Lapillicoccus jejuensis gen. nov., sp. nov., a novel actinobacterium of the family *Intrasporangiaceae*, isolated from stone

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A novel, yellow-pigmented actinobacterium was isolated from a small stone collected in Jeju, Republic of Korea. The cells of the organism, designated strain R-Ac013^T, were Gram-positive, aerobic, non-motile cocci that occurred singly or in pairs. The strain showed growth at 10–37 °C and pH 4.1–11.1, and tolerated 2% NaCl. On the basis of the 16S rRNA gene sequence, the organism was phylogenetically related to members of the genera *Tetrasphaera* (94.6–97.1 % sequence similarity), *Terrabacter* (96.5 %), *Knoellia* (96.4 %), *Terracoccus* (96.4 %), *Oryzihumus* (96.4 %), *Janibacter* (96.1–96.4 %) and *Intrasporangium* (96.2 %). The chemotaxonomic results for the organism were as follows: LL-diaminopimelic acid as the diagnostic diamino acid in the peptidoglycan, acetyl-type murein, MK-8(H₄) as the major menaquinone, a DNA G + C content of 74.1 mol%, and a polar lipid profile that comprised diphosphatidylglycerol and phosphatidylinositol. The fatty acid profile consisted of iso- and anteiso-methyl-branched, straight-chain saturated and monounsaturated types, the major components being iso-C_{16:0}, C_{17:1ω8c} and iso-C_{15:0}. The combination of the phenotypic and phylogenetic data revealed that this strain represents a novel genus and species of the family *Intrasporangiaceae*, for which the name *Lapillicoccus jejuensis* gen. nov., sp. nov. is proposed. The type strain is strain R-Ac013^T (=KCTC 19200^T=DSM 18607^T).

The family *Intrasporangiaceae* (Stackebrandt *et al.*, 1997; Stackebrandt & Schumann, 2000) contains a variety of actinomycetes that have LL-diaminopimelic acid (LL-DAP), meso-diaminopimelic acid (meso-DAP) or L-ornithine as the diagnostic diamino acid in the cell-wall peptidoglycan (Martin *et al.*, 1997; Maszenan *et al.*, 2000; Hanada *et al.*, 2002; Groth *et al.*, 2002; Kageyama *et al.*, 2005). At present, this family contains 13 genera with validly published names, including the recently described genera *Phycococcus* (Lee, 2006) and *Kribbia* (Jung *et al.*, 2006); these genera can be readily differentiated from one another on the basis of a combination of phenotypic and genotypic features. Of these genera, *Arsenicococcus* (Collins *et al.*, 2004), *Intrasporangium* (Kalakoutskii *et al.*, 1967), *Terrabacter* (Collins *et al.*, 1989) and *Terracoccus* (Prauser *et al.*, 1997) contain LL-DAP as the diagnostic diamino acid in the cell-wall peptidoglycan. In this study, we describe the classification of an LL-DAP-containing actinomycete (isolated from a small stone) by

means of a polyphasic approach; we propose that it represents a novel genus and species of the family *Intrasporangiaceae*.

Strain R-Ac013^T was isolated from a small stone collected from an agricultural field in Jeju, Republic of Korea. For the bacterial isolation, a piece of the stone was crushed into a powder (using a pestle) and suspended in 10 ml sterilized, distilled water. Serial diluents of the sample were transferred onto starch-casein agar (Kuester & Williams, 1964) and the plates then incubated at 30 °C for 14 days. The pure culture was maintained as a 20 % glycerol suspension at –20 and –80 °C.

DNA extraction, PCR-amplification of the 16S rRNA gene and sequencing were performed as described by Lee & Jeong (2006). An almost-complete 16S rRNA gene sequence (1412 nt) for strain R-Ac013^T was determined in this study and aligned with respect to the corresponding sequences of representatives of the family *Intrasporangiaceae*, using the program CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic analyses were performed by using three tree-making algorithms, namely the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. A phylogenetic tree was constructed using

Abbreviations: LL-DAP, LL-diaminopimelic acid; meso-DAP, meso-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain R-Ac013^T is AM398397.

A transmission electron micrograph of cells of strain R-Ac013^T is available as supplementary material with the online version of this paper.

the Jukes–Cantor coefficient (Jukes & Cantor, 1969) and the neighbour-joining method (Saitou & Nei, 1987). A bootstrap analysis (Felsenstein, 1985) was performed by using 1000 replicated datasets.

In total, 1342 unambiguous aligned nucleotides present in all strains were used for the phylogenetic analyses. A neighbour-joining tree (Fig. 1) based on the 16S rRNA gene sequence revealed that strain R-Ac013^T occupied a phylogenetically distinct lineage within the family *Intrasporangiaceae* and formed a sub-branch between a *Janibacter*–*Knoellia*–*Tetrasphaera* cluster and an *Intrasporangium*–*Phycoccus*–*Terrabacter*–*Terracoccus* cluster. However, this relationship was not supported by a high bootstrap value or with the two other tree-making methods used in this study. When the maximum-parsimony method was applied, strain R-Ac013^T was positioned at the periphery of an *Intrasporangium*–*Phycoccus*–*Terrabacter*–*Terracoccus* cluster, but formed a sub-branch between an *Ornithinococcus hortensis*–*Oryzihumus leptocrescens* cluster and *Tetrasphaera*

japonica in the maximum-likelihood tree (data not shown). Strain R-Ac013^T revealed the highest 16S rRNA gene sequence similarity value (97.1%) with respect to *Tetrasphaera japonica* ACM 5116^T. Members of the genera *Terrabacter* (96.5% sequence similarity), *Tetrasphaera* (94.6–96.5%), *Knoellia* (96.4%), *Terracoccus* (96.4%), *Oryzihumus* (96.4%), *Janibacter* (96.1–96.4%) and *Intrasporangium* (96.2%) were also revealed as phylogenetic neighbours of strain R-Ac013^T.

Temperature (4–42 °C) and pH (4.1–12.1) ranges for growth were determined on ISP 2 medium (Shirling & Gottlieb, 1966). The pH of the medium was adjusted (using increments of 1.0 pH unit) before sterilization. NaCl tolerance for growth was tested at final concentrations of 1–9% (w/v). For these tests, plates were incubated for 5 days at 28 °C. Protease and cellulase activities were determined on ISP 2 medium supplemented with 1% (w/v) skimmed milk and 0.5% (w/v) CM-cellulose (Sigma), respectively. Degradation of hypoxanthine,

Fig. 1. Phylogenetic tree showing the relationships between strain R-Ac013^T and representatives of the family *Intrasporangiaceae*. The tree was constructed, using the neighbour-joining method, from evolutionary distances calculated with the Jukes–Cantor coefficient. Asterisks indicate branches that were also found in the maximum-likelihood and maximum-parsimony trees. Bootstrap percentages (>50%), based on a neighbour-joining analysis of 1000 resampled datasets, are shown at nodes. Bar, 0.01 substitutions per nucleotide position.

tyrosine and xanthine was investigated as described previously (Lee, 2006); the results were recorded after 14 days incubation at 28 °C. The ability to hydrolyse DNA and starch was examined using DNase test agar (Difco) and starch agar (Difco), respectively, and was recorded as positive by the presence of transparent zones when plates were flooded with 1 M HCl and iodine solution, respectively. Gram staining was performed using a Gram 2 kit (bioMérieux) according to the manufacturer's instructions. Oxidase and catalase activities were tested as described previously (Lee, 2006). Tests with API 20NE and API ZYM strips (bioMérieux) were performed according to the manufacturer's instructions. Cell morphology and motility were observed using phase-contrast and transmission electron microscopy, using cells grown on trypticase soy agar (TSA; Difco) for 3 days at 28 °C. To check for the presence of flagella, cells were negatively stained with 1 % (w/v) phosphotungstic acid and observed with a JEOL 1200EXII transmission electron microscope. Colony morphology and pigmentation were usually observed using cells grown on TSA for 7 days at 28 °C. The cells of strain R-Ac013^T were found to comprise Gram-positive, aerobic, catalase-positive, oxidase-negative, non-endospore-forming, non-motile cocci that occurred singly or in pairs (see Supplementary Fig. S1, available in IJSEM Online). Colonies were circular, flat, undulate and bright yellow in colour, reaching 0.6–1.1 mm in diameter after 7 days incubation. Other physiological and biochemical properties are given in the genus/species descriptions.

Cell biomass for chemotaxonomic analyses was obtained from cells grown in trypticase soy broth (Difco) for 3 days at 28 °C. The following chemotaxonomic characteristics of strain R-Ac013^T were determined as described by Lee (2006): the diaminopimelic acid isomer in the peptidoglycan, the acyl type of the murein, the menaquinones, the polar lipids and the presence of any mycolic acids. Strain R-Ac013^T contained LL-DAP as the diagnostic diamino acid in the peptidoglycan. The phospholipids were diphosphatidylglycerol and phosphatidylinositol. The major menaquinone (66 % of the total) was MK-8(H₄); small amounts of MK-9(H₀) (12 %), MK-7(H₀) (12 %) and MK-8(H₂) (8 %) were also detected. The DNA G + C content of strain R-Ac013^T, determined by HPLC (as described by Mesbah *et al.*, 1989), was 74.1 mol%.

The cellular fatty acid composition was determined by using the Sherlock Microbial Identification System (version 6.0; MIDI) according to the manufacturer's instructions. The cells were grown on TSA at 28 °C for 3 days. The fatty acid profile of the organism was characterized by the presence of straight-chain saturated, branched and unsaturated components, as follows (where each of the following constituted >1 % of the total): iso-C_{16:0} (21.2 %), C_{17:1}ω8c (15.5 %), iso-C_{15:0} (13.7 %), C_{15:0} (7.2 %), 10-methyl C_{17:0} (6.5 %), anteiso-C_{17:0} (5.5 %), C_{15:1}ω6c (4.4 %), C_{17:0} (4.1 %), anteiso-C_{15:0} (2.9 %), C_{16:0} (2.8 %), C_{18:0} (2.8 %), C_{16:1} (2.6 %), 10-methyl C_{16:0} (1.8 %), iso-C_{14:0} (1.4 %), C_{17:0} 3-OH (1.3 %),

anteiso-C_{17:1}ω9c (1.1 %), C_{12:1} (1.1 %) and a mixture of C_{16:1}ω7c and/or iso-C_{15:0} 2-OH (1.7 %).

The results of the phylogenetic analyses show that the phylogenetic position of strain R-Ac013^T within the family *Intrasporangiaceae* varies according to the method applied. In terms of 16S rRNA gene sequence similarity, the closest phylogenetic neighbour was *Tetrasphaera japonica* (97.1 % similarity), but it differs significantly from strain R-Ac013^T in that it has *meso*-DAP in its peptidoglycan and has an unknown aminophospholipid and phosphatidylglycerol in its polar lipid profile, but does not have C_{17:1}ω8c and iso-C_{15:0} as its major fatty acids (Table 1). Of the other phylogenetically close neighbours, the other species of the genus *Tetrasphaera* (Hanada *et al.*, 2002; McKenzie *et al.*, 2006) and the genera *Janibacter*, *Knoellia* and *Oryzihumus* are clearly distinguishable from strain R-Ac013^T by the fact that they contain *meso*-DAP as the diagnostic diamino acid and/or from the polar lipid profiles (Table 1). On the other hand, the LL-DAP-containing strain *Intrasporangium calvum* DSM 43043^T (Kalakoutsii *et al.*, 1967; Schumann *et al.*, 1997) differs from the isolate in showing hyphal growth, in having MK-8 as the major menaquinone and in terms of its polar lipid profile. The genus *Terrabacter* (Collins *et al.*, 1989; Montero-Barrientos *et al.*, 2005) comprises long rods or shows a rod/coccus life cycle, whereas the cells of strain R-Ac013^T are coccoid in shape. In addition, the genus *Terrabacter*, together with *Terracoccus luteus*, can be differentiated from strain R-Ac013^T in that they have phosphatidylethanolamine as the diagnostic phospholipid and lack C_{17:1}ω8c as a major fatty acid (Prauser *et al.*, 1997). Differential phenotypic characteristics of strain R-Ac013^T and related taxa in the family *Intrasporangiaceae* are given in Table 1. The results of the chemotaxonomic and phylogenetic analyses strongly suggest that strain R-Ac013^T does not belong to any genera (with validly published names) within the family *Intrasporangiaceae*.

On the basis of the phenotypic and phylogenetic data presented in this study, strain R-Ac013^T represents a novel genus and species within the family *Intrasporangiaceae*, for which the name *Lapillicoccus jejuensis* gen. nov., sp. nov. is proposed.

Description of *Lapillicoccus* gen. nov.

Lapillicoccus (La.pil.li.coc'cus. L. masc. n. *lapillus* a little stone; N.L. masc. n. *coccus* coccus; N.L. masc. n. *Lapillicoccus* a coccus attached to a little stone).

Cells are aerobic, Gram-positive, oxidase-negative, catalase-positive, non-endospore-forming, non-motile cocci (0.2–0.3 µm in diameter) that occur singly or in pairs. The diagnostic diamino acid in the cell wall is LL-DAP. The acyl type of the muramic acid is acetylated. Mycolic acids are not present. MK-8(H₄) is the major menaquinone. Polar lipid profile comprises diphosphatidylglycerol and phosphatidylinositol. Phylogenetically, the genus belongs to the family *Intrasporangiaceae*, suborder *Micrococcineae*. The type species is *Lapillicoccus jejuensis*.

Table 1. Differential characteristics between strain R-Ac013^T and related taxa of the family *Intrasporangiaceae*

Taxa: 1, strain R-Ac013^T (*Lapillicoccus jejuensis* sp. nov.); 2, *Intrasporangium* (data from Kalakoutsii *et al.*, 1967; Schumann *et al.*, 1997; Groth *et al.*, 2001); 3, *Janibacter* (Martin *et al.*, 1997; Yoon *et al.*, 2004; Kämpfer *et al.*, 2006); 4, *Knoellia* (Groth *et al.*, 2002); 5, *Oryzihumus leptocrescens* KV-628^T (Kageyama *et al.*, 2005); 6, *Terrabacter* (Collins *et al.*, 1989; Montero-Barrientos *et al.*, 2005); 7, *Terracoccus* (Prauser *et al.*, 1997); 8, *Tetrasphaera japonica* T1-X7^T (Maszenan *et al.*, 2000). ND, Not determined.

Characteristic	1	2	3	4	5	6	7	8
Cell morphology	Cocci	Hyphae	Cocci or rods	Cocci or rods	Irregular rods	Rods or rod/coccus cycle	Cocci in packets	Cocci
Diamino acid	LL-DAP	LL-DAP	meso-DAP	meso-DAP	meso-DAP	LL-DAP	LL-DAP	meso-DAP
Polar lipids*	DPG, PI	DPG, PG, PI, PIM	DPG, PG, PI	DPG, PE, PG, PI	ND	DPG, PE, PI, PL	DPG, PE, PG, PI	DPG, PG, PI, APL, PL
Major menaquinone	MK-8(H ₄)	MK-8	MK-8(H ₄)	MK-8(H ₄)	MK-8(H ₄)	MK-8(H ₄)	MK-8(H ₄)	MK-8(H ₄)
Major fatty acid(s) (>10% of total)	iso-C _{16:0} , C _{17:1} ω8c, iso-C _{15:0}	iso-C _{15:0} , anteiso-C _{15:0} , iso-C _{16:0}	iso-C _{16:0} , C _{17:1} , C _{18:1} †, C _{15:0} †	iso-C _{15:0} , iso-C _{16:0} , iso-C _{17:0} , anteiso-C _{17:0} , anteiso-C _{17:1} †	iso-C _{16:0} , iso-C _{15:0} , iso-C _{14:0}	iso-C _{15:0} , anteiso-C _{15:0} †, iso-C _{14:0} †, iso-C _{16:0} †	iso-C _{15:0} , anteiso-C _{15:0} , C _{16:0}	iso-C _{16:0} , anteiso-C _{17:0}
DNA G + C content (mol%)	74.1	68	69–73	68–69	72–73	71–73	73	71

*APL, Unknown aminophospholipid; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside; PL, unknown phospholipid.

†May also be present, depending on the species.

Description of *Lapillicoccus jejuensis* sp. nov.

Lapillicoccus jejuensis (je.ju.en'sis. N.L. masc. adj. *jejuensis* of Jeju, Republic of Korea, referring to the site from which the type strain was isolated).

The morphological and chemotaxonomic characteristics are the same as those given in the genus description. Colonies are circular, flat, undulate and bright yellow in colour. Temperature and pH ranges for growth are 20–37 °C and pH 4.1–11.1, with optima at 30 °C and pH 7.1. No growth occurs below 10 °C, above 42 °C or at pH 12.1. Growth occurs in the presence of 0–2 % NaCl, but not with 3 % NaCl. β-Galactosidase is present, but urease and arginine dihydrolase are absent. Nitrate is not reduced to nitrite. Aesculin degradation is observed, but indole production, glucose fermentation and gelatin hydrolysis are not. D-Arabinose, D-mannitol and malate are assimilated or weakly assimilated, but the following substrates are not assimilated: D-glucose, D-mannose, N-acetyl-D-glucosamine, maltose, gluconate, caprate, adipate, citrate and phenylacetate (API 20NE). Protease, cellulase and amylase activities are present. DNA is hydrolysed. Hypoxanthine, tyrosine and xanthine are degraded. In API ZYM tests, the results for leucine arylamidase, acid phosphatase, α-glucosidase and N-acetyl-β-glucosaminidase are positive. Results for esterase lipase (C8) and α-chymotrypsin are weakly positive. Results for alkaline phosphatase, esterase (C4), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucosidase, β-glucuronidase, α-mannosidase and α-fucosidase are negative. Major fatty acids are iso-C_{16:0}

(21.2 %), C_{17:1}ω8c (15.5 %) and iso-C_{15:0} (13.7 %). The DNA G + C content of the type strain is 74.1 mol%.

The type strain, R-Ac013^T (=KCTC 19200^T=DSM 18607^T), was isolated from a small stone collected from an agricultural field in Jeju, Republic of Korea.

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