Kribbia dieselivorans gen. nov., sp. nov., a novel member of the family *Intrasporangiaceae*

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Two Gram-positive, catalase-positive, irregular short rod- or coccoid-shaped bacterial strains, N113^T and R33, were isolated from an enrichment culture with diesel oil-degradation activity and their taxonomic positions were investigated using a polyphasic approach. Phenotypic, phylogenetic and genetic similarities indicated that strains N113^T and R33 were representatives of the same species. Phylogenetic analysis based on 16S rRNA gene sequences showed that strains N113^T and R33 form a lineage independent from those of members of the family *Intrasporangiaceae*. The novel isolates had cell-wall peptidoglycan based on *meso*-diaminopimelic acid, MK-8(H₄) as the predominant menaquinone and 10-methyl-C_{18:0}, iso-C_{16:0}, C_{18:1}ω9c, C_{16:0} and C_{18:0} as the major cellular fatty acids. The DNA G + C contents were 69.6–69.9 mol%. These chemotaxonomic properties, together with phylogenetic distinctiveness, distinguish the two novel strains from recognized members of the family *Intrasporangiaceae*. On the basis of phenotypic, phylogenetic and genetic data, strains N113^T (=KCTC 19143^T = JCM 13585^T) and R33 are classified as representatives of a novel genus and species, *Kribbia dieselivorans* gen. nov., sp. nov., within the family *Intrasporangiaceae*.

The family *Intrasporangiaceae* was proposed as a family of the suborder *Micrococcineae* by Stackebrandt *et al.* (1997) on the basis of 16S rRNA gene sequence analysis and the family currently consists of 11 genera. The family *Intrasporangiaceae* is a phenotypically heterogeneous taxon which can be divided into three groups on the basis of the diagnostic diamino acid in the cell-wall peptidoglycan. The genera *Intrasporangium* (Kalakoutskii *et al.*, 1967), *Terrabacter* (Collins *et al.*, 1989), *Terracoccus* (Prauser *et al.*, 1997) and *Arsenicicoccus* (Collins *et al.*, 2004) contain LL-diaminopimelic acid, the genera *Janibacter* (Martin *et al.*, 1997), *Tetrasphaera* (Maszenan *et al.*, 2000), *Knoellia* (Groth *et al.*, 2002) and *Oryzihumus* (Kageyama *et al.*, 2005) contain *meso*-diaminopimelic acid and the genera *Ornithinicoccus* (Groth *et al.*, 1999), *Ornithinimicrobium* (Groth *et al.*, 2001) and *Serinicoccus* (Yi *et al.*, 2004) contain L-ornithine. In this study, we describe two *meso*-diaminopimelic acid-containing bacterial strains, N113^T and R33, which are phylogenetically related to the family *Intrasporangiaceae*. The aim of the present study was to determine the exact taxonomic

position of strains N113^T and R33 using a polyphasic approach that included analysis of phenotypic properties, detailed phylogenetic analysis based on 16S rRNA gene sequences and genetic relatedness.

Tidal flat sediment collected from Kwangyang, Korea, was used as the source for the isolation of diesel oil-degrading bacteria. Strains N113^T and R33 were isolated from an enrichment culture with diesel oil-degradation activity by dilution plating on 10 × diluted nutrient agar (Difco) and R2A agar (Difco), respectively. To investigate their morphological, physiological and biochemical characteristics, strains N113^T and R33 were routinely cultivated at 30 °C on trypticase soy agar (TSA; Difco). Growth at various temperatures from 4 to 50 °C was measured on TSA. The pH range for growth was determined in nutrient broth (NB; Difco) that was adjusted to various pH values (initial pH 4.5–11.5 at intervals of 0.5 pH units). Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on TSA and on TSA supplemented with nitrate, both of which had been prepared anaerobically using nitrogen. Cell morphology and presence of flagella were examined by light microscopy (E600; Nikon) and transmission electron microscopy by using cells grown on TSA. Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Catalase and oxidase activities and hydrolysis of casein, hypoxanthine, tyrosine, xanthine, starch and

Abbreviation: FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains N113^T and R33 are DQ372707 and DQ062659, respectively.

A supplementary table detailing the cellular fatty acid contents of *Kribbia dieselivorans* sp. nov. strains N113^T and R33 is available in IJSEM Online.

Tweens 20, 40, 60 and 80 were determined as described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin and urea and nitrate reduction were studied as described previously (Lanyi, 1987). Acid production from carbohydrates was determined according to Leifson (1963). Utilization of various substrates for growth was tested as described by Yurkov *et al.* (1994). The API ZYM system (bioMérieux) was used to determine enzyme activity. Other physiological and biochemical tests were performed with the API 20E and API 20NE systems (bioMérieux). Antibiotic sensitivity was tested by spreading bacterial suspension on TSA and applying discs impregnated with the following antibiotics (concentration per disc): ampicillin (10 µg), carbenicillin (100 µg), cephalothin (30 µg), chloramphenicol (100 µg), gentamicin (30 µg), lincomycin (15 µg), kanamycin (30 µg), neomycin (30 µg), novobiocin (5 µg), oleandomycin (15 µg), penicillin G (20 U), polymyxin B (100 U), streptomycin (50 µg) and tetracycline (30 µg).

Strains N113^T and R33 were cultivated for 3 days in trypticase soy broth (Difco) at 30 °C to obtain the cell mass required for isoprenoid quinone and cell wall analyses and DNA extraction. Isoprenoid quinones were analysed as described previously (Komagata & Suzuki, 1987) using reverse-phase HPLC. The isomer type of the cell-wall diamino acid was analysed according to the method of Komagata & Suzuki (1987). For fatty acid methyl ester (FAME) analysis, cell mass of strains N113^T and R33 was harvested from TSA plates after cultivation for 3 days at 30 °C. FAMES were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). Chromosomal DNA was extracted and purified by the procedure described previously (Yoon *et al.*, 1996). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984) with a modification that DNA was hydrolysed and the resultant nucleotides were analysed by reverse-phase HPLC. The 16S rRNA gene amplification was performed according to the method of Yoon *et al.* (1998) using two universal primers. Sequencing of the amplified 16S rRNA gene was performed as described by Yoon *et al.* (2003). Alignment of sequences was carried out with CLUSTAL W (Thompson *et al.*, 1994) and gaps at the 5' and 3' ends of the alignment were omitted from further analysis. The evolutionary distances were calculated using the Kimura two-parameter correction with the CLUSTAL W package (Thompson *et al.*, 1994). A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) on the basis of distance matrix data. The reliability of the grouping was assessed by 1000 bootstrap resamplings of the neighbour-joining dataset by using the CLUSTAL W package. DNA–DNA hybridization was determined by the microplate hybridization method (Ezaki *et al.*, 1989) using photobiotin-labelled DNA probes.

Morphological, cultural, physiological and biochemical characteristics of strains N113^T and R33 are given in the genus and species descriptions (see below) or shown in

Table 1. Strains N113^T and R33 had *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. They contained MK-8(H₄) as the predominant menaquinone. Strains N113^T and R33 had cellular fatty acid profiles that contained large amounts of straight-chain, branched, unsaturated and 10-methyl fatty acids. The major components (>10% of total fatty acids) were 10-methyl-C_{18:0}, iso-C_{16:0}, C_{18:1}ω9c, C_{16:0} and C_{18:0} (see Supplementary Table S1 in IJSEM Online). The DNA G + C contents of strains N113^T and R33 were 69.9 and 69.6 mol%, respectively. The 16S rRNA gene sequences of strains N113^T and R33 determined in this study each comprised 1476 nucleotides and were 99.9% similar (one nucleotide difference). Comparative 16S rRNA gene sequence analyses revealed that strains N113^T and R33 were phylogenetically most closely related to the family *Intrasporangiaceae*. In a phylogenetic tree based on the neighbour-joining algorithm, strains N113^T and R33 formed a distinct phylogenetic lineage within the family *Intrasporangiaceae* (Fig. 1). 16S rRNA gene sequence similarity values between strains N113^T and R33 and genera within the family *Intrasporangiaceae* were as follows: *Janibacter* (95.3–96.0%), *Intrasporangium* (95.6%), *Teracoccus* (95.6%), *Terrabacter* (95.7–95.9%), *Arsenicicoccus* (95.5%), *Knoellia* (94.9–95.0%), *Ornithinicoccus* (94.8%), *Ornithinimicrobium* (94.3%), *Oryzihumus* (94.2%), *Serinicoccus* (94.2%) and *Tetrasphaera* (93.8–94.5%). Strains N113^T and R33 exhibited a mean DNA–DNA relatedness value of 90% when their DNAs were used individually as labelled DNA probes for cross-hybridization, indicating that the two strains are members of the same genomic species (Wayne *et al.*, 1987). In view of their phenotypic, phylogenetic and genetic similarities, strains N113^T and R33 are representatives of the same species.

Strains N113^T and R33 could be clearly distinguished from recognized members of the family *Intrasporangiaceae* by differences in chemotaxonomic properties, particularly by the diamino acid type in position 3 of the peptidoglycan and by fatty acid profiles (Table 1). Strains N113^T and R33 are distinguishable from the genera which contain LL-diaminopimelic acid or L-ornithine as the diagnostic diamino acid (Table 1). The fatty acid profiles, particularly the major fatty acids, distinguish strains N113^T and R33 from recognized genera of the family *Intrasporangiaceae*, although there may be differences in the proportions of some fatty acids probably due to differences in cultivation conditions and extraction procedures (Table 1). The iso-C_{15:0} fatty acid was a minor component in strains N113^T and R33, whereas it was a major component in all genera of the family *Intrasporangiaceae*, except the genus *Janibacter*. The 10-methyl fatty acids were major fatty acids in strains N113^T and R33, but they were absent or present as minor components in other genera of the family *Intrasporangiaceae*. On the basis of the phylogenetic data and differential chemotaxonomic properties reported here, it is suggested that strains N113^T and R33 represent a new genus

Table 1. Differential characteristics of *Kribbia dieselivorans* gen. nov., sp. nov. and genera of the family *Intrasporangiaceae*

Genera: 1, *Kribbia*; 2, *Arsenicicoccus* (data from Collins *et al.*, 2004); 3, *Ornithinimicrobium* (Groth *et al.*, 2001); 4, *Serinicoccus* (Yi *et al.*, 2004); 5, *Janibacter* (Martin *et al.*, 1997; Imamura *et al.*, 2000; Yoon *et al.*, 2000, 2004; Kämpfer *et al.*, 2006); 6, *Knoellia* (Groth *et al.*, 2002); 7, *Tetrasphaera* (Maszenan *et al.*, 2000; Hanada *et al.*, 2002); 8, *Ornithinicoccus* (Groth *et al.*, 1999); 9, *Oryzihumus* (Kageyama *et al.*, 2005); 10, *Intrasporangium* (Kalakoutskii *et al.*, 1967; Schumann *et al.*, 1997; Groth *et al.*, 2001); 11, *Terracoccus* (Prauser *et al.*, 1997); 12, *Terrabacter* (Collins *et al.*, 1989; Montero-Barrientos *et al.*, 2005). L-Orn, L-ornithine; meso-A₂pm, meso-diaminopimelic acid; LL-A₂pm, LL-diaminopimelic acid.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Cell morphology	Irregular short rods or cocci	Cocci	Irregular rods and cocci	Cocci	Coccoid to rod-shaped	Irregular rods and cocci	Cocci or short rods	Cocci	Irregular rods	Hyphae	Cocci	Irregular rods
Cell wall diamino acid	meso-A ₂ pm	LL-A ₂ pm	L-Orn	L-Orn	meso-A ₂ pm	meso-A ₂ pm	meso-A ₂ pm	L-Orn	meso-A ₂ pm	LL-A ₂ pm	LL-A ₂ pm	LL-A ₂ pm
Major menaquinone	MK-8(H ₄)	MK-8(H ₄)	MK-8(H ₄)	MK-8(H ₄)	MK-8(H ₄)	MK-8(H ₄)	MK-8(H ₄)	MK-8(H ₄)	MK-8(H ₄)	MK-8	MK-8(H ₄)	MK-8(H ₄)
Major fatty acid (> 10 % of total fatty acids)	10-methyl-C _{18:0} , iso-C _{16:0} , C _{18:1ω9c} , C _{16:0} , C _{18:0}	C _{16:1ω7c} , iso-C _{15:0} , C _{18:1ω9c} , anteiso-C _{15:0}	iso-C _{16:0} , iso-C _{15:0} , anteiso-C _{15:0}	iso-C _{15:0} , iso-C _{16:0} , iso-C _{17:1ω9c}	iso-C _{16:0} , C _{17:1ω8c} , C _{18:1ω9c} , C _{17:0}	iso-C _{15:0} , iso-C _{17:0} , iso-C _{16:0} , anteiso-C _{17:0}	(anteiso-C _{15:0} , iso-C _{15:0})* or (iso-C _{16:0} , anteiso-C _{17:0} , iso-C _{15:0})† or (iso-C _{16:0} , anteiso-C _{17:0})‡	iso-C _{15:0} , anteiso-C _{15:0}	iso-C _{16:0} , iso-C _{15:0} , iso-C _{14:0}	iso-C _{15:0} , anteiso-C _{15:0} , iso-C _{16:0}	iso-C _{15:0} , anteiso-C _{15:0} , C _{16:0}	(iso-C _{15:0} , anteiso-C _{15:0})\$ or (iso-C _{15:0} , iso-C _{14:0} , iso-C _{16:0})
DNA G+C (mol%)	69–70	72.2	70	72	69–73	68–69	68–71	72	72–73	68.2	73	69.8–72.4

Data for **Tetrasphaera elongata*, †*Tetrasphaera australiensis*, ‡*Tetrasphaera japonica*, \$*Terrabacter terrae* and ||*Terrabacter tumescens*.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of *Kribbia dieselivorans* gen. nov., sp. nov. and some other related taxa. Bar, 0.01 substitutions per nucleotide position.

and novel species within the family *Intrasporangiaceae*, for which the name *Kribbia dieselivorans* gen. nov., sp. nov. is proposed.

Description of *Kribbia* gen. nov.

Kribbia (Kribb.i'a. N.L. fem. n. *Kribbia* arbitrary name formed from the acronym of the Korea Research Institute of Bioscience and Biotechnology, KRIBB, where taxonomic studies of this taxon were performed).

Cells are Gram-positive, non-flagellated, irregular short rods or cocci. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant menaquinone is MK-8(H₄). Major cellular fatty acids (>10% of total fatty acids) are 10-methyl-C_{18:0}, iso-C_{16:0}, C_{18:1}ω9c, C_{16:0} and C_{18:0}. The DNA G+C content is 69–70 mol%. Phylogenetically, the genus is a member of the family *Intrasporangiaceae* within the suborder *Micrococccineae* of the order *Actinomycetales*. The type species is *Kribbia dieselivorans*.

Description of *Kribbia dieselivorans* sp. nov.

Kribbia dieselivorans (die.se.li.vo'rans. N.L. n. *dieselum* diesel; L. v. *vorare* to devour; N.L. part. adj. *dieselivorans* diesel oil-devouring).

Exhibits the following properties in addition to those given in the genus description. Cells are 0.4–0.6 × 1.0–1.5 μm. Catalase-positive, oxidase-negative and urease-negative. Growth occurs between 8 and 42 °C with an optimum temperature of 30 °C. Optimal pH for growth is 6.5–7.5. Anaerobic growth occurs on TSA and on TSA with nitrate.

Casein, gelatin, aesculin and Tweens 20, 40, 60 and 80 are hydrolysed, but starch, hypoxanthine, xanthine and tyrosine are not. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are absent. H₂S and indole are not produced. Nitrate is reduced to nitrite. Acetate, benzoate, D-cellobiose and pyruvate are utilized as sole carbon and energy sources; D-glucose, D-trehalose, sucrose and maltose are weakly utilized. D-Fructose, D-galactose, D-mannose, D-xylose, L-arabinose, citrate, formate, salicin, succinate, L-glutamate and L-malate are not utilized. Acid is produced from D-glucose, D-cellobiose, sucrose, maltose and D-trehalose, but not from D-sorbitol, *myo*-inositol, D-ribose, D-fructose, D-mannitol, melibiose, L-arabinose, D-melezitose, D-galactose, L-rhamnose, lactose or D-raffinose. Susceptible to streptomycin, penicillin G, chloramphenicol, ampicillin, cephalothin, gentamicin, novobiocin, tetracycline, lincomycin, oleandomycin, neomycin and carbenicillin, but not to polymyxin B or kanamycin. In assays with the API ZYM system (bioMérieux), esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase and β-glucosidase are present, but alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. The major cellular fatty acids (>10% of total fatty acids) are 10-methyl-C_{18:0}, iso-C_{16:0}, C_{18:1}ω9c, C_{16:0} and C_{18:0}. The DNA G+C content is 69.6–69.9 mol%.

The type strain, N113^T (= KCTC 19143^T = JCM 13585^T), was isolated from tidal flat sediment from Kwangyang, Korea.

Note added in proof

Another new genus has been described in the *Intrasporangiaceae* with the same diagnostic diamino acid, *Phycococcus jejuensis* (see Lee, 2006, this issue IJSEM, pp. 2369–2373).

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