Actinomadura apis sp. nov., isolated from a honey bee (Apis mellifera) hive, and the reclassification of Actinomadura cremea subsp. rifamycini Gauze et al. 1987 as Actinomadura rifamycini (Gauze et al. 1987) sp. nov., comb. nov.

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A Gram-reaction-positive aerobic actinomycete, designated strain IM17-1T, was isolated from a honey bee (Apis mellifera) hive in Chiang Mai Province, Thailand. The strain formed a branched substrate mycelium and mature aerial mycelium bore short chains of arthrospores with warty surfaces. The cell wall contained meso-2,6-diaminopimelic acid (cell-wall type III) and the whole cell sugars were fucose, galactose, glucose, madurose, mannose and ribose. The major isoprenoid quinone was hexahydrogenated menaquinone with nine isoprene units and the predominant cellular fatty acids were C16:0 (33.8 %), C18:1ω9c (32.7 %), summed feature 3 (C16:1ω7c and/or iso-C15:0 2-OH) (8.7 %) and 10-methyl C18:0 (8.2 %). The phospholipids were diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. These morphological and chemotaxonomic characteristics were consistent with the classification of IM17-1T within the genus Actinomadura. Based on 16S rRNA gene sequence analysis, strain IM17-1T was closely related to the type strains of Actinomadura cremea subsp. cremea (98.1 %) and Actinomadura cremea subsp. rifamycini (98.6 %); however, it represented a distinct phylogenetic lineage from the other species within this genus. The unique genetic characteristics were reaffirmed by low levels of DNA–DNA relatedness between strain IM17-1T and the two most closely related type strains, A. cremea subsp. cremea JCM 3308T (56.5 ± 4.9 %) and A. cremea subsp. rifamycini JCM 3309T (31.0 ± 22.6 %), and further supported the proposal of IM17-1T as a novel species. Strain IM17-1T (JCM 16576T =TISTR 1980T) thus represents a novel species of the genus Actinomadura, for which the name Actinomadura apis sp. nov. is proposed. In addition, the genotypic and phenotypic data suggested the reclassification of Actinomadura cremea subsp. rifamycini Gauze et al. 1987 as a separate species, Actinomadura rifamycini sp. nov., comb. nov.

The genus Actinomadura was established by Lechevalier & Lechevalier (1968) and, at the time of writing, encompassed 42 species with validly published names including two subspecies, Actinomadura cremea subsp. cremea (Preobrazhenskaya et al., 1975) and Actinomadura cremea subsp. rifamycini (Gauze et al., 1975, 1987) (http://www.bacterio.cict.fr/). Members of this genus are aerobic Gram-positive actinomycetes that can grow at 10–60 °C and form non-fragmenting branched substrate mycelia, which give rise to aerial hyphae bearing spore chains up to 15 spores long with various shapes and ornamentations. Other characteristics of the genus are as follows. The cell-wall peptidoglycan is type III sensu Lechevalier & Lechevalier (1970) containing meso-diaminopimelic acid (A2pm) without glycine; the major isoprenoid quinone is hexahydrogenated menaquinone with nine isoprene units; whole-cell hydrolysates mostly contain madurose; the fatty acid profiles are type 3a sensu Kroppenstedt (1985) containing saturated, unsaturated and branched fatty acids plus tuberculostearic acid, with the exception of the thermophilic actinomadurae, which differ in their fatty acid composition; and the phospholipid pattern is type PI sensu Lechevalier et al. (1981), in which no nitrogenous

The GenBank/EMBL/DBJ accession numbers for the partial 16S rRNA gene sequences of strain IM17-1T and Actinomadura rifamycini JCM 3309T are AB557596 and AB557595, respectively.

A supplementary table is available with the online version of this paper.
phospholipids are present (Kroppenstedt et al., 1990; Kroppenstedt & Goodfellow, 2006).

Most actinomadurae are widespread in soil (Cook et al., 2005; Kroppenstedt & Goodfellow, 2006; Ara et al., 2008), a few are found in clinical materials (Yassin et al., 2010) and one was isolated as an endophyte from the leaves of a medicinal plant in China (Qin et al., 2009). During the course of a study on the microflora of the European honey bee (Apis mellifera Linnaeus, 1758) in Thailand, actinomycetes were isolated from honey bee hive materials. Most of the isolates belonged to the genus Streptomyces and some less common isolates were classified as belonging to the genera Nonomuraea and Nocardiosis. The remaining isolate, strain IM17-1T, was identified as belonging to the genus Actinomadura but could not be classified in any known species (Promnuan et al., 2009). Here, we describe the characterization and classification of strain IM17-1T, which is, to our knowledge, the first member of the genus Actinomadura isolated from a hive of the European honey bee.

The bacterial strain was isolated using the standard dilution plate method on starch-casein agar (Küster & Williams, 1964) supplemented with 25 μg ml⁻¹ each of nystatin and nalidixic acid. After being incubated at 30 °C for 21 days, the isolate was transferred to, and purified and maintained on, oatmeal agar (International Streptomyces Project Medium 3; ISP 3) (Shirling & Gottlieb, 1966) supplemented with 0.1 % yeast extract.

The morphological and physiological characteristics of strain IM17-1T were determined and compared with those of phylogenetically closely related type strains, A. cremea subsp. cremea JCM 3308T and A. cremea subsp. rifamycinii JCM 3309T. The temperature range for growth and NaCl tolerance were determined by culturing on ISP 3 medium and incubating for 14 days. Carbon utilization and melanoid pigment production were tested using the methods described by Shirling & Gottlieb (1966). Biomass for chemotaxonomic studies was prepared from cultures grown in yeast extract–malt extract (ISP 2) broth on a rotary shaker for 2–4 weeks at 30 °C. The isomers of A3pm were determined by TLC of whole-cell hydrolysates according to the method of Staneck & Roberts (1974). Reducing sugars in whole-cell hydrolysates were analysed by using the HPLC method of Mikami & Ishida (1983). Phospholipids were extracted and identified following the methods of Innikin et al. (1984). Methyl esters of cellular fatty acids were prepared and analysed according to the instructions for the Sherlock Microbial Identification System (version 2.998, MIDI) using GC (model HP6890; Hewlett Packard) with the TSBA40 method (Sasser, 1990). Isoprenoid quinones were extracted according to the method of Collins et al. (1977, 1984) and were analysed in an HPLC system (model LC-10AD VP; Shimadzu) equipped with a Cosmosil 5C18 column (Nacalai Tesque) (Tamaoka et al., 1983).

Cultural characteristics of strain IM17-1T were determined after incubation at 30 °C for 21 days on various media as described by Shirling & Gottlieb (1966) and Asano & Kawamoto (1986). Determination of colony colours was based on Jacobson et al. (1958). After incubation on ISP 2 and ISP 3 agar at 30 °C for 21 days, morphological properties were examined using a light microscope and a scanning electron microscope (model S-2400; Hitachi). Samples for scanning electron microscopy were prepared as described by Itoh et al. (1989). Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and sequencing of the PCR products were carried out as described by Nakajima et al. (1999). CLUSTAL_X (Thompson et al., 1997) was used to perform a multiple alignment of sequences downloaded from GenBank and the sequences obtained in this study. The alignment was verified manually and adjusted prior to the reconstruction of a phylogenetic tree. For reconstruction of phylogenetic trees, the software packages MEGA version 4 (Tamura et al., 2007) and CLUSTAL_X were used. Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution and maximum-parsimony methods (Takahashi & Nei, 2000), based on a comparison of 1172 nt present in all of the strains after elimination of gaps and ambiguous nucleotides from the sequences corresponding to positions 34 and 1491 in Escherichia coli Streptosporangium album DSM 43023T was used as an outgroup. Confidence values for the branches of the phylogenetic tree were determined using bootstrap analysis based on 1000 resamplings (Felsenstein, 1985). Sequence similarities between strains of the genus Actinomadura were calculated manually after pairwise alignment was performed using CLUSTAL_X.

For preparation of chromosomal DNA for G+C content analysis and DNA–DNA hybridization experiments, cells were freeze-dried and mechanically ground as described by Reader & Broda (1985). The G+C content of the DNA was determined using the HPLC method of Tamaoka & Komagata (1984). An equimolar mixture of nucleotides for analysis of the DNA base composition (Yamasawa Shoyu) was digested by bacterial alkaline phosphatase and used as the quantitative standard. Levels of DNA–DNA relatedness were measured fluorometrically using the microplate hybridization method devised by Ezaki et al. (1989). Hybridization was carried out at 60 °C for 12 h.

Strain IM17-1T was found to be a Gram-positive aerobic actinomycete that produced well-developed and branched substrate hyphae. Spore chains were short, curved and consisted of ~3–5 non-motile spores with warty surfaces. The spores were borne on aerial hyphae as shown in Fig. 1a. The spore morphology was similar to that of A. cremea subsp. rifamycinii, the most closely related species, phylogenetically (Fig. 1b).

The cultural characteristics of strain IM17-1T were also examined. The isolate showed good growth on glucose–yeast extract agar and moderate growth on ISP 2, ISP 3, ISP 4, ISP 5 and ISP 7 media, Bennett agar, glucose–asparagine agar, Hickey–Tresner agar, nutrient agar and oatmeal–nitrate agar. Strain IM17-1T formed white to grey aerial
mycelia on the various media tested and the substrate mycelia showed different colours on different media (Supplementary Table S1, available in IJSEM Online). A greenish-black pigment was produced on ISP 2 and ISP 7 media. Strain IM17-1T grew at 20–45 °C, at pH 6.0–12.0 and in the presence of 4 % (w/v) NaCl.

Chemotaxonomic analyses revealed that strain IM17-1T exhibited characteristics that were typical of members of the genus *Actinomadura*. The cell wall contained meso-A2pm and whole-cell sugars including fucose, galactose, glucose, madurose, mannos and ribose. The phospholipids were diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. The predominant menaquinones were MK-9(H6) (53 %) and MK-9(H4) (26 %); MK-9(H4) (8 %) was detected as a minor component. The major cellular fatty acids were C16:0 (33.8 %), C18:1ω9c (32.7 %), summed feature 3 (C16:1ω7c and/or iso-C15:0 2-OH) (8.7 %) and 10-methyl C18:0 (8.2 %); minor amounts of C18:1ω9c (6.9 %), iso-C16:1ω0 (1.9 %), C14:0 (1.7 %), C17:0 (1 %) and C16:1ω9c (<1 %) were also present (Table 1).

The 16S rRNA gene sequence of strain IM17-1T (1495 nt) was compared with sequences of all species with validly published names within the genus *Actinomadura* and related taxa in the family Thermomonosporaceae (Rainey et al., 1997 in Stackebrandt et al., 1997; emend. Zhi et al., 2009). Based on the comparative study of 16S rRNA gene sequences, strain IM17-1T shared the closest sequence similarity values with *A. cremea* subsp. *cremea* (98.1 %) and *A. cremea* subsp. *rifamycinici* (98.6 %). The neighbour-joining tree (Fig. 2) showed that strain IM17-1T was a member of the genus *Actinomadura* and was most closely related to *A. cremea* subsp. *cremea* and *A. cremea* subsp. *rifamycinici* but it was also readily distinguishable from the two subspecies (95 % bootstrap support). To confirm the taxonomic position of the isolate, DNA–DNA hybridization studies were carried out. The mean of DNA–DNA relatedness values of strain IM17-1T with *A. cremea* subsp. *cremea* JCM 3308T and *A. cremea* subsp. *rifamycinici* JCM 3309T were 56.5 ± 4.9 and 31.0 ± 22.6 %, respectively. According to the 70 % DNA–DNA relatedness cut-off point recommended for the delineation of bacterial species (Wayne et al., 1987), strain IM17-1T represents a novel species. This assumption was also confirmed by differences in phenotypic characteristics observed between strains IM17-1T, *A. cremea* subsp. *cremea* JCM 3308T and *A. cremea* subsp. *rifamycinici* JCM 3309T, such as the production of melanoid pigments, the composition of polar lipids, menaquinones, whole-cell sugars and fatty acids, and carbon utilization patterns (Table 1).

On the basis of the cultural, morphological, physiological and chemotaxonomic characteristics, 16S rRNA gene sequence similarity and DNA–DNA hybridization results, strain IM17-1T represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura apis* sp. nov. is proposed.

*A. cremea* subsp. *rifamycinici* appeared on validation list no. 23 (Gauze et al., 1987) as a new subspecies and *A. cremea* subsp. *cremea* was automatically created by Rule 46 (Howey et al., 1990). Our findings suggested that taxonomic revision of these subspecies is necessary. We found that the level of DNA–DNA relatedness between *A. cremea* subsp. *cremea* JCM 3308T and *A. cremea* subsp. *rifamycinici* JCM 3309T was 34 ± 4.2 %, which was much lower than the threshold recommended for members of a single genomic species (Wayne et al., 1987). The partial 16S rRNA gene sequence of *A. cremea* subsp. *rifamycinici* JCM 3309T was also analysed to confirm the previously determined data and to obtain a longer 16S rRNA gene sequence. *A. cremea* subsp. *rifamycinici* JCM 3309T was also confirmed by differences in the production of melanoid pigments, the composition of polar lipids, menaquinones, whole-cell sugars and fatty acids, and carbon utilization patterns (Table 1).

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novel species of the genus Actinomadura, for which the name Actinomadura rifamycini sp. nov., comb. nov. is proposed.

Description of Actinomadura apis sp. nov.

Actinomadura apis (a’pis L. gen. apis of/from a honey bee, the genus name of the true honey bee Apis mellifera (Linnaeus, 1758), a hive of which was the source of the type strain).

Cells are aerobic Gram-reaction-positive actinomyces that form a branched substrate mycelium. Aerial mycelium at maturity forms short branched spore chains with 3–5 spores. Spore shape is oval and the spore surface is warty at maturity. On ISP 2 agar, colonies are lacking in aerial mycelia and have a cartilaginous appearance. Good growth occurs on glucose–yeast extract agar and moderate growth occurs on ISP 2, 3, 4, 5 and 7 media, Bennett agar, glucose–asparagine agar, Hickey–Tresner agar, oatmeal–nitrate agar and nutrient agar. Substrate mycelia are dark-olive to light-brown on various agar media. Aerial mycelia and sporulation are found on ISP3, 4, 5 and 7 media, Hickey–Tresner agar and oatmeal–nitrate agar. A soluble greenish-black pigment is produced on ISP 2 and ISP 7 media. Growth occurs at 20–45°C and pH 6–12 and moderate growth occurs in the presence of 4 % (w/v) NaCl. D-Glucose, L-arabinose and rhamnose are utilized as sole carbon sources but sucrose, D-xylose, inositol, D-mannitol, D-fructose and raffinose are not. Negative for the production of melanoid pigments. The dominant diamino acid isomer in the cell wall is meso-2,6-diaminopimelic acid. Fucose, galactose, glucose, madurose, mannose and ribose occur in whole-cell hydrolysates. Diphosphatidylglycerol, phosphatidylglycerol, PI, phosphatidylinositol; PIMs, phosphatidylinositol mannosides; Fuc, fucose; Gal, galactose; Glu, glucose; Mad, madurose; Man, mannose and moderate Rib, ribose.

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<th>Characteristics</th>
<th>1</th>
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<td>+</td>
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<td>Growth at 45 °C</td>
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<td>DPG, PI, PG*</td>
<td>DPG, PI, PIMs*</td>
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*Data from Kroppenstedt et al. (1990).
†Summed feature 3 comprises C16:1ω7c and/or iso-C15:0 2-OH.

Table 1. Characteristics of strain IM17-1T and the type strains Actinomadura cremea subsp. cremea JCM 3308T and Actinomadura cremea subsp. rifamycini JCM 3309T

All data are from this study, except where indicated otherwise. Strains: 1, IM17-1T; 2, A. cremea subsp. cremea JCM 3308T; 3, A. cremea subsp. rifamycini JCM 3309T. +, Positive; (+), moderate; ±, poor; −, negative; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIMs, phosphatidylinositol mannosides; Fuc, fucose; Gal, galactose; Glu, glucose; Mad, madurose; Man, mannose and moderate Rib, ribose.

Hickey–Tresner agar, oatmeal–nitrate agar and nutrient agar. Substrate mycelia are dark-olive to light-brown on various agar media. Aerial mycelia and sporulation are found on ISP3, 4, 5 and 7 media, Hickey–Tresner agar and oatmeal–nitrate agar. A soluble greenish-black pigment is produced on ISP 2 and ISP 7 media. Growth occurs at 20–45°C and pH 6–12 and moderate growth occurs in the presence of 4 % (w/v) NaCl. D-Glucose, L-arabinose and rhamnose are utilized as sole carbon sources but sucrose, D-xylose, inositol, D-mannitol, D-fructose and raffinose are not. Negative for the production of melanoid pigments. The dominant diamino acid isomer in the cell wall is meso-2,6-diaminopimelic acid. Fucose, galactose, glucose, madurose, mannose and ribose occur in whole-cell hydrolysates. Diphosphatidylglycerol, phosphatidylglycerol, PI, phosphatidylinositol; PIMs, phosphatidylinositol mannosides; Fuc, fucose; Gal, galactose; Glu, glucose; Mad, madurose; Man, mannose and moderate Rib, ribose.
is characterized by the presence of significant amounts (>7% of the total) of C₁₆:₀, C₁₈:₁ᵥ₉c, summed feature 3 (C₁₆:₁ᵥ₇c and/or iso-C₁₅:₀2-OH), and 10-methyl C₁₈:₀.

The type strain, IM17-¹T (JCM 16576T = JISTR 1980T), was isolated from a honey bee (Apis mellifera) hive in Chiang Mai Province, Thailand. The DNA G+C content of the type strain is 73.7 mol%.

**Description of Actinomadura rifamycini**

**Gauze et al. 1987** sp. nov., comb. nov.

Actinomadura rifamycini (ri.fa.my.ci’ni. N.L. gen. n. rifamycini of rifamycin, referring to an antibiotic, rifamycin).


Forms branched substrate mycelia. Aerial mycelia at maturity form short spore chains with 3–8 spores per chain. Spore shape is oval and the spore surface is warty.

Good growth occurs on ISP 2 medium; poor growth on ISP 3 and 4 media. Substrate mycelia are brown on ISP 2 medium and colourless on ISP 3 and 4 media. Aerial mycelia are white to pale-pink and sporulation occurs in ISP 2, 3 and 4 media. A soluble brown pigment is produced in ISP 2 medium. Growth occurs at 20–50 °C and pH 6–12; moderate growth occurs in the presence of 4% (w/v) NaCl. D-Glucose, L-arabinose, sucrose, inositol and rhamnose are utilized as sole carbon sources but D-mannitol, D-fructose, D-xylose and raffinose are not. Production of melanoid pigments is positive only in ISP 6 medium after 4 days of growth. The dominant diaminopimelic acid isomer in the cell wall is meso-2,6-diaminopimelic acid. Galactose, mannose and ribose occur in whole-cell hydrolysates. Polar lipids detected include diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. The major menaquinones are MK-9(H₈) and MK-9(H₆); minor amounts of MK-9(H₁₀) are also found. The fatty acid profile is characterized by the presence of significant amounts (>7% of the total) of C₁₆:₀, C₁₈:₁ᵥ₉c, 10-methyl C₁₈:₀ and summed feature 3 (C₁₆:₁ᵥ₇c and/or iso-C₁₅:₀2-OH).

The type strain is JCM 3309T (ATCC 33264T = DSM 43936T = INIA 1349T = KCTC 9248T = NBRC 14183T).
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