Halophilic Actinomycetes in Saharan Soils of Algeria: Isolation, Taxonomy and Antagonistic Properties

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Running title: HALOPHILIC ACTINOMYCETES IN SAHARAN SOILS OF ALGERIA

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Abstract

The diversity of a population of 52 halophilic actinomycetes was evaluated by a polyphasic approach, which showed the presence of *Actinopolyspora*, *Nocardiopsis*, *Saccharomonospora*, *Streptomonospora* and *Saccharopolyspora* genera. One strain was considered to be a new member of the last genus and several other strains seem to be new species. Furthermore, 50% of strains were active against a broad range of indicators and contained genes encoding polyketide synthetases and nonribosomal peptide synthetases.

Actinomycetes are economically and biotechnologically priceless prokaryotes. They have provided many important bioactive compounds (27). Several studies of the ecology of actinomycetes have shown that these microorganisms are widespread in nature and may occur in extreme environments. Thus, groups of acidophilic and alkaliphilic, psychrophilic and thermophilic, halophilic and haloalkaliphilic, and xerophilic actinomycetes have been described (1, 12, 25). Several genera and species of novel halophilic actinomycetes have been mentioned in the literature (1, 9, 14, 29, 30). Despite these findings, little is known about the diversity of actinomycetes in saline environments. A broad range of biologically active molecules are synthesized by polyketide synthetase (PKS) and nonribosomal peptide synthetase (NRPS) pathways (2, 18). The exploration of new soils and habitats has been recommended to screen for rare microorganisms able to produce several antibiotics (21). The Algerian Sahara is one such extreme environment. The aims of the present work are to study the biodiversity of halophilic actinomycetes in Saharan soils and to highlight their potential to produce bioactive substances.

Isolation and phenotypical identification of actinomycetes. Eighteen non-rhizospheric soil samples were collected from various Saharan regions in the south of Algeria: Adrar, Bechar, Djelfa, El Golea, El Oued, Ghardaia, Laghouat, Ouargla and Tolga. Most of them were saline, with an electrical conductivity (1/5 at 25°C) between 2 and 55 mS/cm. Actinomycetes strains were isolated at 30°C for 2 to 6 weeks by dilution on two selective media: complex medium agar (7) and humic acid-vitamin agar (11) supplemented with 20% NaCl, plus 50 mg/liter of actidione. The morphological characteristics of actinomycetes were studied on ISP medium 2, ISP medium 4 and complex medium agar (7, 26) containing 20% NaCl. Sixty-two physiological tests were used to characterize actinomycete strains. Degradation of different organic substrates and resistance to some chemical and physical agents were determined as described by Locci (17). The results of the physiological study

were analyzed by numerical taxonomy. The degree of similarity between strains was calculated by simple matching (SM) and clustering was performed by the unweighted average linkage UPGMA procedure using the SPSS package (v.16.0.1). For the chemical study of cell constituents, analyses of diaminopimelic acid and whole-cell sugars were carried out using the method of Becker et al. (3) and Lechevalier and Lechevalier (13). Phospholipids were analyzed according to the procedure developed by Minnikin et al. (20).

A variable level of cultivable halophilic actinomycetes was observed, ranging from 10^2 to 10^3 CFU per g (dry weight) of soil, which represented only 0.001 to 0.1% of the total actinomycetes in these soils (23, 24). In total, 52 actinomycetes were collected from 18 soil samples analyzed, and were detected in both the media used. The preliminary investigation based on morphological and chemical analysis of the 52 isolates of actinomycetes allowed some of these strains to be tentatively classified into several genera. Sixteen strains belonged to *Nocardiopsis* (19), six strains to *Saccharomonospora* (22) and three strains to *Streptomonospora* (8). Twenty-seven strains could not be accurately identified at genus level at this stage. The physiological classification based on the S_{SM} coefficient and UPGMA clustering gave 4 cluster-groups, designated I to IV, and one single strain at the 75% similarity (S) level (Fig. 1). Two major (14 and 18 strains) and 6 minor (2 to 6 strains) sub-clusters were circumscribed at the 81% S level (Fig. 1). The clusters II, III and IV contained strains of *Streptomonospora*, *Nocardiopsis* and *Saccharomonospora*, respectively. Cluster I and the single strain H31 included the strains which were not identified at genus level. The numerical taxonomy used in this study enabled rational discrimination between strains but did not permit identification.

Phylogenetic studies of actinomycetes. Genomic DNA of 30 strains, belonging to different clusters established by numerical taxonomy, was extracted for 16S rDNA analysis

according to the method of Liu et al. (16). The 16S rRNA gene was PCR-amplified by the method cited by Zitouni et al. (34). Phylogenetic analysis was performed as described by Boudjelal et al. (4). DNA sequences obtained in this study were deposited in GenBank under the accession numbers HQ918181 to HQ918210 (Fig. 2).

It is interesting to note that the phylogenetic study corresponded well with phenotypic and chemical taxonomy. The *Actinopolyspora* strains (which belonged to cluster I) formed distinct phylogenetic lines in the neighbor-joining tree but were distinct from each other and from members of the genus *Actinopolyspora* (Fig. 2). Seven strains were related to *A. halophila* with 97.8 to 98.5% of similarity. Three strains were assigned to *A. mortivallis* with 98.7 to 98.9% of similarity, and two strains were related to *A. erythraea* with 97.6 to 98.1% of similarity. The highest degrees of similarity among *Actinopolyspora* species were obtained between *A. erythraea* (28) and *A. xinjiangensis* (10) (98.4%), and *A. alba* (28) and *A. erythraea* (97.6%). It seems likely that some strains in this genus represent novel species of *Actinopolyspora*. The *Nocardiopsis* strains were well included in the clade of this genus. They were related to two species, *N. litoralis* and *N. xinjiangensis*. The 16S rRNA gene sequence similarity of our *Nocardiopsis* strains ranged between 99.1 and 99.9%. However, high 16S rDNA similarities have been found between representatives of validly described *Nocardiopsis* species, such as the type strains of *N. valliformis* and *N. exhalans* (99.9%) (32). Thus, several strains of *Nocardiopsis* could be equated with novel species. The *Saccharomonospora* strains were related to *S. paurometabolica* and *S. halophila*. They shared similarities within the range 98.7 to 99.3%. The two strains of *Streptomonospora* were assigned to *S. alba* and *S. amylolytica* at 98.5 and 99.5% of similarity, respectively. These 16S rDNA sequence similarity values are approximately the same or less than the similarity values between closely related *Streptomonospora* species, such as *S. flavalba* with *S. alba* (99.6%) and *S. amycolata* with *S. alba* (99.3%) (6). These data indicate that these strains probably belong to novel

species. The single strain H31 was identified as *Saccharopolysora*. This strain formed a distinct phyletic line within the *Saccharopolyspora* clade, but showed only 96.6% similarity with *S. qijiaojingensis*, the most closely related species. This 16S rDNA sequence similarity value was lower than 97%, which is considered to be the cut-off value for species identity (31). Therefore, the strain H31 represents a novel species of the genus *Saccharopolyspora*. With the exception of the latter strain, DNA pairing studies need to be performed for all other strains to confirm the species affiliation.

Antimicrobial assay and detection of PKSs and NRPS sequences. The antagonistic properties of actinomycete isolates were determined by the cylinder plate method on ISP medium 2 containing 15% NaCl, against several bacteria and filamentous fungi (listed in Table 1). The study of genomic potential for producing bioactive metabolites of the isolates was evaluated by using specific degenerate primers. Polyketide synthetase I (PKS-I) and nonribosomal peptide synthetase (NRPS) genes were amplified from genomic DNA with the primer pairs K1F (5'-TSAAGTCSAACATCGGBCA-3')/M6R (5'-CGCAGGTTSCSGTACC AGTA-3') and A3F (5'-GCSTACSYSATSTACACSTCSGG-3')/A7R (5'-SASGTCVCCSGTS CGGTAS-3') respectively, according to the method of Ayuso-Sacido et al. (2). Polyketide synthetase II (PKS-II) gene was amplified with the primer pair KSα (5'-TSGCSTGCTTGGAYGCSATC-3')/KSβ (5'-TGGAANCCGCCGAABCCTCT-3') as described by Metsä-Ketelä et al. (18). Enediyne polyketide synthase (PKSE) gene was amplified with the primer pair EdyA (5'-CCGCVCACATCACSGSCCTCGCSGTGAACATG CT-3')/EdyE (5'-GCAGGCKCCGTCSACSGTGTABCCGCCGCC-3') according to the method of Liu et al. (15). All the amplification products were examined by 0.8% agarose gel electrophoresis, and bands of 1,200 to 1,400 bp, 600 bp, 1,400 bp and 700 to 800 bp were classified as products of PKS-I, PKS-II, PKSE and NRPS genes respectively.

Twenty-six of the 52 strains (50%) were active against at least one of the pathogenic microorganisms tested. About 11.5% of strains exhibited antibacterial activity, 46.1% only antifungal activity, and 42.3% both antibacterial and antifungal activities. It was interesting to note that the 16 strains of *Nocardiopsis* were active. The activity was present in 27% of *Actinopolyspora*, and 33% of *Saccharomonospora* and *Streptomonospora* strains, whereas the single strain of *Saccharopolyspora* did not show any activity. Although the antagonistic properties of *Nocardiopsis* have often been reported in the literature (35), those of the other rare genera, such as *Actinopolyspora*, *Saccharomonospora* and *Streptomonospora*, are very rarely mentioned. Among 26 strains tested for the presence of PKSs and NRPS genes, 23 strains (88.5%) yielded at least one type of the biosynthetic sequences. NRPS were the most frequent genes; they were detected in 20 strains (76.9%). PKSE were detected in 10 strains (38.5%), PKS-II in 6 strains (23%), and PKS-I in only 3 strains (11.5%). One strain (H37) contained all the genes studied (Table 1). Compared to the later screening of genes associated with secondary metabolites in a large collection of reference actinomycetes, NRPS sequences were extensively distributed among actinomycetes taxa, whereas PKS-I genes were concentrated in fewer genera (2, 18). The low detection of PKS genes (types I and II) may have been caused by the type of degenerate primers used, which were not suitable for amplifying these genes (5). The percentage of isolates that tested positive for PKSE genes correlated well with that reported for soil-derived actinomycetes (33). The absence of amplification products from some active strains tested may reflect the lack of targeted genes or the implication of other genes in the biosynthesis of these bioactive products. The high frequency of NRPS genes in the actinomycetes tested here, mostly in *Nocardiopsis* strains, could be evidence of the high potential of halophilic actinomycetes for producing a large number of biologically active compounds.

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282 TABLE 1. Antimicrobial activities and PKSs/NRPS genes of halophilic actinomycetes isolated from Saharan soils

Strains	Activity[a] (mm) against									presence[b] of gene			
	Mucor ramannianus	*Aspergillus niger*	*Penicillium expansum*	*Penicillium glabrum*	*Bacillus subtilis*	*Staphylococcus aureus*	*Micrococcus luteus*	*Klebsiella pneumoniae*	*Listeria monocytogenes*	PKS-I	PKS-II	PKSE	NRPS
H1	12	–	15	15	–	–	–	–	–	+	–	+	+
H2	12	–	15	–	22	–	23	–	–	–	–	+	+
H3	15	–	15	–	–	–	–	–	–	–	–	–	+
H4	17	14	15	–	15	–	–	–	13	–	–	–	+
H5	20	17	17	15	15	–	–	–	24	–	–	–	+
H6	15	14	17	17	15	–	14	–	24	–	+	–	+
H12	–	–	15	–	–	–	–	–	15	–	–	+	–
H14	12	–	–	–	–	22	–	22	26	–	–	–	+
H15	12	–	–	–	–	22	–	34	32	–	–	–	–
H16	12	–	–	–	–	25	–	36	36	–	+	+	+
H17	17	–	–	–	–	22	–	32	30	–	–	–	+
H18	15	–	–	–	–	–	–	–	–	–	–	–	+
H19	–	–	–	–	15	–	–	–	–	+	–	+	+
H21	15	–	15	–	–	–	15	–	–	–	+	–	–

H28	19	–	18	–	–	–	–	–	–	–	–	–	–
H29	–	14	18	17	–	–	–	–	–	–	–	+	+
H37	–	–	–	–	–	15	–	–	–	+	+	+	+
H41	17	–	17	–	–	–	–	–	22	–	–	–	+
H42	15	–	17	–	–	–	–	–	–	–	–	–	+
H49	–	–	–	–	–	–	15	–	15	–	–	–	–
H51	13	–	–	–	–	–	–	–	–	–	+	+	–
H54	–	–	15	–	–	–	–	–	–	–	–	–	+
H57	17	–	17	–	–	–	–	–	–	–	–	+	+
H58	17	–	16	–	15	–	18	–	16	–	–	–	+
H62	13	–	14	–	–	–	–	–	–	–	–	+	+
H63	13	–	14	–	–	–	–	–	–	–	+	–	+

[a] estimated by measuring the diameter of the clear zone of growth inhibition. Symbols: –, no activity.

[b] +, present, –, absent.

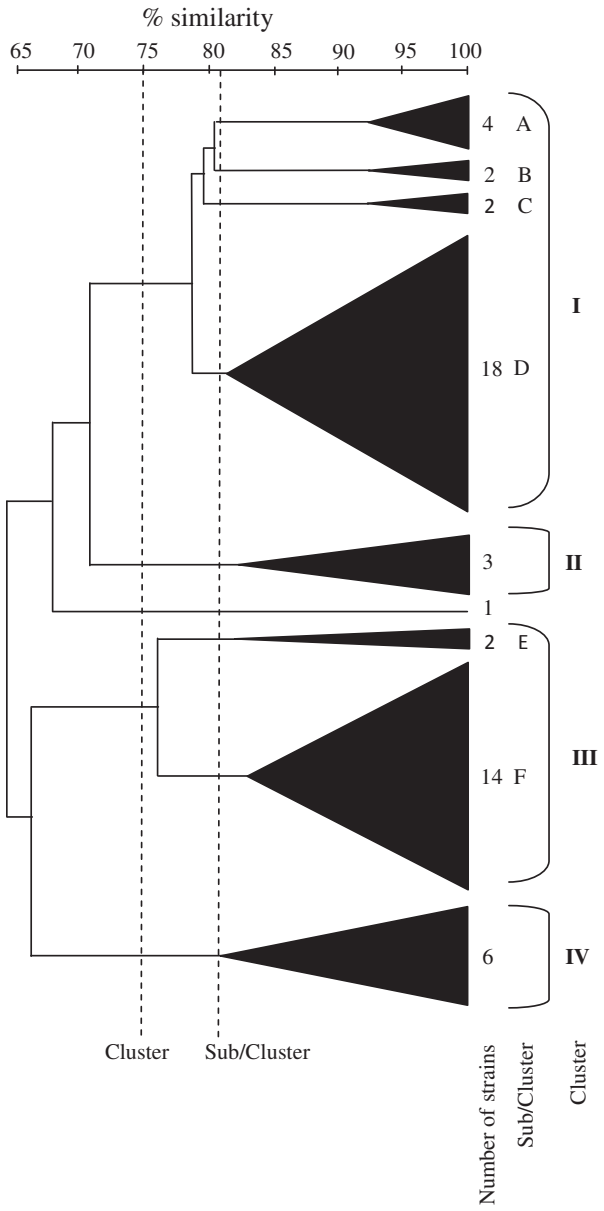

FIG. 1. Dendrogram derived from UPGMA cluster analysis of 62 phenotypic characters showing the relationship between 52 strains of halophilic actinomycetes isolated from Saharan soils.

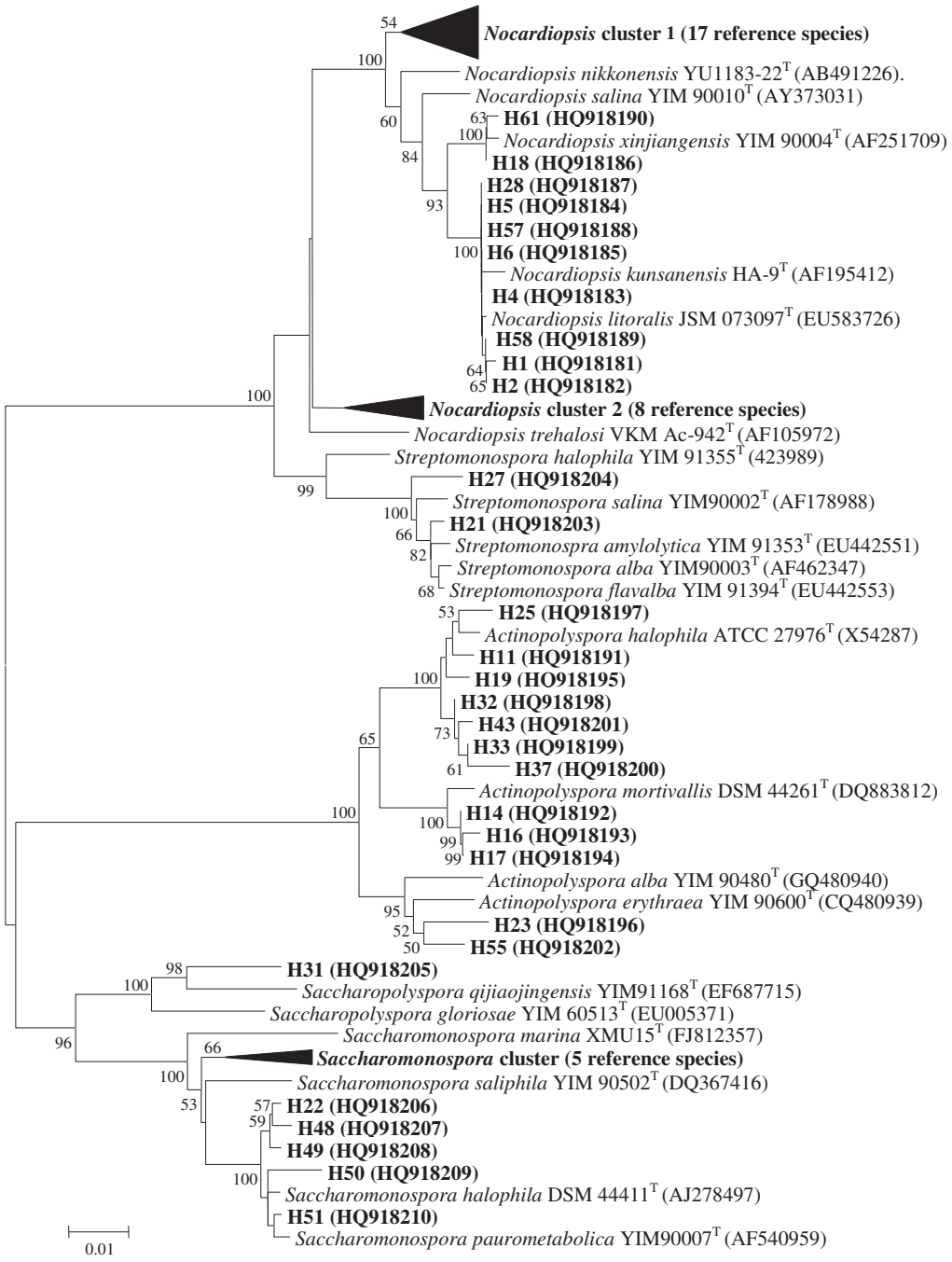

FIG. 2. Phylogenetic tree derived from nearly complete 16S rRNA gene sequences showing relationships between the isolates of actinomycetes and their phylogenetic neighbors. The tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap values greater than 50% are indicated at nodes. Bar, 0.01 substitutions per nucleotide position.