Actinomadura sputi sp. nov., isolated from the sputum of a patient with pulmonary infection

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The taxonomic position of an actinomycete, strain IMMIB L-889^T, isolated from the sputum of a 64-year-old man, was determined using a polyphasic taxonomic approach. The strain had chemical and morphological properties that were consistent with its classification in the genus *Actinomadura*. It formed a distinct phyletic line in the 16S rRNA gene tree of *Actinomadura* and was most closely related to the type strain of *Actinomadura hallensis* (98.4 % sequence similarity), but could be readily distinguished from the latter species using DNA–DNA relatedness and phenotypic data. The combined genotypic and phenotypic data indicate that strain IMMIB L-889^T represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura sputi* sp. nov. is proposed. The type strain is IMMIB L-889^T (=DSM 45233^T=CCUG 56587^T).

The genus *Actinomadura* Lechevalier and Lechevalier 1968 (Lechevalier & Lechevalier, 1968) belongs to the family *Thermomonosporaceae* (Stackebrandt *et al.*, 1997; Zhang *et al.*, 1998, 2001). This taxon also includes the genera *Actinocorallia* (Iinuma *et al.*, 1994), *Spirillospora* (Couch, 1963) and *Thermomonospora* (Henssen, 1957). At the time of writing, the genus *Actinomadura* encompassed 40 species with validly published names (www.bacterio.cict.fr) that can be delineated using a combination of genotypic and phenotypic data (Kroppenstedt & Goodfellow, 1991; Lu *et al.*, 2003; Zhang *et al.*, 2001). The primary reservoir of actinomadurae is soil (Chormonova & Preobrazhenskaya, 1981; Quintana *et al.*, 2003; Lu *et al.*, 2003; Ara *et al.*, 2008), although members of the genus are better known as causal agents of actinomycetoma (McNeil & Brown, 1994; Trujillo & Goodfellow, 1997, 2003), superficial or deep suppurating tumefactions of the skin and subcutaneous tissues that result from soil contamination of a penetrating wound. *Actinomadura* should also be considered as causative agents for non-mycetomic infections such as pneumonia. *Actinomadura madurae* accounted for 11.5 % of the aerobic actinomycetes from clinical specimens that were referred to the Actinomycete Laboratory of the Centers for Disease Control and Prevention (CDC, USA) during the period October 1985 to February 1988 and is suggested to play some role in pneumonitis or bronchitis (McNeil *et al.*, 1990). *A. madurae* was isolated originally

from a 46-year-old alcoholic man with pneumonia suspected of Tbc reactivation (Bär *et al.*, 2003). The majority of clinical isolates from sputa or bronchoalveolar lavage fluid in Japan are *A. madurae* (Hanafy *et al.*, 2006). In the present study, the isolation and identification of an actinomycete from the sputum of a 64-year-old man suspected to be infected with *Mycobacterium tuberculosis* are reported.

For isolation, sputum of a 64-year-old man was treated using a NaOH–N-acetyl cysteine decontamination procedure (Master, 1994). After decontamination, the sample was centrifuged at 3000 r.p.m. for 20 min. The supernatant was discarded and the sediment was inoculated into a Mycobacteria Growth Indicator tube (MGIT; Becton Dickinson) and on Löwenstein–Jensen medium. The organism, designated strain IMMIB L-889^T, was recovered on Löwenstein–Jensen and was subsequently cultivated on brain heart infusion (BHI) agar, glucose-yeast extract-malt extract agar (GYM; medium 65 DSMZ Catalogue) and Columbia agar supplemented with 5 % sheep blood. Gram and Ziehl–Neelsen stains were applied to mycelia grown on Columbia agar. To determine the morphological characteristics of the isolate, it was grown on yeast extract/malt extract agar (ISP medium 2), oatmeal agar (ISP medium 3) and inorganic salts/starch agar (ISP medium 4) as described by Shirling & Gottlieb (1966) and was examined for pigmentation and colour of aerial and substrate mycelia. Growth temperatures were determined by incubating the organism at 27, 37 and 42 °C on Columbia agar.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMMIB L-889^T is FM957483.

Strain IMMIB L-889^T was examined for a range of phenotypic characteristics using standard procedures (Gordon, 1966, 1967; Gordon & Mihm, 1957; Yassin *et al.*, 1995). Biomass for chemotaxonomic studies was derived from a 7-day-old BHI broth shake culture incubated at 37 °C, harvested by centrifugation and washed with distilled water. Standard procedures were used to determine the isomeric form of the diaminopimelic acid (Becker *et al.*, 1964), whole-cell sugars (Lechevalier, 1968), non-hydroxylated fatty acids (Minnikin *et al.*, 1980; Yassin *et al.*, 2007), polar lipids (Yassin *et al.*, 1993) and isoprenoid quinones (Collins *et al.*, 1977; Yassin & Hupfer, 2006).

Genomic DNA extraction and PCR-mediated amplification of the 16S rRNA gene were carried out using established procedures (Rainey *et al.*, 1996). The purified PCR products were sequenced using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) as described by the manufacturer. A Genetic Analyzer (310; Applied Biosystems) was used for electrophoresis of the sequence reaction products. The 16S rRNA gene sequence of strain IMMIB L-889^T, as well as those of species of the genus *Actinomadura* with validly published names (retrieved from GenBank), were added to the ARB database (Ludwig *et al.*, 2004) and aligned using the respective tool from the ARB package. The resulting alignment was corrected manually and evolutionary trees were inferred using maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. The evolutionary distance matrix for the neighbour-joining method was calculated using the correction of Jukes & Cantor (1969). Topologies of the neighbour-joining tree were evaluated using bootstrap analyses (Felsenstein, 1985) based on 500 resamplings. DNA–DNA relatedness studies were performed between strain IMMIB L-889^T and *Actinomadura hallensis* DSM 45043^T. 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) under consideration of the modifications described by Huß *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multi-cell changer and a temperature controller with *in situ* temperature probe (Varian).

To establish the phylogenetic position of strain IMMIB L-889^T, its 16S rRNA gene sequence was determined in this study (1509 nt). Sequence database searches revealed that strain IMMIB L-889^T was most closely related to species of the genus *Actinomadura* (data not shown). Phylogenetic analysis confirmed the placement of strain IMMIB L-889^T within the genus *Actinomadura*. A tree constructed using the neighbour-joining method showing the nearest phylogenetic relatives of strain IMMIB L-889^T is shown in Fig. 1. It is evident from the tree that strain IMMIB L-889^T is most closely related to the type strain of *A. hallensis*. The association of strain IMMIB L-889^T with *A. hallensis* was

supported by all of the treeing algorithms and by a bootstrap value of 100 %. Comparative 16S rRNA gene sequence analysis demonstrated that strain IMMIB L-889^T displayed sequence similarity values of less than 98.4 % to the type strains of recognized members of the genus *Actinomadura*. Highest sequence similarity was shown to *A. hallensis* DSM 45043^T (98.4 %). DNA–DNA relatedness studies between strain IMMIB L-889^T and *A. hallensis* DSM 45043^T clearly demonstrated that both of them belong to separate genomic species. The mean DNA–DNA relatedness value was 61.3 ± 2.96 %, a value well below the 70 % cut-off point recommended for the assignment of strains to the same genomic species (Wayne *et al.*, 1987). It is also apparent that strain IMMIB L-889^T can be distinguished from *A. hallensis* using a combination of phenotypic properties (Table 1).

Strain IMMIB L-889^T also exhibited typical chemical markers of members of the genus *Actinomadura*. The cell wall of strain IMMIB L-889^T was of type III (Lechevalier & Lechevalier, 1970), contained *meso*-diaminopimelic acid as the wall diamino acid and type B whole-cell sugars (madurose was the diagnostic sugar). The predominant menaquinone was a hexahydrogenated menaquinone with nine isoprene units, MK-9(H₆). In addition, small amounts of MK-9(H₈) and MK-9(H₄) were also detected. The phospholipid pattern was type PI *sensu* Lechevalier *et al.* (1977) and consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside. The fatty acid profile consisted of complex mixtures of fatty acids, notably C_{14:0} (0.9 % of total fatty acids), C_{15:0} (0.7 %), iso-C_{16:0} (9.4 %), C_{16:1ω7c} (0.6 %), C_{16:0} (34.3 %), anteiso-C_{17:0} (0.6 %), C_{17:1ω8c} (1.0 %), C_{17:0} (3.2 %), 10-methyl-C_{17:0} (0.8 %), iso-C_{18:0} (1.9 %), C_{18:1ω9c} (18.0 %), C_{18:1ω7c} (0.6 %), C_{18:0} (9.0 %), 10-methyl-C_{18:0} (14.5 %), C_{20:1} (0.6 %), C_{20:0} (0.4 %), C_{24:1} (0.7 %) and C_{24:0} (0.4 %).

The phenotypic properties of strain IMMIB L-889^T were also consistent with its classification in the genus *Actinomadura*. The isolate was an aerobic, non-motile, Gram-positive, non-acid–alcohol-fast actinomycete that formed extensively branched, non-fragmenting substrate mycelia which bore abundant aerial mycelia. The substrate mycelia appeared grey on Columbia blood agar, yellowish on ISP medium 2, and whitish in colour on ISP medium 3, ISP medium 4 and GYM agar. The aerial mycelia of strain IMMIB L-889^T appeared grey in colour on Columbia blood agar, creamy on ISP medium 2, skin-coloured on both ISP medium 3 and GYM agar, and white on ISP medium 4. The aerial mycelia bore straight chains of two to four spores at the tips of sporulating aerial hyphae. The spores were elongated and had smooth surfaces. Diffusible pigments were not produced. Melanoid pigments were not produced on ISP medium 6 or ISP medium 7. The organism grew at 27–37 °C, but not at 42 °C. Strain IMMIB L-889^T was examined for a range of phenotypic properties, the results of which are listed in Table 1 and cited in detail in the species description.

Fig. 1. Neighbour-joining tree showing the position of isolate IMMIB L-889^T in the genus *Actinomadura*. Numbers at the nodes indicate the level of bootstrap support (%) based on neighbour-joining analyses of 500 resampled datasets; solid circles indicate that the corresponding nodes (groupings) were also recovered in maximum-likelihood and maximum-parsimony trees. Bar, 5.0 % sequence divergence.

On the basis of the genotypic and phenotypic data, strain IMMIB L-889^T is a member of the genus *Actinomadura* and represents a novel species, for which the name *Actinomadura sputi* sp. nov. is proposed.

Description of *Actinomadura sputi* sp. nov.

Actinomadura sputi sp. nov. (spu'ti. L. gen. n. *sputi* of sputum).

Aerobic, Gram-positive, non-acid-alcohol-fast, non-motile actinomycete. Forms non-fragmenting, whitish (on ISP medium 3, ISP medium 4 and GYM agar), yellowish (on ISP medium 2) or grey (on Columbia blood agar) substrate mycelia that bear white (on ISP medium 4), creamy (on ISP medium 2), skin-coloured (on ISP medium 3 and GYM agar) or grey (on Columbia blood agar) aerial mycelia. Aerial mycelia bear straight chains of two to four rod-shaped spores with smooth surfaces. Grows at

Table 1. Differential phenotypic characteristics of strain IMMIB L-889^T and *A. hallensis* DSM 45043^T

Strains: 1, IMMIB L-889^T; 2, *A. hallensis* DSM 45043^T. Both strains are positive for hydrolysis of aesculin, tyrosine and urea, but negative for hydrolysis of adenine, casein, elastin, gelatin, guanine, testosterone and xanthine. Both strains are positive for utilization of cellobiose and L-rhamnose as carbon sources, but negative for utilization of adonitol, adipic acid, 2,3-butanediol, citrate, cellobiose, *meso*-erythritol, *m*-hydroxybenzoate, 1,2-propanediol and raffinose as carbon sources. Both strains are positive for utilization of acetamide, L-alanine, L-arginine, gelatin, proline, ornithine and serine as simultaneous sources of carbon and nitrogen. +, Positive; –, negative; w, weak utilization after 3 weeks.

Characteristic	1	2
Hydrolysis of:		
Hypoxanthine	–	+
Utilization as sole sources of carbon and energy:		
Acetate	–	+
<i>iso</i> -Amylalcohol	–	+
L-Arabinose	–	+
D-Galactose	–	w
D-Glucose	–	+
D-Gluconate	–	+
<i>p</i> -Hydroxybenzoate	w	–
<i>myo</i> -Inositol	–	+
L-Lactate	+	–
Lactose	+	–
Maltose	–	+
D-Mannitol	–	+
Melezitose	–	+
Sucrose	–	+
D-Sorbitol	+	–
Trehalose	–	+
D-Xylose	–	+

27–37 °C, but not at 42 °C. Diffusible pigments are not produced. Melanoid pigment is not produced on ISP medium 6 or ISP medium 7. Hydrolyses aesculin, tyrosine and urea, but not adenine, casein, elastin, gelatin, guanine, hypoxanthine, testosterone or xanthine. Assimilates cellobiose, L-lactate, lactose, L-rhamnose and D-sorbitol as carbon sources, but not acetate, adonitol, adipate, *iso*-amylalcohol, L-arabinose, 2,3-butanediol, citrate, *meso*-erythritol, D-galactose, D-gluconate, glucose, *m*-hydroxybenzoate, *p*-hydroxybenzoate, *myo*-inositol, maltose, D-mannitol, melezitose, 1,2-propanediol, raffinose, sucrose, trehalose or D-xylose. Utilizes acetamide, L-alanine, arginine, gelatin, proline, ornithine and serine as simultaneous carbon and nitrogen sources. Whole-organism hydrolysates contain *meso*-diaminopimelic acid and madurose as characteristic sugar. The major phospholipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside. The major menaquinone is MK-9(H₆). The major fatty acids are C_{16:0}, *iso*-C_{16:0}, C_{18:0}, C_{18:1}ω9c and 10-methyl-C_{18:0}.

The type strain is IMMIB L-889^T (=DSM 45233^T=CCUG 56587^T), which was isolated from the sputum of a 64-year-old man suspected to be infected with *Mycobacterium tuberculosis*.

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