Kocuria gwangalliensis sp. nov., an actinobacterium isolated from seawater

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A novel aerobic, non-motile, Gram-positive, pink–orange-pigmented, coccoid marine bacterium, designated strain SJ2^T, was isolated from the Gwangalli coast of Korea. The taxonomic position of strain SJ2^T was determined based on 16S rRNA gene sequence analysis, fatty acid patterns and physiological reaction profiles. The full-length 16S rRNA gene sequence of strain SJ2^T showed highest similarity to those of the type strains of *Kocuria carniphila* and *Kocuria marina*. Strain SJ2^T exhibited mean levels of DNA–DNA relatedness of 17 and 35 % to the type strains of *K. carniphila* and *K. marina*, respectively. Based on these results, strain SJ2^T is considered to represent a novel species of the genus *Kocuria*, for which the name *Kocuria gwangalliensis* sp. nov. is proposed. The type strain is SJ2^T (=KCCM 42914^T =LMG 24672^T).

Members of the genus *Kocuria* are Gram-positive, coccoid, non-endospore-forming, aerobic bacteria. At the time of writing, the genus comprises 12 recognized species (Zhou *et al.*, 2008). Phylogenetically, the genus belongs to the family *Micrococcaceae*, class *Actinobacteria*. Here we describe the results of phenotypic and phylogenetic analyses of a novel marine bacterium and show that this strain represents a novel species of the genus *Kocuria*.

A pink–orange-pigmented bacterial strain, SJ2^T, was isolated from seawater collected on the Gwangalli coast of Korea. The strain was isolated on nutrient agar medium (Difco) and was maintained on PPES-II medium [containing (per litre distilled water) 1 g Bacto tryptone, 1 g Bacto soytone, 1 g Bacto yeast extract (all Difco), 0.01 g ferric citrate, 2 g polypeptone and 30 g NaCl (pH 6.8)] by serial inoculation. Cells of strain SJ2^T were Gram-positive,

spherical (0.6–1.2 µm in diameter) and occurred in pairs. Cells were non-motile and non-spore-forming. Colonies grown on agar were smooth, convex and pink–orange.

Cultured cells were suspended in 0.1 M phosphate buffer (pH 7.2). Cells were fixed with 2 % glutaraldehyde, washed with 0.05 M cacodylate buffer and post-fixed with 1 % osmium tetroxide. Fixed cells were dehydrated in ethanol, and the ethanol was replaced with propylene oxide before embedding in Epon resin and sectioning with an ultramicrotome. Sections were examined with a JEM 1200EX-II transmission electron microscope (see Supplementary Fig. S1 in IJSEM Online).

Strain SJ2^T was cultivated for 2 days at 25 °C on modified trypticase soy agar (2 % NaCl added) adjusted to pH 8.0. Harvesting, saponification, methylation and extraction of fatty acid methyl esters were performed according to Sasser (1990). Fatty acid methyl esters were identified by GC-MS, as described by Lipski & Altendorf (1997). Menaquinone profiles were examined as described by Li *et al.* (2004).

The fatty acids of strain SJ2^T were anteiso-C_{15:0} (65.2 % of the total), anteiso-C_{17:0} (11.4 %), C_{16:0} (7.6 %), iso-C_{16:0} (4.6 %), iso-C_{15:0} (2.5 %), iso-C_{14:0} (2.0 %), C_{14:0} (2.0 %),

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SJ2^T is EU286964.

A transmission electron micrograph of cells of strain SJ2^T and maximum-parsimony tree based on 16S rRNA gene sequences showing the phylogenetic position of strain SJ2^T among members of the genus *Kocuria* are available as supplementary material with the online version of this paper.

C_{18:0} (1.4%), C_{16:1} (1.3%), iso-C_{17:0} (0.4%), C_{18:3 ω 6c} (0.3%), anteiso-C_{13:0} (0.2%) and C_{18:1 ω 9c} (0.2%). Strain SJ2^T contained menaquinones MK-7(H₂) and MK-6(H₂) at a peak area ratio of 75:21.

Strain SJ2^T was grown on PPES-II medium and subjected to physiological characterization. The temperature range for growth was determined by incubating cells for 3 days on nutrient agar plates at 4, 10, 15, 20, 25, 27, 30, 32, 37 and 42 °C. The pH range for growth was determined by incubating cells in PPES-II broth medium at 27 °C for 3 days at pH 4.0, 5.0, 5.5, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0. NaCl tolerance was measured in trypticase soy broth, with NaCl at concentrations of 0–15% (w/v). Motility was determined via light microscopy by using the hanging-drop technique (Skerman, 1967). To investigate morphological and physiological characteristics, strain SJ2^T was cultivated at 27 °C on trypticase soy agar or trypticase soy broth with 3% NaCl added. Acid production from carbohydrates was detected by using the method of Hugh & Leifson (1953). Growth was monitored by measuring turbidity after 5 days incubation at 27 °C with shaking. Starch hydrolysis was determined as described by Cowan & Steel (1965). Catalase activity was determined based on the presence of bubbles in a 3% H₂O₂ solution. Oxidase activity was determined based on oxidation of 1% *p*-aminodimethylaniline oxalate. Urease production was determined as described by Lányi (1987).

Strain SJ2^T grew at 10–37 °C, but not at 4 or 42 °C. Optimal growth was at 27 °C. Growth was observed in the presence of 0–7% NaCl, with optimum growth at 4% NaCl. The optimal pH for growth was pH 8. Growth was very slow below pH 5 and was inhibited at pH 11. Acid was produced from D-fructose, D-galactose, maltose, D-mannitol, D-mannose, melibiose and trehalose, but not from N-acetylglucosamine, D-arabinose, cellobiose, D-glucose, lactose, methyl α -D-glucoside, pullulan, pyruvate, raffinose, sucrose, xylitol or D-xylose. Starch was not hydrolysed. β -Galactosidase and urease activities were not detected. No indole was produced from tryptophan. The citrate utilization test was negative. L-Lysine dihydrolase and L-ornithine dihydrolase were detected. The methyl red test was positive but the Voges–Proskauer test was negative. Strain SJ2^T was catalase-positive but oxidase-negative. Glucose and lactose were not fermented. Metabolism was aerobic.

Extraction of genomic DNA and amplification of the 16S rRNA gene were carried out as described by Rainey *et al.* (1992). The PCR product was ligated into pGEM-T vector (Promega) and sequenced by using a termination sequencing ready reaction kit (Perkin Elmer) and sequencing reaction products were separated by electrophoresis by using an ABI 377 genetic analyser (Perkin Elmer). The resulting sequence data were analysed with the programs BLASTN and BLASTX from GenBank. Sequences were aligned by using the program CLUSTAL X. Positions with gaps in the alignment were omitted from further analyses. A phylo-

genetic tree was constructed with the neighbour-joining method (Fig. 1) by using MEGA version 4.01 (Tamura *et al.*, 2007). A phylogenetic tree was also constructed via the maximum-parsimony method by using MEGA version 4.01 (Supplementary Fig. S2). The topology of the neighbour-joining phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) based on 1000 replications. On the basis of 16S rRNA gene sequence analysis, strain SJ2^T was related most closely to the type strains of species of the genus *Kocuria* (96.1–99.3% similarity). The full-length 16S rRNA gene sequence of strain SJ2^T shared highest similarity with that of the type strain of *Kocuria carniphila* (99.3%) (Tvrzová *et al.*, 2005).

The G+C content of the genomic DNA was determined according to the method of Tamaoka & Komagata (1984). The DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically according to the method of Ezaki *et al.* (1989) by using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded, and the mean of the remaining three values is quoted as the level of DNA–DNA relatedness. The DNA G+C content of strain SJ2^T was 65.2 mol% (by HPLC analysis). DNA–DNA hybridization was performed to determine the genomic relatedness between strain SJ2^T and the type strains of three *Kocuria* species. Strain SJ2^T exhibited mean levels of DNA–DNA relatedness of 17.3% (repetition 18.5%), 35.1% (repetition 36.2%) and 21.3% (repetition 20.5%) to *K. carniphila* CCM 132^T, *Kocuria marina* KMM 3905^T (Kim *et al.*, 2004) and *Kocuria rosea* DSM 20447^T, respectively, when each of their DNAs was used separately as a labelled DNA probe. Levels of DNA–DNA relatedness between strain SJ2^T and the type strains of other *Kocuria* species were not determined as it has been shown that organisms

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequence analysis of strain SJ2^T among members of the genus *Kocuria*. Numbers at branch nodes are bootstrap percentages (based on 1000 resamplings). The sequence of *Micrococcus luteus* DSM 20030^T was used as an outgroup. Bar, 0.5% sequence divergence.

showing more than 3% 16S rRNA gene sequence dissimilarity generally belong to different genomic species (Stackebrandt & Goebel, 1994). Phenotypic properties that can be used to differentiate strain SJ2^T from other *Kocuria* species are summarized in Table 1.

On the basis of the data presented, strain SJ2^T is considered to represent a novel species of the genus *Kocuria*, for which the name *Kocuria gwangalliensis* sp. nov. is proposed.

Description of *Kocuria gwangalliensis* sp. nov.

Kocuria gwangalliensis (gwan.gal.li.en'sis. N.L. fem. adj. *gwangalliensis* referring to Gwangalli beach in Busan, Korea, where the type strain was isolated).

Cells are Gram-positive, non-motile, spherical (0.6–1.2 µm in diameter), non-spore-forming and occur in pairs. Colonies are pink–orange pigmented on nutrient agar. Grows at 10–37 °C, but not at 4 or 42 °C. The optimal growth temperature is 27 °C. The NaCl concentration

range for growth is 0–7%. No growth occurs in the presence of more than 10% (w/v) NaCl. The optimal pH for growth is pH 8. The major fatty acid is anteiso-C_{15:0}. Acid is produced from D-fructose, D-galactose, maltose, D-mannitol, D-mannose, melibiose and trehalose, but not from N-acetylglucosamine, D-arabinose, cellobiose, D-glucose, lactose, methyl α-D-glucoside, pullulan, pyruvate, raffinose, sucrose, xylitol or D-xylose. β-Galactosidase and urease activities are not detected. L-Lysine dihydrolase and L-ornithine dihydrolase are detected. The citrate utilization test is negative. Starch is not hydrolysed. No indole is produced from tryptophan. Nitrate is reduced to nitrite. Catalase-positive but oxidase-negative. Glucose and lactose are not fermented. Metabolism is aerobic. The major isoprenoid quinones are MK-7(H₂) and MK-6(H₂). The DNA G+C content of the type strain is 65.2 mol%.

The type strain, SJ2^T (=KCCM 42914^T =LMG 24672^T), was isolated from the Gwangalli coast of Korea.

Table 1. Differential phenotypic properties between strain SJ2^T and members of the genus *Kocuria*

Strains: 1, SJ2^T; 2, *K. carniphila* CCM 132^T; 3, *K. marina* KMM 3905^T; 4, *K. rosea* DSM 20447^T (data for 1–4 are from the present study); 5, *K. kristinae* DSM 20032^T (Stackebrandt *et al.*, 1995); 6, *K. palustris* DSM 11925^T (Kovács *et al.*, 1999); 7, *K. polaris* MTCC3702^T (Reddy *et al.*, 2003); 8, *K. rhizophila* DSM 11926^T (Kovács *et al.*, 1999); 9, *K. varians* DSM 20033^T (Stackebrandt *et al.*, 1995); 10, *K. aegyptia* YIM 70003^T (Li *et al.*, 2006); 11, *K. flava* HO-9041^T (Zhou *et al.*, 2008); 12, *K. turfanensis* HO-9042^T (Zhou *et al.*, 2008); 13, *K. himachalensis* K07-05^T (Mayilraj *et al.*, 2006). +, Positive; –, negative; w, weak; v, variable; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Major pigment colour*	P–O	Y	O	P/R	PO	PY	Y	O	Y	P	Y	O–R	RO
Oxygen requirement†	A	A	A	A	FAN	A	A	A	A	A	A	A	A
Citrate utilization	–	–	+	–	ND	ND	+	ND	+	ND	ND	ND	–
Acid production from:													
D-Arabinose	–	–	–	+	+	–	–	–	–	–	ND	ND	–
D-Glucose	–	+	–	+	+	+	+	+	v	–	ND	ND	+
Lactose	–	+	–	–	–	+	–	–	+	–	ND	ND	–
Maltose	+	+	–	+	+	–	+	–	–	–	ND	ND	+
D-Mannitol	+	–	–	+	–	–	+	–	–	ND	ND	ND	+
D-Mannose	+	–	–	–	+	–	–	+	–	–	ND	ND	+
Melibiose	+	–	–	+	+	ND	+	ND	+	ND	ND	ND	–
Sucrose	–	+	–	–	+	+	–	+	–	ND	ND	ND	–
Trehalose	+	–	–	–	+	–	w	–	–	–	ND	ND	+
D-Xylose	–	–	–	–	–	–	+	–	–	ND	ND	ND	–
Hydrolysis of:													
Starch	–	–	+‡	–	–	–	+	–	v	–	ND	ND	–
Urea	–	–	+	–	v	+	–	–	+	–	+	–	–
β-Galactosidase	–	+	+	–	–	+	–	–	+	–	w	+	ND
Major menaquinone(s)	7(H ₂), 6(H ₂)	7(H ₂)	8(H ₂), 7(H ₂)	8(H ₂)	7(H ₂), 8(H ₂)	7(H ₂)	7(H ₂), 8(H ₂)	7(H ₂), 8(H ₂)	7(H ₂)	8(H ₂), 9(H ₂)	8(H ₂), 9(H ₂)	8(H ₂), 9(H ₂)	8(H ₂)
DNA G+C content (mol%)	65.2	71.0	60.0	70.2	67.0	69.6	72.5	69.4	66–72§	73.0	71.0	65.0	75.3

*O, Orange; P, pink; PO, pale orange; PY, pale yellow; R, red; RO, reddish orange; Y, yellow.

†A, Aerobic; FAN, facultatively anaerobic.

‡Reported as negative by Kim *et al.* (2004).

§Range of values for a number of strains including the type strain.

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References

- Cowan, S. T. & Steel, K. J. (1965). *Manual for the Identification of Medical Bacteria*. London: Cambridge University Press.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Hugh, R. & Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J Bacteriol* **66**, 24–26.
- Kim, S. B., Nedashkovskaya, O. I., Mikhailov, V. V., Han, S. K., Kim, K.-O., Rhee, M. S. & Bae, K. S. (2004). *Kocuria marina* sp. nov., a novel actinobacterium isolated from marine sediment. *Int J Syst Evol Microbiol* **54**, 1617–1620.
- Kovács, G., Burghardt, J., Pradella, S., Schumann, P., Stackebrandt, E. & Märialigeti, K. (1999). *Kocuria palustris* sp. nov. and *Kocuria rhizophila* sp. nov., isolated from the rhizoplane of the narrow-leaved cattail (*Typha angustifolia*). *Int J Syst Bacteriol* **49**, 167–173.
- Lányi, B. (1987). Classical and rapid identification methods for medically important bacteria. *Methods Microbiol* **19**, 1–67.
- Li, W.-J., Chen, H.-H., Xu, P., Zhang, Y.-O., Schumann, P., Tang, S.-K., Xu, L.-H. & Jiang, C.-L. (2004). *Yania halotolerans* gen. nov., sp. nov., a novel member of the suborder *Micrococcineae* from saline soil in China. *Int J Syst Evol Microbiol* **54**, 525–531.
- Li, W.-J., Zhang, Y.-Q., Schumann, P., Chen, H.-H., Hozzein, W. N., Tian, X.-P., Xu, L.-H. & Jiang, C.-L. (2006). *Kocuria aegyptia* sp. nov., a novel actinobacterium isolated from a saline, alkaline desert soil in Egypt. *Int J Syst Evol Microbiol* **56**, 733–737.
- Lipski, A. & Altendorf, K. (1997). Identification of heterotrophic bacteria isolated from ammonia-supplied experimental biofilters. *Syst Appl Microbiol* **20**, 448–457.
- Mayilraj, S., Kroppenstedt, R. M., Suresh, K. & Saini, H. S. (2006). *Kocuria himachalensis* sp. nov., an actinobacterium isolated from the Indian Himalayas. *Int J Syst Evol Microbiol* **56**, 1971–1975.
- Rainey, F. A., Dorsch, M., Morgan, H. W. & Stackebrandt, E. (1992). 16S rDNA analysis of *Spirochaeta thermophila*: its phylogenetic position and implications for the systematics of the order *Spirochaetales*. *Syst Appl Microbiol* **15**, 197–202.
- Reddy, G. S. N., Prakash, J. S. S., Prabakar, V., Matsumoto, G. I., Stackebrandt, E. & Shivaji, S. (2003). *Kocuria polaris* sp. nov., an orange-pigmented psychrophilic bacterium isolated from an Antarctic cyanobacterial mat sample. *Int J Syst Evol Microbiol* **53**, 183–187.
- Sasser, M. (1990). Identification of bacteria through fatty acid analysis. In *Methods in Phytobacteriology*, pp. 199–204. Edited by Z. Klement, K. Rudolph & D. C. Sands. Budapest: Akademiai Kiado.
- Skerman, V. B. D. (1967). *A Guide to the Identification of the Genera of Bacteria*, 2nd edn. Baltimore: Williams & Wilkins.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Stackebrandt, E., Koch, C., Gvozdiak, O. & Schumann, P. (1995). Taxonomic dissection of the genus *Micrococcus*: *Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. *Int J Syst Bacteriol* **45**, 628–692.
- Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Tvrzová, L., Schumann, P., Sedláček, I., Páčová, Z., Spröer, C., Verburg, S. & Kroppenstedt, R. M. (2005). Reclassification of strain CCM 132, previously classified as *Kocuria varians*, as *Kocuria carniphila* sp. nov. *Int J Syst Evol Microbiol* **55**, 139–142.
- Zhou, G., Luo, X., Tang, Y., Zhang, L., Yang, Q., Qiu, Y. & Fang, C. (2008). *Kocuria flava* sp. nov. and *Kocuria turfanensis* sp. nov., airborne actinobacteria isolated from Xinjiang, China. *Int J Syst Evol Microbiol* **58**, 1304–1307.