS

Isolation of the actinobacterial strain

Saharan soil samples were collected aseptically from the region of Mzab, Ghardaïa (32°29′00″N, 3°41′00″E) at a depth of 20 cm. The samples were placed in clean dry sterile polythene bags and closed tightly. Samples were then serially diluted up to 3×10^{-2} and 0.1 mL of each dilution was spread over the surface of chitin-vitamin B agar medium supplemented with the antifungal cycloheximide (50 µg/mL) to avoid the development of unwanted micro-fungi. The plates were incubated at 55°C for 15 days. One colony was selected and streaked in a new plate containing ISP2 (International *Streptomyces* Project medium 2) [47] to obtain a pure culture named NT-T1 and then was maintained at 4°C.

Morphological study

The morphological and cultural characteristics were determined after 10 days of incubation on ISP2, ISP4, nutrient agar (NA) and R8 culture media [1]. The rate

of growth, the colors of the substrate and aerial mycelia and any soluble pigments produced were determined by comparison with chips from ISCC-NBS color charts [23]. The micromorphology of the thermophilic strain and the spores chain were observed by light microscope (Motic BA210) and scanning electron microscope (JSM-7100F, JEOL).

Chemotaxonomic analysis

For chemotaxonomic analysis, a biomass of the strain NT-T1 was grown in nutrient broth, on a rotary shaker (250 r.p.m., New Brunswick Scientific Co., NJ, USA) at 30°C for one week. Biomass was harvested by centrifugation and washed twice with distilled water. Determination of the isomer of diaminopimelic acid (DAP), whole-cell sugars, phospholipids and fatty acids profile were performed according to the procedures of Becker et al. (1964) [4], Lechevalier and Lechevalier (1970) [30], Minnikin et al. (1977) [36] and Sasser (1990) [46], respectively.

Physiological characterization

Sixty two (62) physiological tests were carried out to characterize the actinobacterial thermophilic strain. The degradation of different organic substrates was evaluated following the method of Goodfellow (1971, 1989) [13, 14]. Growth and production of acid from carbohydrates and decarboxylation of organic acids were studied as described by Gordon et al. (1974) [15]. Tolerance to different temperatures (30, 40, 50, 55, 60, 65 and 70°C) and pH (5, 7 and 9) were tested, and visible growth was recorded as a positive result. Strains were checked for salt requirement or tolerance by inoculation on NA containing 0, 5 and 10% w/v of NaCl as described by Kushner (1993) [29]. Lysozyme sensitivity was examined according to the methods of Gordon and Barnett (1977) [16]. Growth in the presence of antibiotics (chloramphenicol, erythromycin, kanamycin, streptomycin and penicillin) was also studied. All physiological tests were repeated three times.

Molecular study using 16S rRNA sequence analysis

Actinobacterial colonies were picked up aseptically from ISP2 medium and transferred to 1.5 mL sterile Eppendorf tubes. Genomic DNA was extracted according to the method of Liu et al. (2000) [32]. The 16S rRNA gene was PCR amplified using the primer pair 10-30F (5′-GAGTTTGATCCTGGCTCA-3′) and 1500R (5′-AGAAAGGAGGTGATCCAGCC-3′) (Integrated DNA Technologies).

The PCR reactions were carried out in a final volume of 50 µL of reaction mixture containing approximately 50 ng of genomic DNA, 0.5 µM of each primer, 1X PCR buffer, 1.5 mM of $MgCl_2$, 200 µM of each dNTP (*Taq* Core kit 10, MP Biomedicals) and 1U *Taq* DNA polymerase (Silver- Star). The PCR thermal cycling program included an initial denaturation at 96°C for 4 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 52°C for 1 min and extension at 72°C for 2 min, and then final extension at 72°C for 10 min. The amplification product was checked by electrophoresis on 0.8% agarose stained with EZ-Vision, and then submitted to the Beckman Coulter Genomics Company (United Kingdom) for purification and sequencing.

The obtained 16S rRNA gene sequence of the strain was compared for similarity level with sequences present in public sequence databases as well as in the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) [57]. Phylogenetic analysis was conducted using MEGA version 7 [28]. The multiple alignment of the obtained 16S rRNA gene sequence against nucleotide sequences, recovered from EzTaxon, was generated using the CLUSTAL W program [51]. A phylogenetic tree was constructed using the neighbor-joining method [40] with the Kimura 2-parameter [27] model. Tree topologies were evaluated using the bootstrap resampling method with 1000 replicates [12].

Antifungal activity assay

Strain NT-T1 was tested for antagonistic activity against several micro-fungi (*Fusarium culmorum*, *Fusarium sporotrichioides*, *Penicillium expansum* and *Aspergillus flavus*) using the agar plug diffusion method on NA medium. These target micro-fungi were provided by LBSM (Laboratoire de Biologie des Systèmes Microbiens), located at Ecole Normale Supérieure, Algeirs, Algeria. The method consists of inoculating the NT-T1 strain in tight streaks on the surface of the NA medium and incubating at 55°C for 6 days. Agar cylinders (10 mm in diameter) recovered from the actinobacteria culture were cut aseptically with a sterile cork borer and deposited on the agar surface of new NA plates freshly seeded by target fungi (10^7 UFC/mL), then incubated for 48 h (at 25°C). After incubation, the antifungal activity was evaluated by measuring the inhibition zone around the agar cylinder [5].

RESULTS

Morphological observation of a 5 days old culture of strain NT-T1 revealed that the strain was found to show good growth on R8 and nutrient agar (NA) culture media, but no growth on ISP2 and ISP4 media. The aerial mycelium was observed to be grey in color. Spore chain morphology was *spiral* (type S) and *rectiflexible* (type RF) (Fig. 1a). Aerial mycelium form spores that are non-motile, smooth-surfaced, and rod in shape (0.5–0.7 × 0.9–1 µm) (Fig. 1b). The substrate mycelium was observed to be a light yellow, branched and not fragmented on the same culture media. The melanin pigments were not produced.

Whole-cell hydrolysates of strain NT-T1 contained LL-diaminopimelic acid and glycine as the main components (Fig. 2a). Moreover, galactose was detected, but no characteristic sugars were found in cell hydrolysates components (Fig. 2b). The fatty acids profile was found to be composed as follows: iso-$C_{14:0}$

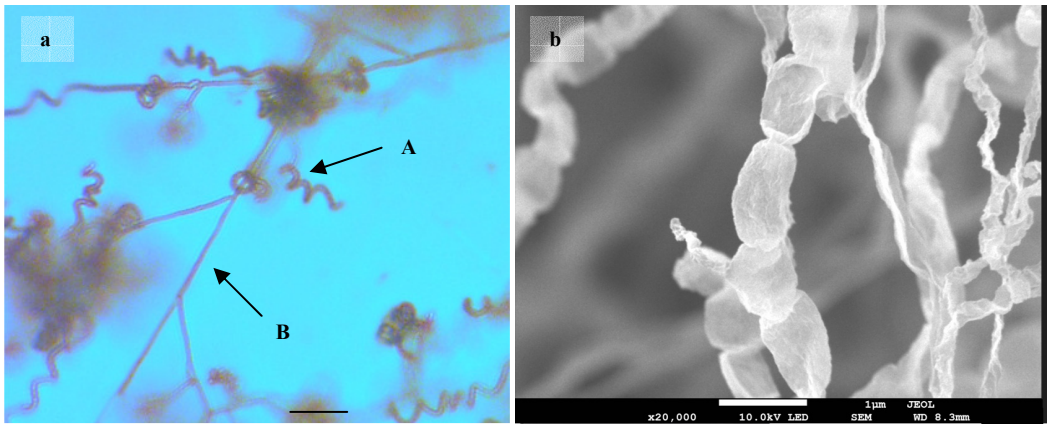

Figure 1. Morphological caracteristics of *Streptomyces* NT-T1 strain on nutrient agar medium after 5 days of incubation at 55°C: **(a)** light microscopy (A = spiral spore chain; B = rectiflexible spore chain), bar: 5 µm; and **(b)** Scanning electron microscopy, bar: 1 µm.

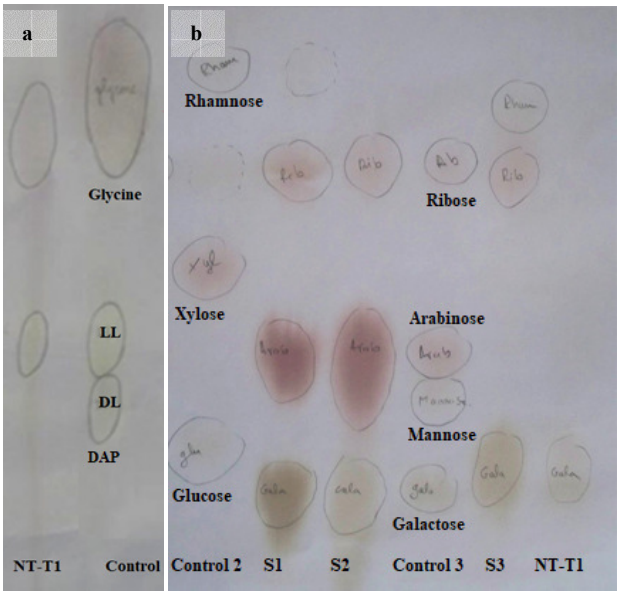

Figure 2. Chromatogram for cell wall amino-acides (a) and whole cell sugars (b) content stained with naphtoresorcinol – sulfuric acid reagent for the strain NT-T1. Control 1: mixture of DL- diaminopimelic acid (DL-DAP), LL-DAP and glycine; control 2: mixture of glucose, xylose and rhamnose; control 3: mixture of galactose, mannose, arabinose and ribose. S1, S2, S3: Strains of non-identified actinobacteria.

(10.49%); anteiso-$C_{14:0}$ (17.63); iso-$C_{15:0}$ (65.93%), iso-$C_{16:0}$ (1.87%) and anteiso-$C_{16:0}$ (4.08%).

Good growth was found to occur at 40-65°C with an optimum at 50-60°C and pH 7. The NaCl concentration range for growth was 0-5%, with optimal growth occurring at 0%. Strain NT-T1 was found to be able to use the majority of the sugars and other organic compounds tested for its growth. It degrades casein, gelatin, starch, cellobiose, D-fructose, D-galactose, *meso*-inositol, mannitol, D-mannose and D-xylose as sole carbon sources, and degrades tyrosine, L-serine, proline, and xanthine as sole nitrogen sources. The detailed physiological and biochemical characteristics of the NT-T1 strain are given in Table 1.

The 16S rRNA gene sequence analysis indicated that the strain NT-T1 is closely related to *Streptomyces thermovulgaris* NRRL B-12375^T and *S. thermogriseus* NBRC 100772^T with 100% of similarity. The strain NT-T1 was clustered in the same clade with *S. thermovulgaris* and *S. thermogriseus* (Fig. 3). The 16S rRNA gene sequence of strain NT-T1 has been deposited in GenBank under the accession number OM838400.

The antifungal activity of strain NT-T1 against various target micro-fungi is shown in Table 2. NT-T1 showed antifungal activity against two phytopathogenic fungi, with a strong activity against *Fusarium culmorum* (40 mm) and a moderate inhibition (16 mm) was detected against *Fusarium sporotrichioides*; however, no activity was observed against mycotoxigenic fungi (*Aspergillus flavus* and *Penicillium expansum*) tested in this study using the agar plug diffusion method on NA medium.

Table 1. Phenotypic properties of strain NT-T1 and closely related *Streptomyces* species.

Characteristics	Strains		
	1	2	3
Temperature range for growth (°C)	55-65	25-55	25-55
pH range for growth	6-7	6-10	6-10
Maximum NaCl concentration (%, w/v)	5	nd	7
Utilization of:			
D-Arabinose	-	-	-
Glycerol	+	nd	nd
Lactose, D-Mannose, Galactose,	+	+	nd
Inositol, Cellobiose	+	+	-
Mannitol, Glucose, D-Fructose	+	+	+
Maltose	+	nd	+
L-Rhamnose	-	-	-
D-Ribose	+	nd	
Adonitol	-	+	nd
Erythritol	-	nd	nd
Salicine	+	nd	nd
Xylose	+	-	+
Sorbitol, Melibiose	-	-	nd
Melezitose	-	+	nd
Raffinose	-	+	-
Saccharose	-	nd	+
Degradation of:			
Casein	+	+	nd
Gelatin, Starch	+	+	+
Acetate, Alanine	+	nd	nd
Butyrate	-	+	nd
Oxalate, Propionate	-	nd	nd
Tartrate	+	-	nd
Succinate	+	nd	nd
Citrate	-	nd	nd
Benzoate	-	-	nd
Tyrosine, Proline, Serine, Tween 80, Adenine	+	+	nd
Guanine, Hipoxanthine	-	-	-
Testosterone	-	-	nd
Xanthine	+	-	-
Growth in the presence of:			
Chloramphenicol, Kanamycine and Erythromycin	+	nd	nd
Streptomycin	-	-	nd
Penicillin	+	+	nd
Lysozyme	-	nd	nd

Srains: 1, NT-T1; 2, S. thermovulgaris DSM 40579^T; 3, S. thermogriseus NBRC 100772^T;; +: Positive, -: negative, nd: not determined.

Table 2. *In-vitro* antifungal activity of strain NT-T1 by the agar plug diffusion method on NA medium

Target micro-fungi	Inhibition zone (mm)
Fusarium culmorum	40
Fusarium sporotrichioides	16
Penicillium expansum	-
Aspergillus flavus	-

DISCUSSION

Strain NT-T1 has morphological characteristics that were consistent with members of the genus *Streptomyces*. From light microscopic and scanning electron microscope observations, it was found that studied strain has *spirales* and *rectiflexible*-type spore chains. The chemotaxonomic characteristics of strain NT-T1 supported its classification as a member of the genus *Streptomyces.* LL-diaminopimelic acid and glycine were detected in the cell wall peptidoglycan corresponding to chemotype pattern IC of Lechevalier and Lechevalier (1970) [30], and was found to possess phosphatidylethanolamine corresponding to phospholipid type PII of Lechevalier et al. (1977) [31]. Phospholipid patterns can be important for the recognition of actinobacteria genera [14, 53]. As usually found for the *Streptomyces* genus, the fatty acid profile was comprised mainly of fatty acids with a length of 14–16 carbon atoms, in particular saturated iso-$C_{15:0}$ and anteiso-branched chain fatty acids anteiso-$C_{14:0}$. Functional plasma membrane requires the presence of a suitable mix of both relatively fluid and solid fatty acids esterified to polar lipid head groups. Several different types of fatty acid mixtures are found in actinobacteria. *Streptomyces* strains contain iso-fatty acids as their main relatively solid base, smaller amounts of straight chain fatty acids and anteiso-fatty acids provide the fluid element in these microorganisms [37]. The morphological and chemotaxonomic properties of strain NT-T1 are consistent with those shared by members of the genus *Streptomyces* [25].

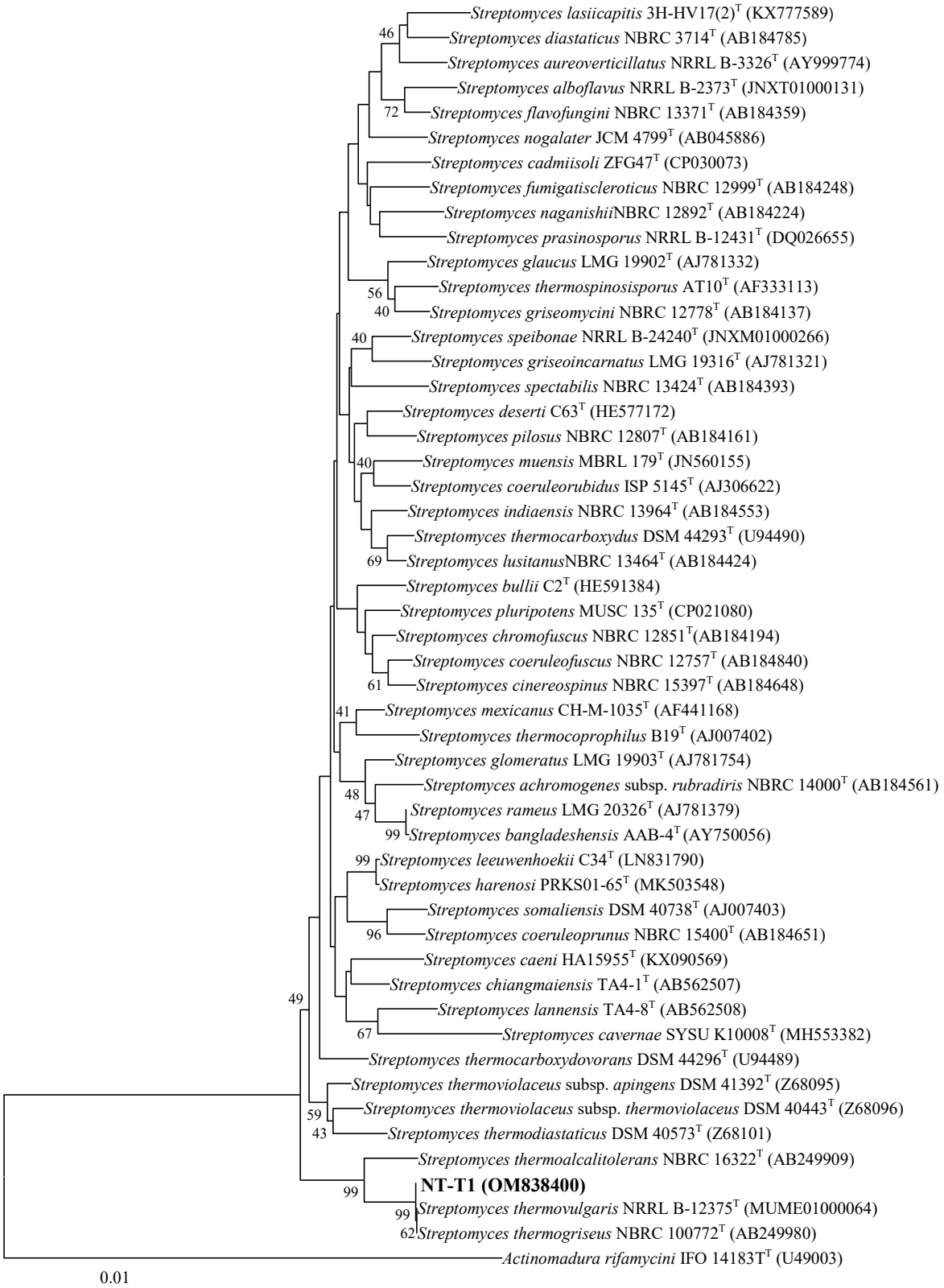

Figure 3. Phylogenetic tree derived from 16S rRNA gene sequences showing the position of strain NT-T1 and its phylogenetic neighbors. Bootstrap values (40%) based on 1000 resamplings are shown at branch nodes. *Actinomadura rifamycini* was used as the out-group.

The optimum temperature range of strain NT-T1 (50-60°C) lets it be classified as a strictly thermophilic actinobacterium [21, 55]. The biochemical results showed that the NT-T1 strain is physiologically very close to the species *S. thermovulgaris* [19] and *S. thermogriseus* [54] as they share several physiological characteristics and a high degree (100%) of similarity of 16S rRNA gene sequences. However, some differences were observed between the NT-T1 strain and the closest species; for example, NT-T1 is able to

degrade xanthine and enable to use melezitose and adonitol.

Micro-fungi are responsible for a large number of diseases in humans, animals and plants [18], and cause damage to wood products, fruits, vegetables and other foodstuffs [6, 26]. To find novel bioactive compound producers, the exploration of ecosystems exposed to extreme environmental conditions is an interesting approach. Hence, research in recent years has been oriented towards the screening and isolation of actinobacteria from untapped habitats [48]. The exploration of such habitats could even provide new taxa which, in turn, could be promising sources of novel bioactive compounds [35, 59]. In this sense, Algerian Sahara soils, exposed to harsh climatic conditions, represent particular ecosystems worthy of being explored. In addition, the Algerian Sahara soils have significant biodiversity [7, 39]. The screening for the antifungal activity showed that the strain NT-T1 exhibited a good to moderate activity against *Fusarium culmorum* and *F. sporotrichioides*. This might be supported by the fact that this actinobacterium strain produced antimicrobial substances and exhibited strong antifungal activity against phytopathogenic micro-fungi. Different microbial species such as bacteria, micro-fungi and *Actinobacteria* have been successfully used for controlling plant pathogens [38, 58]. Especially, *Actinobacteria* are well-known prolific producers of bioactive secondary metabolites [20]. In diverse crop production systems, *Streptomyces* species have been shown to suppress soil-borne diseases caused by plant pathogenic micro-fungi, bacteria, and nematodes [44].

Streptomyces species have often been described in the scientific literature for their important antifungal properties. Some Saharan *Streptomyces* strains are a good biocontrol agent for some phytopathogenic diseases such as damping-off and seedling blight of cereals crops [17, 56]. *Streptomyces* are also efficient plant colonizers and able to employ different mechanisms of control against toxigenic micro-fungi on cereals [11]. Furthermore, the *Streptomyces* genus is responsible for the production of numerous drug molecules and represents a huge resource for the discovery of new bioactive molecules.

The strain NT-T1 was related to *Streptomyces thermovulgaris* and *S. thermogriseus* with a high percentage of similarity. Nevertheless, this strain differs from the closest species in several physiological characteristics. Hence, the deep evaluation of taxonomic status of this strain at the species level should be completed. Therefore, DNA/DNA hybridization experimental analysis needs to be performed between the studied strain NT-T1 and related closely related *Streptomyces* species.

The present study provides further evidence that thermophilic actinobacteria notably *Streptomyces* strains widely distributed in nature, and also in the Algerian Sahara may be an excellent source of novel interesting antimicrobial compounds. Future research will be required to identify the produced antimicrobial compounds which will involve their purification and the use of different chemical analysis such as HPLC-MS, FTIR and NMR techniques.

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Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

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