Actinomadura hallensis sp. nov., a novel actinomycete isolated from Mt. Halla in Korea

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A novel actinomycete, strain H647-1^T, which was isolated from soil of Mt. Halla, Jeju Island, Republic of Korea, was subjected to phenotypic and genotypic characterization. The organism, which produces warty spore chains arranged in hooks or spirals on the aerial mycelium, was shown to have chemotaxonomic characteristics consistent with assignment to the genus *Actinomadura*. A comparative analysis of 16S rRNA gene sequences indicated that the organism formed a distinct clade within the evolutionary radiation of the family *Thermomonosporaceae* and that it was closely associated with members of the genus *Actinomadura*, but possessed a 22 nt extended loop between positions 453 and 479 of the 16S rRNA gene sequence (*Escherichia coli* numbering). A broad range of phenotypic and genetic data supported the suggestion that this organism represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura hallensis* sp. nov. is proposed. The type strain is strain H647-1^T (=IMSNU 50760^T=KCTC 9992^T=KCCM 42245^T=NRRL B-24436^T).

As a result of phylogenetic and chemotaxonomic analyses, the description of the family *Thermomonosporaceae* was emended by Zhang *et al.* (2001) and the family currently contains four genera: *Actinomadura*, *Actinocorallia*, *Spirillospora* and *Thermomonospora*. Although the members of these genera are phylogenetically intermixed and share major phenotypic characteristics (Goodfellow, 1992; Kroppenstedt & Goodfellow, 1991; Zhang *et al.*, 1998, 2001), they differ in terms of morphology. They are phylogenetically distinct from the members of the other two families in the sub-order *Streptosporangineae*, namely *Streptosporangiaceae* and *Nocardiopsaceae* (Zhang *et al.*, 1998, 2001). In their extensive study, Zhang *et al.* (2001) proposed that four *Actinomadura* species, *Actinomadura aurantiaca*, *Actinomadura glomerata*, *Actinomadura libanotica* and *Actinomadura longicatena*, be transferred to the genus *Actinocorallia*, and that *Excellospora viridilutea*, the only species of the genus *Excellospora*, be reclassified as *Actinomadura viridilutea*.

The genus *Actinomadura* contains aerobic, Gram-positive, non-acid-fast, non-motile, chemo-organotrophic actinomycetes that produce well-developed, non-fragmenting vegetative mycelia and aerial hyphae that differentiate into surface-ornamented spore chains. These chains are of various lengths and can be straight, hooked or spiral. The genus currently comprises 31 species with validly published names, although the species status of some strains remains

uncertain, and further comparative studies are needed (Zhang *et al.*, 2001). Members of the genus are characterized chemotaxonomically by type III/B cell walls (*meso*-diaminopimelic acid and madurose are present) with peptidoglycan structures of the acetyl type. The predominant menaquinone types are MK-9(H₄), MK-9(H₆) and MK-9(H₈). The phospholipid pattern is PI (diphosphatidylglycerol and phosphatidylinositol are present as major phospholipids) and the fatty acid pattern is type 3a (branched saturated and unsaturated fatty acids plus tuberculostearic acid) (Kroppenstedt *et al.*, 1990; Kroppenstedt & Goodfellow, 1991).

In this paper, we describe the characterization and classification of strain H647-1^T, isolated in 1992 from a soil sample collected on Mt. Halla, Jeju Island, Republic of Korea.

Strain H647-1^T was isolated using starch/casein agar and was maintained on yeast extract/malt extract agar (ISP 2 medium) and as mycelial fragments or spores in 20% (v/v) glycerol or as lyophilized cells. For comparative purposes, the following type strains of the genus *Actinomadura* were used in this study: *Actinomadura livida* DSM 43677^T (=IMSNU 22191^T) and *Actinomadura yumaensis* KCTC 9495^T (=IMSNU 22167^T). To examine the degree of growth and pigmentation, strain H647-1^T was grown on ISP 2 medium, oatmeal agar (ISP 3 medium) and inorganic salts/starch agar (ISP 4 medium) as described by Shirling & Gottlieb (1966). For scanning electron microscopy, the agar blocks containing the organism (cultivated on ISP 4 medium for 3 weeks at 28 °C) were fixed with the vapour of

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain H647-1^T is DQ076484.

Fig. 1. Scanning electron micrograph of strain H647-1^T grown on ISP 4 medium for 3 weeks. Bar, 0.8 µm.

1 % osmium tetroxide. The specimen was dehydrated using a graded ethanol and isoamyl acetate series and then

critical-point-dried. After being sputter-coated with gold, the specimen was observed under a scanning electron microscope (Cambridge Stereoscan 260). The cultural characteristics of strain H647-1^T were recorded after 3 weeks incubation at 28 °C. The isolate showed good growth on all of the media tested and produced well-developed and extensively branched substrate mycelia. The colours of the reverse sides of colonies were variable, according to the media tested, e.g. blackish purple on ISP 2 medium, moderate brown on ISP 3 medium and greyish reddish purple on ISP 4 medium. A dark-brown-coloured, diffusible pigment was produced on ISP 2 medium. Moderately well-developed aerial mycelia were produced on ISP 3 and ISP 4 media, from which, at maturity, short spore chains (in hooks or spirals) were produced. The spore surface was warty (Fig. 1).

Chromosomal DNA was extracted and purified as described previously (Hopwood *et al.*, 1985). Cloning of 16S rRNA genes after PCR-mediated amplification from genomic DNA was carried out as described previously (Lee *et al.*, 2000). The cloned 16S rRNA gene sequence was determined by using a model ALFexpress DNA sequencer (Pharmacia

Fig. 2. Phylogenetic tree showing the position of strain H647-1^T within the radiation of the family *Thermomonosporaceae*. The tree was reconstructed by the neighbour-joining method from evolutionary distances calculated using the Jukes–Cantor coefficient. *Streptosporangium roseum* was used as an outgroup (data not shown). Bootstrap values at branching points are expressed as percentages from 1000 replications (only values greater than 40 % are indicated). Bar, 1 nucleotide substitution per 100 nucleotides.

Biotech) and the Cy5 AutoRead sequencing kit (Pharmacia). The almost-complete 16S rRNA gene sequence of strain H647-1^T determined in this study contained a continuous stretch of 1528 nt. The sequence obtained was aligned with those of the type strains of the family *Thermomonosporaceae* and related actinomycete taxa by using the program CLUSTAL X (Thompson *et al.*, 1997). Evolutionary distance matrices were calculated by using the method of Jukes & Cantor (1969), and a phylogenetic tree was reconstructed by the neighbour-joining method (Saitou & Nei, 1987) based on the comparison of 1352 nt present in all strains between positions 57 and 1480 positions (*Escherichia coli* numbering system). The reliability of the tree topology was evaluated by using the SEQBOOT, DNADIST, NEIGHBOR and CONSENSE programs in the PHYLIP package (Felsenstein, 1985, 1993), based on 1000 replications. The phylogenetic analysis (Fig. 2) indicated that isolate H647-1^T formed a distinct cluster with members of the genus *Actinomadura* within the radiation encompassing members of the family *Thermomonosporaceae*, albeit with low bootstrap values. This relationship was also supported by the presence of all signature nucleotides specific for the family *Thermomonosporaceae*, except for *Escherichia coli* positions 603:635 (Zhang *et al.*, 2001). In our study, most of the members of the family contained G–C pairs, instead of the A–U pairs previously described, for positions 603:635. The levels of 16S rRNA gene sequence similarity between strain H647-1^T and members of the family *Thermomonosporaceae* ranged from 94.3 to 98.2%. The highest levels of 16S rRNA gene sequence similarity were with *A. livida* (98.2%) and *A. yumaensis* (97.9%), whereas the sequence similarity to another phylogenetic relative, *Actinomadura catellatispora*, was 96.6%. Strain H647-1^T could be readily distinguished from all members of the family *Thermomonosporaceae* by the possession of an additional extended helix (22 nt in length) between *Escherichia coli* positions 453 and 479. A similar-sized loop was also found in the sequences of the genera *Actinomyces*, *Glycomyces*, *Jonesia*, *Mycobacterium*, *Nocardiopsis* and *Thermobifida* (data not shown), although this stretch of the 16S rRNA gene sequence of strain H647-1^T showed a unique nucleotide composition, suggesting that the sequence of this region might be useful for species identification.

The G+C content of the DNA of strain H647-1^T (determined by HPLC; Mesbah *et al.*, 1989) was 67.5 mol%.

The chemotaxonomic characteristics of strain H647-1^T were determined by using previously described methods (Kroppenstedt, 1985; Minnikin *et al.*, 1977, 1980, 1984; Saddler *et al.*, 1991; Stanek & Roberts, 1974; Uchida & Aida, 1984) for the analyses of the isomer of diaminopimelic acid, whole-cell sugars, isoprenoid quinones and phospholipids. The cell wall of isolate H647-1^T was of type III (*meso*-diaminopimelic acid was the diagnostic diamino acid) and the whole-cell sugars were of type B (madurose was the characteristic sugar) (Lechevalier & Lechevalier, 1970). The acyl group of the muramic acid in the cell wall was of

the acetyl type. The major menaquinones of isolate H647-1^T were MK-9(H₆) (43%) and MK-9(H₈) (40%). In addition, a small amount of MK-9(H₄) was also detected. The polar lipid profile contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside and unknown phospholipids. Nitrogenous phospholipids and phosphatidylcholine were not detected [the phospholipid pattern was type PI, described by Lechevalier *et al.* (1981)]. Mycolic acids were not detected. Cellular fatty acid methyl esters were prepared from cells grown on tryptic soy broth (Difco) at 30 °C for 3 days (Minnikin, 1988) and were analysed as described previously (Lee *et al.*, 2000). The fatty acid profile of strain H647-1^T was characterized by the predominance of hexadecanoic acid (C_{16:0}) and 10-methyloctadecanoic acid (C_{18:0} 10-methyl, tuberculostearic acid). Significant amounts of saturated, unsaturated and branched fatty acids were also present [fatty acid type 3a, according to the classification of Kroppenstedt & Goodfellow (1991)]. The cellular fatty acid compositions of strain H647-1^T and closely related *Actinomadura* species are shown in Table 1.

The temperature and pH ranges for growth, and the degree of NaCl tolerance, were determined on ISP 2 medium. The decomposition of adenine, hypoxanthine, DL-tyrosine and xanthine was determined as described by Gordon *et al.*

Table 1. Fatty acid composition (%) of strain H647-1^T and the type strains of closely related *Actinomadura* species

Examples of abbreviations for fatty acids: 16:0, hexadecanoic acid; 15:0 3-OH, 3-hydroxypentadecanoic acid; 18:0 10-methyl, 10-methyloctadecanoic acid (tuberculostearic acid); 18:1 *cis*9, *cis*-9-octadecenoic acid.

Fatty acid	Strain H647-1 ^T	<i>A. livida</i> IMSNU 22191 ^T	<i>A. yumaensis</i> IMSNU 22167 ^T
12:0	–	0.6	1.1
14:0	0.5	0.5	1.3
15:0 iso	1.1	–	–
15:0	–	7.4	0.6
16:0 iso	15.9	9.2	5.0
16:1	1.4	–	–
16:0	27.3	20.2	38.2
15:0 3-OH	–	–	3.8
16:0 10-methyl	–	–	1.8
17:0 anteiso	0.6	–	–
17:1	–	12.4	0.7
17:0	0.5	8.7	0.9
18:1 iso	1.6	–	–
17:0 10-methyl	–	17.0	2.0
18:1 <i>cis</i> 9	19.1	3.8	8.6
18:0	4.4	6.9	3.9
18:0 10-methyl	25.6	11.9	29.5
19:1	–	1.0	2.7

Table 2. Phenotypic properties that differentiate strain H647-1^T from the type strains of closely related *Actinomadura* species

+, Positive; −, negative; ND, not determined. Data were taken from the present study, Meyer (1989) and Lu *et al.* (2003).

Characteristic	Strain H647-1 ^T	<i>A. catellatispora</i> IFO 16341 ^T	<i>A. livida</i> IMSNU 22191 ^T	<i>A. yumaensis</i> IMSNU 22167 ^T
Morphology				
Spore-chain arrangement	Hooks, spirals	Straight	Hooks, spirals	Hooks
Spore-surface ornamentation	Warty	Smooth	Irregular	Smooth
Growth on ISP 2 medium				
Aerial mycelium	None	Yellow	None	None
Substrate mycelium	Blackish purple	Light yellow	Brown	Yellowish brown
Diffusible pigment	Dark brown	None	None	Orange
Acid produced from:				
L-Arabinose	+	ND	+	−
Glycerol	+	ND	−	+
D-Mannitol	+	ND	+	−
L-Rhamnose	−	ND	+	−
Salicin	+	ND	−	+
D-Sorbitol	−	ND	+	−
Sucrose	+	ND	−	+
D-Xylose	+	ND	+	−
Decomposition of:				
Casein	+	−	−*	+
DNA	+	ND	+	−
Hypoxanthine	−	−	−	+
Starch	+	−	−*	+
Tween 80	−	ND	+	+
Tyrosine	−	−	−	+
Xanthine	−	−	−	+
Growth conditions				
45 °C	+	−	−	+
3 % NaCl	+	ND	−	+

*Differs from data in a previous description (Meyer, 1989).

(1974). Urease activity was determined by means of a colour change using Bacto urea broth (Difco). The production of hydrogen sulfide was tested using a lead acetate strip. Catalase activity was determined with a 3 % (v/v) hydrogen peroxide solution. Nitrate reductase activity was determined with an equal mixture of 0.5 % α -naphthylamine and 0.8 % sulfanilic acid. The hydrolysis of casein, gelatin and starch was studied by using previously described methods (MacFaddin, 1981). The hydrolysis of DNA was determined using Bacto DNase test agar (Difco). Acid production from carbohydrates was determined by means of a colour change in Bacto OF basal medium (Difco) supplemented with each substrate at a final concentration of 1 % (w/v). All test strains produced acid from D-cellobiose, D-fructose, D-glucose, maltose and D-trehalose but not from D-galactose, D-lactose, D-mannose, D-melezitose, melibiose, methyl α -D-glucoside, methyl α -D-mannoside, D-raffinose, L-sorbose, adonitol, 2,3-butanediol, dulcitol, *meso*-erythritol, *myo*-inositol, 1,2-propanediol and D-xylitol. Decomposition of

aesculin and gelatin and growth in the presence of 2 % NaCl were observed in all test strains. All test strains possessed nitrate reductase activity but not urease activity. A range of phenetic characteristics that distinguish strain H647-1^T from closely related species of the genus *Actinomadura* are listed in Table 2.

A broad range of phenetic and genetic data suggest that strain H647-1^T merits species status in the genus *Actinomadura* of the family *Thermomonosporaceae*. The name *Actinomadura hallensis* sp. nov. is proposed.

Description of *Actinomadura hallensis* sp. nov.

Actinomadura hallensis (hall.en'sis. N.L. fem. adj. *hallensis* of Mt. Halla, Jeju Island, Republic of Korea, the origin of the soil sample from which the type strain was isolated).

Gram-positive. Catalase-positive. Urease-negative. Nitrate is reduced to nitrite. H₂S is produced. Good growth on ISP

2, ISP 3 and ISP 4 media. Forms a well-developed, non-fragmenting substrate mycelium that is blackish purple on ISP 2 medium, moderate brown on ISP 3 medium and greyish reddish purple on ISP 4 medium. A dark-brown-coloured pigment is produced on ISP 2 medium. On ISP 3 and ISP 4 media, a moderate amount of white aerial mycelia is formed, which matures into short spore chains in hooks or spirals. The spore surface is warty. The temperature range for growth is 20–45 °C. The optimum pH for growth is 7.0. Acid is produced from L-arabinose, D-cellobiose, D-fructose, D-glucose, glycerol, maltose, D-mannitol, salicin, sucrose, D-trehalose and D-xylose. Decomposes casein, DNA, aesculin, gelatin and starch. No acid is produced from adonitol, 2,3-butanediol, dulcitol, *meso*-erythritol, D-galactose, *myo*-inositol, D-lactose, D-mannose, D-melezitose, melibiose, methyl α -D-glucoside, methyl α -D-mannoside, 1,2-propanediol, D-raffinose, L-rhamnose, D-sorbitol, L-sorbose or D-xylitol. Does not decompose hypoxanthine, tyrosine, Tween 80 or xanthine. Grows in the presence of 3 % NaCl. Type III/B cell wall (*meso*-diaminopimelic is the diagnostic diamino acid and madurose is the characteristic sugar). The acyl group of the muramic acid is of the acetyl type. The predominant menaquinones are MK-9(H₆) and MK-9(H₈), with a minor amount of MK-9(H₄). Type PI phospholipid pattern (no diagnostic phospholipid). Mycolic acids are absent. The predominant fatty acids are hexadecanoic acid (C_{16:0}) and tuberculostearic acid (C_{18:0}, 10-methyl). Significant amounts of saturated, unsaturated and anteiso-branched fatty acids are also present. The G + C content of the DNA (determined by HPLC) is 67.5 mol%.

The type strain is H647-1^T (=IMSNU 50760^T=KCTC 9992^T=KCCM 42245^T=NRRL B-24436^T), isolated from a soil sample from Mt. Halla, Jeju Island, Republic of Korea.

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