Actinopolyspora iraqiensis sp. nov., a New Halophilic Actinomycete Isolated from Soil

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Halophilic actinomycete strains, IQ-H1^T (T = type strain) and IQ-H2, were isolated from extremely saline soil samples in Iraq. Besides morphological properties, strains IQ-H1^T and IQ-H2 have a type IV cell wall, type PIII phospholipid, and menaquinone MK-9 (H₄, H₆), which are characteristic of *Actinopolyspora* spp. In addition, both strains required high concentrations of sodium chloride (10 to 15% [wt/vol] and 15 to 25% [wt/vol] NaCl, respectively), for optimal growth. However, strains IQ-H1^T and IQ-H2 differed from other previously described species of the genus *Actinopolyspora* in cultural, morphological, and physiological characteristics. In addition, the size of the internal transcribed spacer in the rDNA (gene encoding rRNA) of IQ-H1^T was different from those of known published species, while the size of the internal transcribed spacer of IQ-H2 was similar to that of *Actinopolyspora halophila* JCM 3278^T. Thus, we propose strain IQ-H1^T as a new species and name it *Actinopolyspora iraqiensis* sp. nov. Strain IQ-H1^T has been deposited in the culture collection of the Institute of Microbiology, Academia Sinica, Beijing, the People's Republic of China (strain A.S. 4.1193).

Since 1975, only two species of extremely halophilic actinomycetes, *Actinopolyspora halophila* (7) and *Actinopolyspora mortivallis* (19), have been reported. Recently, over 100 actinomycete strains were isolated from saline soil samples obtained from different regions of Iraq (1). This paper reports the morphological, chemical, and physiological characteristics of strains IQ-H1^T and IQ-H2, with reference to the known species in the genus *Actinopolyspora*. IQ-H1^T differs from previously described species in physiological and biological characteristics and in the size of the internal transcribed spacer (ITS) in the rDNA (gene encoding rRNA). We consider strain IQ-H1^T a new species of the genus *Actinopolyspora*. The name *Actinopolyspora iraqiensis* sp. nov. is proposed for this organism.

MATERIALS AND METHODS

Bacterial strains and cultivation. Two strains, IQ-H1^T and IQ-H2, were isolated from a saline soil sample during a previous study on the occurrence and characterization of actinomycetes in heavily salted soil samples from Iraq. The modified complex medium (SGA) used in this study was based on the previous findings of Al-Tai (1). The medium consisted of (per liter) 7.5 g of Difco Casamino Acids, 10.0 g of Difco yeast extract, 20 g of MgSO₄ · 7H₂O, 3.0 g of sodium citrate, 2.0 g of KCl, and 200.0 g of NaCl (16) plus 1 ml of a trace salts solution (0.1 g of FeSO₄ · 7H₂O, 0.1 g of MnCl₂ · 4H₂O, 0.1 g of ZnSO₄ · 7H₂O, and 1,000.0 ml of distilled water) (17) per liter and Difco agar at 15.0 g/liter. The final pH was adjusted to 7.5 to 7.6 with KOH solution before sterilization. The inoculated plates were incubated at 30°C for 10 to 14 days. Stock cultures were maintained on slant medium (SGA) and stored at 4°C.

The cultures used for comparison were *A. halophila* JCM 3278^T (7), *A. mortivallis* HS-1^T (19), and *Nocardiopsis halophila* IQ-H3^T (1a).

Growth characterization. Strains IQ-H1^T and IQ-H2 were cultivated on SGA medium containing different concentrations of sodium chloride (0, 5, 10, 20, and 25% [wt/vol]). After 14 to 21 days of incubation at 30 or 37°C, the growth and color of both the substrate and aerial mycelium were noted. Production of soluble pigments was also noted. Color standards and color nomenclature were as described in reference 13.

Growth temperature. The ability of the strains to grow at different temperatures (10, 16, 25, 30, 37, 42, and 50°C) was determined after 7 to 28 days. The inoculated SGA plates were supplemented with either 15 or 20% (wt/vol) NaCl.

Morphological characteristics. Morphological observations were made with a light microscope on cultures grown at 30 or 37°C for 7 to 30 days on SGA medium containing various concentrations of NaCl (10, 15, 20, or 25% [wt/vol]). Mycelial growth was observed by staining of cell walls (14). Spore morphology was studied with a transmission electron microscope.

Chemical analysis of cells. The organisms were grown in a 500-ml Erlenmeyer flask containing 100 ml of modified complex medium supplemented with NaCl (15% [wt/vol] and 20% [wt/vol] for strains IQ-H1^T and IQ-H2, respectively). The inoculated broth cultures (3% inoculum) were grown on a rotary shaker (200 rpm at 30°C) for 4 to 5 days. The mycelia were harvested by centrifugation (1,500 × g at 4°C for 10 min) and washed three times with distilled water. The isomers of diaminopimelic acid (DAP), sugars in whole cells, and pure cell wall composition were analyzed by the procedures described by Lechevalier and Lechevalier (10). Isoprenoid quinone analysis was performed by the method of Collins (3), and isoprenoid quinones were identified by high-performance liquid chromatography (11). Phospholipids were extracted and identified by the methods of Lechevalier and Lechevalier (10). Occurrence of mycolic acids was determined by the procedure of Minnikin et al. (11).

Physiological characteristics. The procedures for assessment of carbon utilization, acid production, degradation of tyrosine, xanthine, esculin, and urea, and resistance to lysozyme were those of Berd (2). Lipase production was deter-

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TABLE 1. Morphological and chemical characteristics of strains IQ-H1^T and IQ-H2

Characteristic	Strain IQ-H1 ^T	Strain IQ-H2
Morphology		
Fragmentation of substrate mycelium	Absent	Absent
Chains of arthrospores	Short (1–15 conidia), straight	Long (10–40 conidia), straight to spiral
Spore surface	Smooth	Smooth
Spore shape	Spherical	Elongated with round end
Spore size (µm)	1	1 by 1.5
Cell chemistry		
Cell wall type	IV	IV
Amino acids	meso-DAP (major), L-DAP (tr)	meso-DAP (major), L-DAP (tr)
Whole-cell sugars	Galactose, arabinose, ribose	Galactose, arabinose, ribose
Phospholipid type	PIII	PIII
Predominant phospholipids ^a	PC, Lyso-DPG, PG, DPG	PC, Lyso-DPG, DPG
Predominant menaquinones	MK-9 (H ₄), MK-8 (H ₄) (minor)	MK-9 (H ₄ , H ₆), MK-8
Mycolic acid	Absent	Absent

^a Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol.

mined by the procedure of Gonzalez and Gutierrez (8). Hydrolysis of starch and cellulose was observed on glucose-salt medium (5), which was supplemented with 0.5% soluble starch or carboxymethyl cellulose instead of glucose. Amylolytic cellulolytic activities were determined by clearing zones with Lugol (4) and Congo red (18) solutions as indicators, respectively. Sensitivity to various antimicrobial compounds was determined by the impregnated filter paper disc method. Antagonism towards *Escherichia coli* IBTC 281 and *Schizosaccharomyces pombe* IBTC 18-1-1 was determined by the plug technique. All test media mentioned above were supplemented with 15% (wt/vol) NaCl unless otherwise stated. The inoculated plates or liquid media were incubated at 30 or 37°C. The results were recorded at different time intervals.

PCR amplification of ITS in rDNA (15). The PCR mixture (20 µl) was composed of 19 µM deoxynucleoside triphosphate, 80 pM primer, 1 U of *Taq* polymerase, 2 mM MgCl₂, and 15 ng of template DNA. The sample was denatured at 95°C for 5 min; this was followed by a series of incubations (95°C for 1 min, 45°C for 1 min, and 71°C for 2 min) for 30 cycles and a final incubation at 71°C for 5 min.

RESULTS AND DISCUSSION

The cell chemistry and cultural, morphological, and physiological characteristics of strains IQ-H1^T and IQ-H2 are summarized in Tables 1, 2, 3, and 4. Both strains had a type IV cell wall (meso-DAP, arabinose and galactose, whole-cell sugar pattern A) and type PIII phospholipid. Mycolates were absent. The predominant menaquinones were MK-9 (H₄, H₆). The development of spore chains on aerial mycelium and the

unfragmented vegetative mycelium are shown in Fig. 1. On the basis of the properties described above, strains IQ-H1^T and IQ-H2 should be placed in the genus *Actinopolyspora* (6, 7).

The characterizations of these two strains were done by comparing their characteristics with those of *A. halophila* and *A. mortivallis*, which were previously described. The similarity between strains IQ-H1^T and IQ-H2 and *A. halophila* was in the requirement for high concentrations of sodium chloride for growth. However, strain IQ-H1^T differs from IQ-H2 and *A. halophila* in morphological characteristics (short spore chains), as well as in its ability to grow at 5% (wt/vol) NaCl and not at 25% (wt/vol) NaCl (Table 5). In addition, its physiological characteristics and sensitivity to antibiotics are different from those of strain IQ-H2 and *A. halophila*.

In actinomycetes, between the 16S rDNA and 23S rDNA there is an ITS, which is a divergent domain that can be used to determine the relationships among different species within the same genus (12). The size of the ITS in the rDNA of strain IQ-H1^T was different from those of the known published species, while the ITS size of strain IQ-H2 was similar to that of *A. halophila* (Fig. 2). Thus, we suggest that strain IQ-H1^T is a new species of the genus *Actinopolyspora*. We propose the name *A. iraqiensis* sp. nov. More work is needed to determine whether strain IQ-H2 is a new species. The type strain of *A. iraqiensis* (IQ-H1^T) has been deposited in the culture collection of the Institute of Microbiology, Academia Sinica, as strain A.S. 4.1193.

Description of *Actinopolyspora iraqiensis* sp. nov. *Actinopolyspora iraqiensis* (ira.qi.ensis M.L. adj. *iraqiensis*, referring to Iraq, the source of the soil sample from which the organism was isolated).

TABLE 2. Growth characteristics of strains IQ-H1^T and IQ-H2 on SGA medium containing different concentrations of sodium chloride

Strain	Characteristic	Result with the following NaCl concn (% wt/vol):					
		0	5	10	15	20	25
IQ-H1 ^T	Growth	None	Slight	Very good	Very good	Slight	None
	Aerial mycelium		None	White	White	Sparse, white	
	Substrate mycelium		Orange buff	Yellow ochre	Yellow ochre	Yellow ochre	
IQ-H2	Soluble pigments		None	None	None	None	
	Growth	None	None	Slight	Good	Very good	Very good
	Aerial mycelium			None	White	White	White
	Substrate mycelium			Antimony yellow	Antimony yellow	Light buff	Light buff
	Soluble pigments			None	None	None	None

TABLE 3. Carbon utilization and acid production by strains IQ-H1^T and IQ-H2^a

Carbon source	Strain IQ-H1 ^T		Strain IQ-H2	
	Utilization	Acid production	Utilization	Acid production
L-Arabinose	+	—	+	+
Fructose	+	—	+	+
Galactose	+	+	+	+
Glucose	+	—	+	+
Inositol	+	—	±	—
Maltose	+	—	+	+
Mannitol	+	+	±	—
Mannose	—	—	±	—
Raffinose	+	+	±	—
Rhamnose	—	—	+	+
Ribose	+	—	±	±
Sucrose	+	—	+	+
D-Xylose	—	—	+	+

^a +, positive; —, negative; ±, indeterminate reaction.

Optimal growth occurs when a liquid medium is supplemented with 10 to 15% (wt/vol) NaCl. No growth occurs at 25% (wt/vol) NaCl or when NaCl is absent. Lower growth efficiencies are observed at 5 and 20% (wt/vol) NaCl. The organism is moderately halophilic according to the definition of Kushner (9).

The optimal growth temperature is between 30 and 35°C. Poor growth occurs at 16 and 40°C. At 42°C, no growth occurs.

TABLE 4. Physiological characteristics of strains IQ-H1^T and IQ-H2^a

Characteristic	Strain IQ-H1 ^{Tb}	Strain IQ-H2 ^b
Hydrolysis or decomposition of:		
Carboxymethyl cellulose	—	—
Esculin	—	—
Starch	+	+
Tween 80	+	+
Tyrosine	+	—
Xanthine	—	±
Urea	—	—
Growth at:		
16°C	+	—
42°C	—	+
Optimal growth temp (°C)	30–35	30–42
Maximum growth temp (°C)	40	50
Sensitivity to:		
Lysozyme	S	S
Amoxycillin	S (40)	R
Bacitracin	S (50)	S (42)
Carbenicillin	S (36)	R
Cephalexin	S (50)	S (40)
Chloramphenicol	S (40)	S (40)
Clindamycin	R	S (30)
Oxacillin	R	R
Erythromycin	S (50)	S (50)
Novobiocin	S (50)	S (35)
Vancomycin	R	S (50)
Antimicrobial activity against:		
<i>E. coli</i> IBTC 281	—	—
<i>S. pombe</i> IBTC 18-1-1	+	+

^a +, positive; —, negative; ±, slight reaction; S, sensitive; R, resistant.

^b Numbers in parentheses indicate the diameter of the inhibition zone in millimeters.

FIG. 1. (a) Strain IQ-H1^T (light microscope; magnification, $\times 1,800$). (b) Spores of IQ-H1^T (transmission electron microscope; magnification, $\times 10,000$).

Colonies are small (2 to 4 mm in diameter), thin, elevated or convex. The spore mass is white and appears abundant on solid medium containing NaCl (10 or 15% [wt/vol]). On the reverse side of the culture growth, the colony is yellow to brownish. No distinctive pigment is present. Substrate mycelia (0.5 to 0.7 μ m in diameter) are well developed and branched, rarely fragmented in both solid and liquid media. The aerial mycelium forms short chains of spores (1 to 15 conidia). The spores are spheroidal (diameter of 1 μ m) and have smooth surfaces (Fig. 1).

The cell wall contains primarily *meso*-DAP trace amounts of *L*-DAP. The whole-cell hydrolysate contains arabinose, galactose, and ribose. The predominant menaquinone is MK-9 (H_4). The phospholipids include phosphatidyl choline, lysophosphatidyl glycerol, phosphatidyl glycerol, and diphosphatidyl glycerol. Mycolic acids are absent.

TABLE 5. Characteristics of strain IQ-H1^T, strain IQ-H2, and other species of extremely halophilic actinomycetes

Characteristic	Strain IQ-H1 ^T	Strain IQ-H2	<i>A. halophila</i> JCM 3278 ^T	<i>A. mortivallis</i> HS-1 ^T
Spore chain morphology	Short (not more than 15)	Very long (sometimes 40), elongated	Long (sometimes 20), elongated	Short, (not more than 10), oval to cylindrical
Growth in 10% (wt/vol) NaCl				
Color of colony	Pale brown	Antimony yellow	Black	Yellowish white
Soluble pigments	None	None	Black	Pale brown
Growth in:				
5% (wt/vol) NaCl	+	—	—	+
25% (wt/vol) NaCl	—	+	+	+
NaCl concn (% wt/vol) for optimal growth	10–15	20–25	15–20	10–15
Sensitivity to antibiotics				
Amoxycillin	Sensitive	Resistant	Sensitive	Resistant
Clindamycin	Resistant	Sensitive	Sensitive	Sensitive
Vancomycin	Resistant	Sensitive	Sensitive	Sensitive
Hydrolysis of carboxymethyl cellulose	—	—	+	ND ^a
Acid production from:				
L-Arabinose	—	+	—	—
Fructose	—	+	—	ND
Inositol	—	—	—	—
Mannitol	+	—	—	—
Rhamnose	—	+	—	—
Xylose	—	+	—	+
Growth at 42°C	—	+	+	+
Optimal growth temp (°C)	30–35	30–42	37	45
Maximum growth temp (°C)	40	50	43	50

^a ND, not determined.

The organism utilizes glucose, maltose, mannitol, sucrose, fructose, L-arabinose, inositol, raffinose, and galactose. It does not grow on L-rhamnose, D-xylose, or mannose. Acid production occurs on mannitol and raffinose. Tyrosinase is produced, as is amylase, capable of hydrolyzing starch. Xanthine and Tween 80 are decomposed or hydrolyzed, while urea, esculin, and cellulose are not.

The organism is sensitive to a number of antibiotics, including lysozyme, amoxycillin, bacitracin, carbenicillin, cephalixin,

chloramphenicol, erythromycin, and novobiocin. Resistance is exhibited only against clindamycin, oxacillin, and vancomycin. The organism also shows antimicrobial activity against *S. pombe*. Its habitat is saline soil. The type strain is *Actinopolyspora iraqiensis* sp. nov.

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FIG. 2. Comparison of the sizes of the ITSS in rDNAs from halophilic actinomycetes by the PCR method. Four microliters of each PCR product was analyzed on a 1.5% agarose gel. The sizes of the ITSS in the rDNAs of *A. iraqiensis* and *A. halophila* were approximately 800 and 750 bp, respectively. Lanes: m, size marker (1-kb ladder from Gibco BRL Life Technologies, Inc.); a, strain IQ-H1^T; b, strain IQ-H2; c, *N. halophila* IQ-H3^T; d, *A. mortivallis* HS-1^T; e, *A. halophila* JCM 3278^T.

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