Luteimicrobium subarcticum gen. nov., sp. nov., a new member of the suborder *Micrococcineae*

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A novel Gram-stain-positive bacterium, designated R19-04^T, was isolated from a soil sample from Rishiri Island, Japan, and its taxonomic position was investigated by a polyphasic approach. Cells of strain R19-04^T exhibited a rod–coccus cycle. The peptidoglycan type of the isolate was A4_α; lysine was the diagnostic diamino acid. The predominant menaquinone was MK-8(H₂) and the major fatty acid was anteiso-C_{15:0}. The polar lipids were diphosphatidylglycerol and phosphatidylglycerol. The DNA G+C content was 72.9 mol%. Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain R19-04^T represented a novel member of the suborder *Micrococcineae*. Its nearest phylogenetic neighbours were the members of the genus *Oerskovia*, with 16S rRNA gene sequence similarity of 97.0–97.1 %. However, strain R19-04^T was clearly distinguishable from members of the genus *Oerskovia* and phylogenetically related genera in terms of chemotaxonomic characteristics. On the basis of the chemotaxonomic and phylogenetic characteristics, a novel genus and species are proposed, *Luteimicrobium subarcticum* gen. nov., sp. nov. The type strain of *Luteimicrobium subarcticum* is R19-04^T (=NBRC 105647^T =DSM 22413^T).

The suborder *Micrococcineae* was first proposed by Stackebrandt *et al.* (1997) and its description was later emended by Zhi *et al.* (2009). This suborder is one of the most studied taxa in the class *Actinobacteria*. While isolating bacteria from subarctic and subtropical islands, we isolated a novel actinobacterium (strain R19-04^T) from a soil sample from Rishiri Island (subarctic), Hokkaido, Japan, using the rehydration and centrifugation method (Hayakawa *et al.*, 2000) and humic acid-vitamin (HV) agar (Hayakawa & Nonomura, 1987). Comparative 16S rRNA gene sequence analysis revealed that the isolate is a member of the suborder *Micrococcineae*. The objective of this study was to determine the taxonomic position of strain R19-04^T by using a polyphasic approach.

Maltose-Bennett's medium [NBRC medium 231; containing 0.1 % yeast extract (Difco), 0.1 % beef extract (Difco), 0.2 % NZ Amine type A (Wako), 1.0 % maltose; 2.0 % agar if required; pH 6.0] was used for general laboratory cultivation, morphological studies and determination of optimal growth parameters. Colony appearance and pigment production were examined following growth at 28 °C for 3 days on several culture media: NBRC medium

231, R2A agar (Difco), trypticase soy agar (TSA; Difco), brain heart infusion agar (BHI; Difco) and Todd–Hewitt broth (Difco) supplemented with 1.5 % agar. Cell morphology was investigated by examining cells from cultures over a whole growth cycle (up to 5 days) under a light microscope (BX-51; Olympus) and a scanning electron microscope (JSM-6060; JEOL). Cell motility was determined by observing cells suspended in saline solution under a light microscope. Growth under anaerobic conditions was determined by incubating in an anaerobic chamber with an O₂-absorbing and CO₂-generating agent (AnaeroPack; Mitsubishi Gas Chemical). Growth parameters (temperature, pH and NaCl tolerance) were determined by measuring the turbidity (at 610 nm) of 5 ml of the culture medium in test tubes after 1–2 days of incubation at 28 °C. The temperature range and optimum temperature for growth were determined by incubating the cultures at 5, 10, 15, 20, 25, 28, 35, 45 and 60 °C. The pH range and optimum pH for growth were established by using a medium adjusted to pH 4–10 with either 4 M HCl or 5 M KOH and incubation at 28 °C. NaCl tolerance and optimum concentration (1, 3, 5, 7, 10 and 15 %, w/v) were determined at 28 °C. Gram staining was performed by using Hucker's modification (Gerhardt *et al.*, 1994). DNase test agar (Difco) was used for the DNase assay. Other physiological and biochemical tests were performed using

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain R19-04^T is AB489904.

API ZYM, API Coryne, API 20E and API 50CH systems (bioMérieux) according to the manufacturer's instructions. Biomass for chemotaxonomic and molecular systematic studies was prepared by growing the strain in shake flasks containing NBRC medium 231 for 48 h at 28 °C at 100 r.p.m.

Strain R19-04^T formed yellow, circular, rough colonies that were approximately 0.2 mm in diameter on NBRC medium 231 after 3 days incubation at 28 °C. Growth also occurred on R2A, TSA, BHI and Todd-Hewitt agar. Pigment production was not observed. The cells of the strain were Gram-stain-positive, aerobic, non-motile and non-sporulating and exhibited a rod-coccus cycle. Cells of exponentially growing cultures were irregular rods (0.5–0.7 × 1.0–3.0 µm), while stationary-phase cells were cocci (approximately 0.7 µm in diameter). Growth occurred at 10–35 °C and no growth was observed at 5, 45 or 60 °C. The pH range for growth was pH 5.0–9.0. Optimal growth was noted at 28 °C and pH 6.0. Strain R19-04^T exhibited good growth with NaCl concentrations of 0–3 % and moderate growth with 5 %; no growth was observed with 7, 10 or 15 % NaCl. Optimal growth was noted with 1 % NaCl. Growth under anaerobic conditions was not observed. The results of other physiological and biochemical analyses have been summarized in the species description.

Cell-wall samples were prepared from approximately 1 g wet cells by mechanical disruption with an ultrasonic oscillator and were purified as described by Schleifer & Kandler (1972). The amino acids in cell-wall hydrolysates were analysed using the methods described by Tamura *et al.* (1994). The amino acid isomers in cell-wall hydrolysates were examined using the method described by Nozawa *et al.* (2007) with the following modifications. Separation of 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide and 1-fluoro-2,4-dinitrophenyl-5-D-leucinamide derivatives of amino acids was performed using a model LC-20AB HPLC apparatus (Shimadzu) equipped with a Wakopak Wakosil PTC column (Wako). Cellular fatty acids were processed and analysed as methyl esters, using the protocol of the MIDI Sherlock Microbial Identification System (Sasser, 1990). Menaquinones were extracted from a 300 mg freeze-dried cell sample and analysed according to the method described by Tamura *et al.* (1994). Polar lipids and the DNA G+C content were also analysed as described by Tamura *et al.* (1994).

Purified peptidoglycan of strain R19-04^T contained alanine, glutamic acid and lysine in a molar ratio of 2.0:2.0:0.7. Enantiomeric analysis of the peptidoglycan amino acids revealed the presence of D-Ala, L-Ala, D-Glu and L-Lys. These data indicated that the cell-wall peptidoglycan of strain R19-04^T is of the A4 α type (Schleifer & Kandler, 1972) with an L-Lys←D-Glu interpeptide bridge. The major cellular fatty acid was anteiso-C_{15:0} (71.4 %), while minor components were C_{16:0} (10.9 %), anteiso-C_{17:0} (8.0 %), iso-C_{16:0} (5.1 %),

iso-C_{15:0} (2.3 %), C_{14:0} (1.3 %) and iso-C_{14:0} (1.0 %). The polar lipids were diphosphatidylglycerol and phosphatidylglycerol. Glycolipids were not detected. The predominant menaquinone was MK-8(H₂); MK-9(H₂) was present as a minor component. The DNA G+C content was 72.9 mol%.

PCR amplification of the 16S rRNA gene of strain R19-04^T was performed as described by Tamura & Hatano (2001). This gene was directly sequenced using a BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems) and an automatic DNA sequencer (ABI Prism 3730 Genetic Analyzer; Applied Biosystems). The almost-complete 16S rRNA gene sequence determined in this study was aligned with reference sequences of the genera *Oerskovia* (Prauser *et al.*, 1970) and *Cellulosimicrobium* (Schumann *et al.*, 2001) and some related taxa by using the CLUSTAL X program (Thompson *et al.*, 1997). A phylogenetic tree was constructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood methods (Felsenstein, 1981). The resultant neighbour-joining tree topology was evaluated by bootstrap analysis based on 1000 replicates (Felsenstein, 1985).

Phylogenetic analysis performed on the basis of the 16S rRNA gene sequence revealed that strain R19-04^T belonged to the suborder *Micrococcineae*. However, strain R19-04^T did not form a reliable clade with any members of the suborder *Micrococcineae*. The position of strain R19-04^T relative to its phylogenetic neighbours is shown in Fig. 1. The highest 16S rRNA gene sequence similarity values were obtained with members of the genera *Oerskovia* (97.0–97.1 %), *Cellulosimicrobium* (96.3–97.1 %), *Cellulomonas* (95.1–96.7 %) and *Isoptericola* (95.6–96.3 %).

The predominant menaquinone of strain R19-04^T was different from those of phylogenetically related taxa. The predominant menaquinone of the isolate was MK-8(H₂). In contrast, the predominant menaquinone of the genera *Oerskovia*, *Cellulosimicrobium*, *Cellulomonas* and *Isoptericola* is reported to be MK-9(H₄). In addition, strain R19-04^T possessed a peptidoglycan interpeptide bridge comprising L-Lys←D-Glu. This structure has not been reported for members of the genera *Oerskovia* and *Cellulosimicrobium*. These data indicated that the chemotaxonomic characteristics of strain R19-04^T were distinct from those of its phylogenetic neighbours (Table 1). On the basis of the chemotaxonomic and phylogenetic characteristics, strain R19-04^T represents a novel genus and species, for which the name *Luteimicrobium subarcticum* gen. nov., sp. nov. is proposed.

The results of the phylogenetic analysis of the 16S rRNA gene sequence suggested that the genus *Luteimicrobium* is related to the families *Cellulomonadaceae* and *Promicromonosporaceae*. However, the new genus did not form a reliable clade with any members of these families. Hence, the genus *Luteimicrobium* cannot be accommodated in either of these families at this time. Further studies are required in order to elucidate the taxonomic position of the new genus.

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequences of strain R19-04^T and its taxonomic neighbours, constructed with the neighbour-joining method. The sequence of *Brevibacterium linens* DSM 20425^T (GenBank accession no. X77451) was used as the outgroup (not shown). Bootstrap values (>50%) based on 1000 replicates are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-likelihood algorithm. Bar, 0.01 substitutions per nucleotide position.

Table 1. Differential chemotaxonomic characteristics of strain R19-04^T and the genera *Oerskovia*, *Cellulosimicrobium*, *Cellulomonas* and *Isoptericola*

Data for reference genera were taken from previous studies (Schumann *et al.*, 2001; Stackebrandt *et al.*, 2004, 2006; Yoon *et al.*, 2006, 2007). ND, No data available.

Characteristic	R19-04 ^T	<i>Oerskovia</i>	<i>Cellulosimicrobium</i>	<i>Cellulomonas</i>	<i>Isoptericola</i>
Peptidoglycan type	A4 α	A4 α	A4 α	A4 β	A4 α
Diamino acid	L-Lys	L-Lys	L-Lys	L-Orn	L-Lys
Interpeptide bridge	L-Lys-D-Glu	L-Thr-D-Asp or L-Thr-D-Glu	D-Ser-D-Asp or L-Thr-D-Asp	D-Asp or D-Glu	D-Asp or D-Glu
Major menaquinone(s)	MK-8(H ₂)	MK-9(H ₄)	MK-9(H ₄)	MK-9(H ₄), MK-8(H ₄)	MK-9(H ₄)
Major fatty acid(s)*	ai-C _{15:0}	ai-C _{15:0} , C _{16:0}	ai-C _{15:0} , i-C _{15:0}	ai-C _{15:0} , C _{14:0}	ai-C _{15:0} , i-C _{15:0}
Polar lipids†	PG, DPG	PG, DPG, PI	ND	PG, DPG, PI, PGL	PG, DPG, PI, PL
DNA G+C content (mol%)	72.9	70.5–75.0	72.9–74.5	69–76	70–74

*ai, Anteiso-branched; i, iso-branched.

†DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PGL, unidentified phosphoglycerolipid; PI, phosphatidylinositol; PL, unidentified phospholipid.

Description of *Luteimicrobium* gen. nov.

Luteimicrobium (Lu.te.i.mi.cro'bi.um. L. adj. *luteus* yellow; N.L. neut. n. *microbium* microbe; N.L. neut. n. *Luteimicrobium* a yellow microbe).

Cells exhibit a rod-coccus cycle and are Gram-stain-positive, aerobic and non-sporulating. The cell-wall peptidoglycan is of the A4 α type. The predominant menaquinone is MK-8(H₂) and the major polar lipids are diphosphatidylglycerol and phosphatidylglycerol. The major cellular fatty acid is anteiso-C_{15:0}. Phylogenetically, the genus *Luteimicrobium* belongs to the suborder *Micrococcineae* within the order *Actinomycetales*. The type species is *Luteimicrobium subarcticum*.

Description of *Luteimicrobium subarcticum* sp. nov.

Luteimicrobium subarcticum (sub.arc'ti.cum. L. prep. *sub* low, below, under; L. neut. adj. *arcticum* northern, arctic; N.L. neut. adj. *subarcticum* subarctic, referring to the isolation of the type strain from the subarctic Rishiri Island).

Displays the following properties in addition to those given for the genus. Rods are 0.5–0.7 $\times$ 1.0–3.0 μ m and cocci are approximately 0.7 μ m in diameter. Cells are non-motile. Colonies are circular, rough and yellow. Catalase-positive and oxidase-negative. Growth occurs with NaCl concentrations of 0–5 % but not with 7, 10 or 15 %; optimal NaCl concentration is 1 %. Grows at 10–35 °C (optimum 28 °C) and pH 5.0–9.0 (optimum pH 6.0). Acid is produced from *N*-acetylglucosamine, amygdalin, *L*-arabinose, arbutin, cellobiose, *D*-fructose, *D*-galactose, gentiobiose, *D*-glucose, maltose, *D*-mannose, sucrose and *D*-xylose. Leucine arylamidase, β -galactosidase, α - and β -glucosidases and *N*-acetyl- β -glucosaminidase are present, but alkaline phosphatase, esterase lipase (C8), lipase (C4), valine arylamidase, cysteine arylamidase, trypsin, chymotrypsin, acid phosphatase, phosphohydrolase, α -galactosidase, β -glucuronidase, α -mannosidase, α -fucosidase, pyrazinamidase, pyrrolidonyl arylamidase, arginine dihydrolase, lysine and ornithine decarboxylases and tryptophan deaminase are absent. H₂S and indole are not produced. Aesculin is hydrolysed, but gelatin, urea and DNA are not hydrolysed. Nitrate is reduced. The interpeptide bridge of the cell-wall peptidoglycan is *L*-Lys←*D*-Glu. MK-9(H₂) is present as a minor component. C_{16:0}, anteiso-C_{17:0}, iso-C_{16:0}, iso-C_{15:0}, C_{14:0} and iso-C_{14:0} are found in minor amounts. The DNA G + C content of the type strain is 72.9 mol%.

The type strain R19-04^T (=NBRC 105647^T =DSM 22413^T) was isolated from a soil sample from Rishiri Island, Japan.

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References

- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 738–791.
- Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (editors) (1994). *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.
- Hayakawa, M. & Nonomura, H. (1987). Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. *J Ferment Technol* **65**, 501–509.
- Hayakawa, M., Ootoguro, M., Takeuchi, T., Yamazaki, T. & Iimura, Y. (2000). Application of a method incorporating differential centrifugation for selective isolation of motile actinomycetes in soil and plant litter. *Antonie van Leeuwenhoek* **78**, 171–185.
- Nozawa, Y., Sakai, N., Arai, K., Kawasaki, Y. & Harada, K. (2007). Reliable and sensitive analysis of amino acids in the peptidoglycan of actinomycetes using the advanced Marfey's method. *J Microbiol Methods* **70**, 306–311.
- Prauser, H., Lechevalier, M. P. & Lechevalier, H. A. (1970). Description of *Oerskovia* gen. n. to harbor Ørskov's motile *Nocardia*. *Appl Microbiol* **19**, 534.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sasser, M. (1990). *Identification of bacteria by gas chromatography of cellular fatty acids*, MIDI Technical Note 101. Newark, DE: MIDI Inc.
- Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.
- Schumann, P., Weiss, N. & Stackebrandt, E. (2001). Reclassification of *Cellulomonas cellulans* (Stackebrandt and Keddie 1986) as *Cellulosimicrobium cellulans* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **51**, 1007–1010.
- Stackebrandt, E., Rainey, F. A. & Ward-Rainey, N. L. (1997). Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *Int J Syst Bacteriol* **47**, 479–491.
- Stackebrandt, E., Schumann, P. & Cui, X.-L. (2004). Reclassification of *Cellulosimicrobium variabilis* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **54**, 685–688.
- Stackebrandt, E., Schumann, P. & Prauser, H. (2006). The family *Cellulomonadaceae*. In *The Prokaryotes: a Handbook on the Biology of Bacteria*, 3rd edn, vol. 3, pp. 983–1001. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. New York: Springer.
- Tamura, T. & Hatano, K. (2001). Phylogenetic analysis of the genus *Actinoplanes* and transfer of *Actinoplanes minutisporangius* Ruan *et al.* 1986 and '*Actinoplanes aurantiacus*' to *Cryptosporangium minutisporangium* comb. nov. and *Cryptosporangium aurantiacum* sp. nov. *Int J Syst Evol Microbiol* **51**, 2119–2125.
- Tamura, T., Nakagaito, Y., Nishi, T., Hasegawa, T., Stackebrandt, E. & Yokota, A. (1994). A new genus of the order *Actinomycetales*, *Couchioplanes* gen. nov., with descriptions of *Couchioplanes caeruleus* (Horan and Brodsky 1986) comb. nov. and *Couchioplanes caeruleus* subsp. *azureus* subsp. nov. *Int J Syst Bacteriol* **44**, 193–203.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Yoon, J.-H., Schumann, P., Kang, S.-J., Jung, S.-Y. & Oh, T.-K. (2006). *Isoptericola dokdonensis* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **56**, 2893–2897.

Yoon, J.-H., Kang, S.-J., Schumann, P. & Oh, T.-K. (2007). *Cellulosimicrobium terreum* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **57**, 2493–2497.

Zhi, X.-Y., Li, W.-J. & Stackebrandt, E. (2009). An update of the structure and 16S rRNA gene sequence-based definition of higher ranks of the class *Actinobacteria*, with the proposal of two new suborders and four new families and emended descriptions of the existing higher taxa. *Int J Syst Evol Microbiol* **59**, 589–608.