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Arthrobacter oryzae sp. nov. and *Arthrobacter humicola* sp. nov.

Akiko Kageyama,[1] Kurimi Morisaki,[1] Satoshi Ōmura[1,2] and Yoko Takahashi[1]

[1]Kitasato Institute for Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

[2]The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

Correspondence
Yoko Takahashi
ytakaha@lisci.kitasato-u.ac.jp

Two novel bacterial strains were isolated from a paddy soil sample collected in Japan using GPM agar plates supplemented with superoxide dismutase and/or catalase. The strains were Gram-positive, catalase-positive and motile, with lysine as the peptidoglycan diagnostic diamino acid and acetyl as the peptidoglycan acyl type. The major menaquinone was MK-9(H_2). Mycolic acids were not detected. The G+C content of the DNA was 66–68 mol%. On the basis of phenotypic analysis, 16S rRNA gene sequence comparisons and DNA–DNA hybridization data, it is proposed that these strains represent two novel species, *Arthrobacter oryzae* sp. nov. (type strain is KV-651^T=NRRL B-24478^T=NBRC 102055^T) and *Arthrobacter humicola* sp. nov. (type strain is KV-653^T=NRRL B-24479^T=NBRC 102056^T), respectively.

The genus *Arthrobacter* was proposed by Conn & Dimmick (1947). The type species of the genus, *Arthrobacter globiformis*, and the closely related species assigned to group III (Stackebrandt & Schumann, 2000) are characterized by peptidoglycan type A3α with an interpeptide bridge containing one to four molecules of L-alanine (Schleifer & Kandler, 1972) and MK-9(H_2) as the major menaquinone. In this paper, the characterization of two novel species that are closely related to *A. globiformis* is reported.

Strains KV-651^T and KV-653^T were isolated from soil samples collected from a paddy in Saitama prefecture, Japan. Soil (2 g) was suspended in 18 ml sterile water and mixed well. Soil particles were allowed to sediment, the liquid phase was diluted 10^5-fold, and 100 μl diluted liquid phase was spread onto the surface of GPM agar plates (1.0 % glucose, 0.5 % peptone, 0.5 % meat extract, 0.3 % NaCl and 1.2 % agar, pH 7.0) (Takahashi *et al.*, 2003) supplemented with superoxide dismutase (300 U per plate) and catalase (2100 U per plate) for KV-651^T or catalase (2100 U per plate) for KV-653^T.

Morphological observations were carried out using a scanning electron microscope (JSM-5600; JEOL) for cultures grown on 1/5 nutrient agar medium at 27 °C for 11–108 h. Motility was determined by microscopic analysis and flagella were observed using a transmission electron microscope (JEM-1200EXII; JEOL) after incubation for 24 h at 27 °C on GPM agar medium. Negative staining of cells was performed with 1 % uranyl acetate. Gram-staining was performed by using a Gram-stain reagent kit (Nacalai Tesque). The ability to utilize various carbon sources was tested on basal medium (Pridham & Gottlieb, 1948) supplemented with 1 % (w/v) of each carbon source. Tolerance to NaCl (0–5 % at intervals of 1 %) was determined on YD agar (1.0 % yeast extract, 1.0 % glucose and 1.2 % agar, pH not adjusted). pH tolerance was determined on the same medium adjusted to various pH values (pH 4–11 at intervals of 1 pH unit) by the addition of HCl or NaOH. The temperature range for growth was determined on the same medium at 4–45 °C. Biochemical characteristics were determined using the API ZYM (bioMérieux) and API Coryne (bioMérieux) systems in accordance with the manufacturer's instructions.

N-Acyl types of muramic acid were determined by using the method of Uchida & Aida (1977). Purified cell walls of the isolated strains were prepared as described by Kawamoto *et al.* (1981) and hydrolysed at 100 °C with 1 ml 6 M HCl for 16 h. Amino acids were derivatized using phenylisothiocyanate and detected using the Pico Tag method (Waters). Cell-wall sugars were prepared according to the method described by Kawamoto *et al.* (1981) and analysed by cellulose TLC (n-butanol : toluene : pyridine : distilled water at 10 : 1 : 6 : 6). The presence of mycolic acids was examined by using the TLC method of Tomiyasu (1982), and phospholipids were extracted and identified by following the method of Minnikin *et al.*

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Arthrobacter oryzae* KV-651^T and *Arthrobacter humicola* KV-653^T are AB279889 and AB279890, respectively.

Supplementary tables detailing 16S rRNA gene sequence similarity values and DNA–DNA relatedness values between the two isolated strains and related species and scanning electron micrographs (Fig. S1) and transmission electron micrographs (Fig. S2) of cells of strain KV-653^T are available with the online version of this paper.

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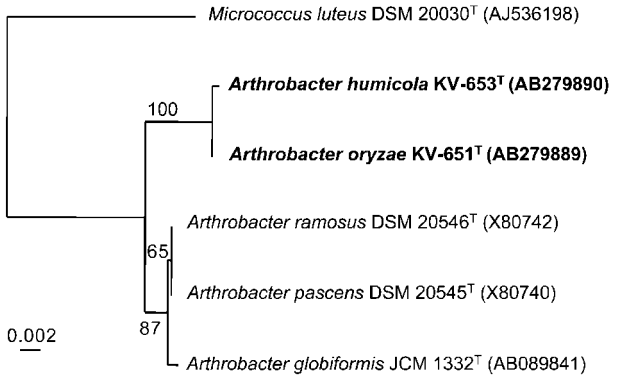

Fig. 1. Phylogenetic tree constructed on the basis of 16S rRNA gene sequences using the neighbour-joining method and K_{nuc} values. Numbers at branching points refer to bootstrap values (1000 resamplings). The tree was unrooted and *Micrococcus luteus* was used as an outgroup. Bar, 0.002 K_{nuc}.

(1977). Menaquinones were extracted and purified according to Collins *et al.* (1977) and then analysed by HPLC (802-SC; Jasco) on a chromatograph equipped with a CAPCELL PAK C18 column (Shiseido) (Tamaoka *et al.*, 1983). Methyl esters of cellular fatty acids were analysed by GLC (HP6890; Hewlett Packard). The method in the manual for the Sherlock Microbial Identification System (version 5.0) (MIDI) was used for sample preparation and analysis.

DNA was isolated as described by Saito & Miura (1963). DNA base composition was estimated by HPLC (Tamaoka & Komagata, 1984). Levels of DNA–DNA relatedness were determined by using the method of Ezaki *et al.* (1989).

For 16S rRNA gene sequence analysis, DNA was prepared and amplified as reported by Yu *et al.* (2002) and Takahashi *et al.* (2003), respectively, and was sequenced with an automatic sequence analyser (ABI Prism 3130; PE Applied Biosystems) using a dye terminator cycle sequencing kit (PE Applied Biosystems). Sequence data for related species were retrieved from GenBank. Phylogenetic analysis was performed using CLUSTAL W software (Thompson *et al.*, 1994). Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura & Ohta, 1972) and phylogenetic trees were constructed by using the neighbour-joining method (Saitou & Nei, 1987). Sequence similarity values were determined by visual comparison and manual calculation.

Nearly complete 16S rRNA gene sequences (1465 bp for strain KV-651^T and 1463 bp for strain KV-653^T) were determined. The sequence similarity between the two strains was 99.5 %. Phylogenetic analysis demonstrated that the strains belonged to the genus *Arthrobacter* and were most closely related to *A. globiformis*, *Arthrobacter ramosus* and *Arthrobacter pascens* (Fig. 1) with 98.3–98.9 % sequence similarity (see Supplementary Table S1, available with the online version of this paper).

Both strains KV-651^T and KV-653^T had a rod–coccus cycle. Their cells were cocci in the stationary growth phase, irregular rods in 11 h-old cultures, predominantly short rods or oval-shaped after 60 h and had a coccoid shape again after 108 h (Fig. 2 and Supplementary Fig. S1 in IJSEM Online). Both strains had flagella (Fig. 3 and Supplementary Fig. S2). The cell-wall peptidoglycan of both strains KV-651^T and KV-653^T contained lysine, glutamic acid and alanine in the molar ratio of approximately 1 : 1 : >4, which suggested that the isolates contained the A3α peptidoglycan type, as observed in *A.*

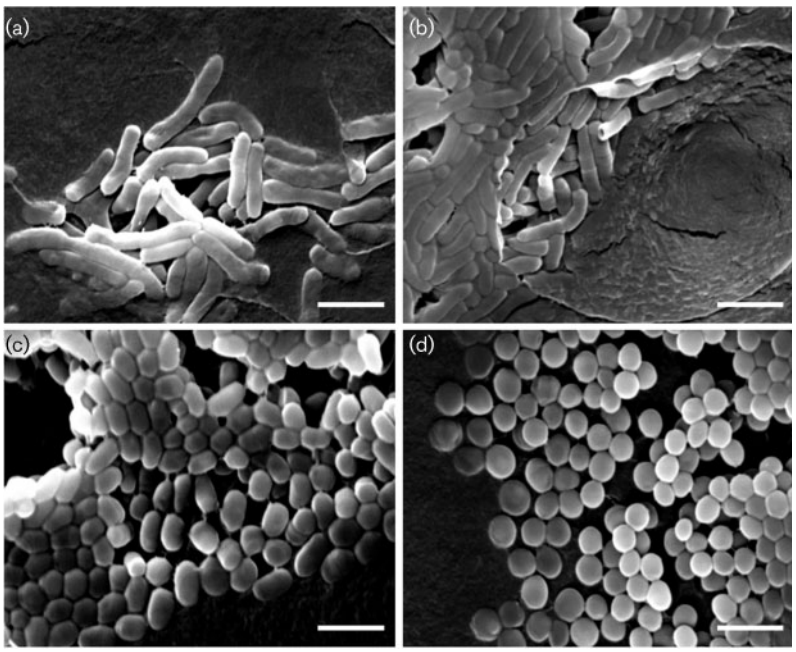

Fig. 2. Scanning electron micrographs of cells of strains KV-651^T (a–d) grown on 1/5 nutrient agar medium at 27 °C. Cells are shown after growth for: (a) 11 h; (b) 24 h; (c) 60 h; (d) 108 h. Bars, 2 μm.

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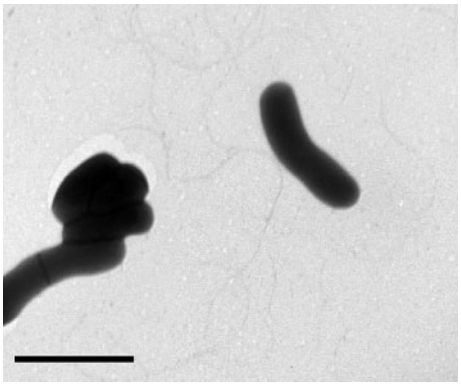

Fig. 3. Transmission electron micrograph of negatively stained cells of strain KV-651^T. Bar, 2 μm.

globiformis and related species. The predominant menaquinone was MK-9(H_2) and the acyl type was acetyl. Mycolic acids were not detected. The cellular fatty acid components of strains KV-651^T and KV-653^T were iso-$C_{15:0}$ (3.86 and 5.32 % of total, respectively), anteiso-$C_{15:0}$ (68.94 and 73.50 %, respectively), iso-$C_{16:0}$ (3.72 and 6.61 %, respectively), $C_{16:0}$ (2.97 and 1.15 %, respectively) and anteiso-$C_{17:0}$ (16.61 and 9.33 %, respectively). In addition, iso-$C_{14:0}$ (1.54 %) was detected in strain KV-653^T. Other phenotypic characteristics are given in the species descriptions.

DNA–DNA hybridization values between the two isolates and related *Arthrobacter* species were less than 50 % (see Supplementary Table S2 in IJSEM Online), well below the 70 % cut-off point for species delineation recommended by Wayne *et al.* (1987).

A range of phenotypic characters that distinguish strains KV-651^T and KV-653^T from each other and from their nearest phylogenetic neighbours is presented in Table 1. For instance, strains KV-651^T and KV-653^T differed from the other three *Arthrobacter* species in their inability to utilize L-arabinose and from each other in their cell-wall sugar compositions, NaCl tolerance and enzymic activities (nitrate reductase, pyrrolidonyl arylamidase and α-galactosidase).

Given the results of this study, it is apparent that the isolated strains represent two distinct novel species within the genus *Arthrobacter*, for which the names *Arthrobacter oryzae* sp. nov. (strain KV-651^T) and *Arthrobacter humicola* sp. nov. (strain KV-653^T) are proposed.

Description of *Arthrobacter oryzae* sp. nov.

Arthrobacter oryzae (ory.za′e. L. fem. n. *oryzae* of rice).

Cells have a rod–coccus cycle. Gram-positive, motile by flagella and aerobic. Colonies are cream coloured on YD agar. Growth occurs on YD agar at initial pH values between 6 and 11 and at temperatures between 4 and 34 °C. In YD agar medium, up to 2 % NaCl is tolerated. D-Glucose, raffinose, melibiose, D-mannitol, L-inositol and sucrose are assimilated, but L-arabinose and cellulose are not. Leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucuronidase and α-glucosidase are detected by the API ZYM enzyme assay; the assay is negative for alkaline phosphatase, esterase lipase (C8), trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Weak reactions are detected for esterase (C4), lipase (C14), valine arylamidase and cystine arylamidase. Nitrate reductase, pyrrolidonyl arylamidase

Table 1. Characteristics of isolates KV-651^T and KV-653^T and related *Arthrobacter* species

Strains: 1, KV-651^T; 2, KV-653^T; 3, *Arthrobacter globiformis* NBRC 12137^T; 4, *Arthrobacter pascens* NBRC 12139^T; 5, *Arthrobacter ramosus* NBRC 12958^T. Data for *A. globiformis* NBRC 12137^T, *A. pascens* NBRC 12139^T and *A. ramosus* NBRC 12958^T are from Keddie *et al.* (1986). +, Positive; w, weakly positive; −, negative; glu, glucose; gal, galactose; man, mannose; rha, rhamnose.

Characteristic	1	2	3	4	5
Motility	+	+	−	−	+
Cell-wall sugars	gal, glu	gal, rha	gal, glu	gal, glu	gal, man, rha
NaCl range for growth (%, w/v)	0–2	0–3	0–5	0–5	0–3
Utilization of L-arabinose	−	−	+	+	+
Enzyme assay (API Coryne):					
Nitrate reductase	+	−	−	−	−
Pyrrolidonyl arylamidase	+	−	−	−	−
Urease	−	−	−	−	+
Enzyme assay (API ZYM):					
Esterase lipase (C8)	−	w	−	−	+
Acid phosphatase	+	+	−	−	+
α-Galactosidase	−	+	−	+	−
β-Glucuronidase	+	w	−	−	+
α-Glucosidase	+	+	+	+	−
α-Mannosidase	−	w	+	+	−

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and catalase are detected by the API Coryne enzyme assay, but urease is negative. The diagnostic diamino acid of the peptidoglycan is lysine. The acyl type of the peptidoglycan is acetyl. The major menaquinone is MK-9(H_2). The major cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{15:0}$. Cell-wall sugars contain galactose and glucose. The DNA G+C content of the type strain is 67 mol%.

The type strain, KV-651^T (=NRRL B-24478^T=NBRC 102055^T), was isolated from paddy soil, Japan.

Description of *Arthrobacter humicola* sp. nov.

Arthrobacter humicola (hu.mi.co′la. L. masc. n. *humus* soil; L. suff. -*cola* dweller; N.L. masc. or fem. n. *humicola* soil dweller).

Cells have a rod–coccus cycle. Gram-positive, motile by flagella and aerobic. Colonies are cream coloured on YD agar. Growth occurs on YD agar at initial pH values between 6 and 10 and at temperatures between 4 and 34 °C. In YD agar medium, up to 3 % NaCl is tolerated. D-Glucose, D-xylose, raffinose, melibiose, D-mannitol, L-rhamnose, L-inositol and sucrose are assimilated, but L-arabinose and cellulose are not. Esterase (C4), leucine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase and α-glucosidase are detected by the API ZYM enzyme assay; the assay is negative for alkaline phosphatase, trypsin, chymotrypsin, β-glucuronidase, *N*-acetyl-β-glucosaminidase and α-fucosidase. Weak reactions are detected for esterase lipase (C8), lipase (C14), valine arylamidase, β-galactosidase, β-glucosidase and α-mannosidase. Catalase is detected by the API Coryne enzyme assay, but nitrate reductase, pyrrolidonyl arylamidase and urease are negative. The diagnostic diamino acid of the peptidoglycan is lysine. The acyl type of the peptidoglycan is acetyl. The major menaquinone is MK-9(H_2). The major cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$. Cell-wall sugars contain galactose and rhamnose. The DNA G+C content of the type strain is 67 mol%.

The type strain, KV-653^T (=NRRL B-24479^T=NBRC 102056^T), was isolated from paddy soil, Japan.

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