Arthrobacter koreensis sp. nov., a novel alkalitolerant bacterium from soil

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Two *Arthrobacter* strains, CA15-8^T and CA15-9, were isolated from soil, under alkali conditions, and characterized phenotypically, chemotaxonomically and genetically. These alkalitolerant organisms grew over a wide pH range (pH 7.0–12.0), with an optimum at pH 7.0–8.0. The mean G + C content of the DNA of these strains was 63 ± 2 mol%. The strains contained MK-8(H₂) and MK-9(H₂) as the main respiratory quinones. The cell-wall peptidoglycan was Lys-Thr-Ala₂ and the whole-cell sugar was rhamnose. The major cellular fatty acids of the isolates were anteiso-C_{15:0} and iso-C_{15:0}. A phylogenetic analysis, based on 16S rDNA sequence data, revealed that strains CA15-8^T and CA15-9 formed an evolutionary lineage distinct from other *Arthrobacter* species. On the basis of morphological, physiological and chemotaxonomic characteristics, 16S rDNA sequence comparisons and DNA–DNA hybridization data, a novel species of *Arthrobacter* is proposed, namely *Arthrobacter koreensis* (type strain CA15-8^T = KCTC 9922^T = IFO 16787^T).

The genus *Arthrobacter* was first proposed by Conn & Dimmick (1947) and its description was emended by Koch *et al.* (1995). At the time of writing, there are 35 recognized species of the genus *Arthrobacter* (<http://www.bacterio.cict.fr/a/arthrobacter.html>), which are reported as being phenotypically heterogeneous. Members of the genus *Arthrobacter* are Gram-positive, aerobic, catalase-positive, coryneform bacteria. They have L-lysine as the diamino acid in their cell walls and have branched cellular fatty acids (Funke *et al.*, 1997). They have been divided into two groups on the basis of their peptidoglycan (Schleifer & Kandler, 1972). Group I, designated A3 α , contains peptidoglycan variations where the murein cross-linkage is by interpeptide bridges involving monocarboxylic L-amino acids or glycine or both. *Arthrobacter globiformis*, the type species of the genus, and most other *Arthrobacter* species are included in group I. By contrast, several other *Arthrobacter* species with A4 peptidoglycan are included in group II. The A4 α variation involves cross-linkage of peptidoglycan by interpeptide bridges containing a dicarboxylic acid such as L-Lys-Ala-Glu or L-Lys-Glu.

The genus *Arthrobacter* constitutes a predominant group of micro-organisms found in soils from various environments all over the world. Many mesophilic and psychrophilic arthrobacters have been reported. However, alkaliphilic or alkalitolerant arthrobacters have rarely been reported. During taxonomic studies, and isolation under alkali conditions, we characterized *Arthrobacter*-like isolates from soil. In this study, we describe the morphological, biochemical and phylogenetic characteristics of alkalitolerant bacteria isolated from soil. On the basis of these data, we propose the description of a novel species of *Arthrobacter*, *Arthrobacter koreensis*.

Strains CA15-8^T and CA15-9 were isolated from soil from Daejeon City, Korea. They were isolated on trypticase/soy agar (TSA) at pH 10.0 and cultured on TSA (neutral pH) for 48 h at 30 °C, for use in morphological, biochemical and molecular studies.

The morphology and motility of cells were examined by light microscopy using the hanging-drop technique (Skerman, 1967). API tests (bioMérieux) 20NE, API 20E and API Coryne, performed in accordance with the manufacturer's directions, were used for physiological and biochemical characterizations. Catalase activity was determined by the production of bubbles from 3% (v/v) H₂O₂, and oxidase activity was determined using 1% (w/v) *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. A GP Biolog microplate, containing 95 different carbon compounds, was used to test for substrate oxidation.

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The GenBank accession numbers for the 16S rDNA sequences of *Arthrobacter koreensis* strains CA15-8^T and CA15-9 are AY116496 and AY116497, respectively.

A more-detailed phylogenetic tree showing the position of strains CA15-8^T and CA15-9 within the genus *Arthrobacter* is available in IJSEM Online.

Cells of the isolates were Gram-positive, had a rod-coccus life cycle and were motile. Strains CA15-8^T and CA15-9 formed round, smooth, yellow colonies on TSA. Both strains tested positive for nitrate reduction, catalase, indole production, acetoin production, pyrazinamidase, alkaline phosphatase and α -glucosidase, and for the assimilation of glucose, mannose, mannitol, maltose, gluconate and malate. In the Biolog microplates, the organisms oxidized Tween 40, Tween 80, D-arabitol, D-ribose, xylitol, acetic acid, *p*-hydroxyphenylacetic acid, methyl pyruvate, pyruvic acid, adenosine, 2'-deoxyadenosine, inosine, thymidine, uridine and thymidine 5'-monophosphate. *Arthrobacter luteolus* KCTC 9907^T, which is closely related to strains CA15-8^T and CA15-9, showed some differences in the Biolog test. It oxidized L-rhamnose and glycerol but not D-arabitol or *p*-hydroxyphenylacetic acid.

Growth experiments were performed using screw-cap tubes (20 × 150 mm) containing 10 ml trypticase soy broth (TSB) adjusted to pH values ranging from 6.0 to 13.0. Growth was estimated by monitoring the OD₆₅₀ value. TSB media containing 100 mM Na₂HPO₄/NaH₂PO₄ buffer at pH 6.0–8.0, 100 mM NaHCO₃/Na₂CO₃ buffer at pH 9.0–10.0, 50 mM Na₂HPO₄/100 mM NaOH buffer at pH 11.0 or 100 mM KCl/NaOH buffer at pH 12.0–13.0 were used. These experiments were performed on the basis of the methods of Yumoto *et al.* (1998). Strains CA15-8^T and CA15-9 did not grow at pH 6.0, but grew over a wide pH range (pH 7.0–12.0). Growth was more rapid at pH 7.0–8.0 than at the other pH values tested. This was an important characteristic for distinguishing these isolates from most of the other *Arthrobacter* species, and indicated they were alkalitolerant organisms (Yumoto *et al.*, 1998).

For analysis of their total cellular fatty acid content, cells of strains CA15-8^T and CA15-9 were cultured on TSA for

48 h at 30 °C. The fatty acids were extracted by following the Microbial Identification System protocol, as described previously (Lee *et al.*, 1996; Yang *et al.*, 1993). Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), then purified by TLC on Merck Kieselgel 60 F₂₅₄ plates (20 × 20 cm, 0.5 mm thickness) using petroleum ether/diethyl ether (9:1, v/v) as the eluting solvent. The identity of the quinone was determined by HPLC analysis, as described by Shin *et al.* (1996). Peptidoglycan and whole-cell sugars were determined by the method of Komagata & Suzuki (1987). The peptidoglycan was hydrolysed, and the amino acids were subjected to HPLC (using Waters equipment).

Strains CA15-8^T and CA15-9 had anteiso-C_{15:0} and iso-C_{15:0} as their major fatty acids, MK-8(H₂) and MK-9(H₂) as their main respiratory quinones, rhamnose as their whole-cell sugar and Lys-Thr-Ala₂ as their cell-wall peptidoglycan. Of the *Arthrobacter* species, *Arthrobacter citreus* and *A. luteolus* are the only two species known to have Lys-Thr-Ala₂ peptidoglycans. A comparison between the phenotypic and chemotaxonomic characteristics of our isolates and those of *A. citreus* and *A. luteolus* is shown in Table 1. As shown in Fig. 1, strains CA15-8^T and CA15-9 were phylogenetically closely related to *A. citreus* and *A. luteolus* [a more-detailed phylogenetic tree is available in IJSEM Online (<http://ijs.sgmjournals.org>)]. Under identical growth conditions (TSA media at 30 °C and pH 7.3), *A. luteolus* KCTC 9907^T had the same cellular fatty acids and menaquinone profile as our novel strains, but the relative contents were different. *A. luteolus* KCTC 9907^T had anteiso-C_{15:0} (72.6 %) and iso-C_{15:0} (14.1 %), but strains CA15-8^T and CA15-9 had anteiso-C_{15:0} (40.0 %) and iso-C_{15:0} (34.4 %). With regard to its menaquinones, *A. luteolus* KCTC 9907^T was found to have 68 % MK-9(H₂) and 22 % MK-8(H₂). In contrast, strains CA15-8^T and

Table 1. Some characteristics of *Arthrobacter* species with Lys-Thr-Ala₂ type peptidoglycan

Data for *A. citreus* SAM 0302^T are from Kodama *et al.* (1992). All strains are motile, reduce nitrate and produce α -glucosidase. None of the strains have activities for pyrrolidonyl peptidase, N-acetylglucosaminidase or urease. All of the strains utilize D-glucose and D-ribose. ND, Not determined; w, weak reaction.

Characteristic	<i>A. citreus</i> SAM 0302 ^T	<i>A. luteolus</i> KCTC 9907 ^T	CA15-8 ^T and CA15-9
Cell-wall sugar	Galactose	Rhamnose	Rhamnose
Major fatty acids	anteiso-C _{15:0} and C _{16:0}	anteiso-C _{15:0} and iso-C _{15:0}	anteiso-C _{15:0} and iso-C _{15:0}
β -Galactosidase	+	–	–
Utilization of:			
D-Arabitol	ND	–	+
<i>p</i> -Hydroxyphenylacetic acid	ND	–	+
Glycerol	ND	+	–
Tween 80	+	–	+
L-Arabinose	+	–	–
D-Galactose	+	–	–
Inositol	–	–	–
L-Rhamnose	w	+	–

Fig. 1. Phylogenetic tree, based on 16S rDNA gene sequences, showing the position of strains CA15-8^T and CA15-9 with respect to other species of the genus *Arthrobacter*. The branching pattern was generated by the neighbour-joining method. Accession numbers are given in parentheses. The numbers at the nodes indicate bootstrap values (1000 replications). Bar, 0.01 accumulated changes per nucleotide.

CA15-9 had 43 ± 2 % MK-8(H₂) and 35 ± 1 % MK-9(H₂). The other percentage (22 ± 3 %) showed the sum of minor peaks in the analysis of quinone by HPLC. The minor peaks appeared in less than 4 % and were not studied in detail. It has been reported that the *Arthrobacter* species contain MK-9(H₂) or MK-8 and MK-9 as the predominant menaquinone(s). However, strains CA15-8^T and CA15-9 contain MK-8(H₂) as the predominant menaquinone together with significant amounts of MK-9(H₂). This characteristic distinguishes these strains from other species of *Arthrobacter*.

DNA was extracted and then purified by a modification of the method of Marmur (1961). The G+C content of the DNA was determined by the reversed-phase HPLC method described by Tamaoka & Komagata (1984). The DNA G+C content of strains CA15-8^T and CA15-9 was 63 ± 2 mol%, while that of *A. luteolus* KCTC 9907^T was 63 mol%.

Two universal primers (9F and 1542R) described by Stackebrandt & Liesack (1993) were used for PCR amplification of the 16S rDNA gene. The amplified PCR product was purified using a QIAquick PCR purification kit (Qiagen). The purified 16S rDNA was sequenced using the ABI Prism BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems), with an automated DNA sequencer (model 377; Applied Biosystems). Nearly complete 16S rDNA sequences (1459 bp) of strains CA15-8^T and CA15-9 were determined. The 16S rDNA sequences of these strains were aligned with the 16S rDNA and rDNA

sequences of representatives of the genus *Arthrobacter* and related taxa, using CLUSTAL W software (Thompson *et al.*, 1994). A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) based on distance-matrix data. The evolutionary distances were calculated using the Jukes-Cantor model (Jukes & Cantor, 1969). The PHYLIP software package (Felsenstein, 1993) was used for all analyses. The topology of the phylogenetic tree was evaluated with bootstrap analysis (Felsenstein, 1985) of the neighbour-joining data, based on 1000 replications.

The primary structures of the 16S rDNA sequences of strains CA15-8^T and CA15-9 were compared with those of closely related reference strains. A phylogenetic tree, based on the K_{nuc} value (Fig. 1), indicates that these strains belong to the genus *Arthrobacter*.

Strains CA15-8^T and CA15-9 had identical 16S rRNA gene sequences and morphological, physiological and chemotaxonomic characteristics, implying that they may be members of the same species. Our novel strains were phylogenetically most closely related to *A. luteolus* KCTC 9907^T (98.9 % sequence similarity), but formed a distinct cluster with this strain (Fig. 1). The levels of sequence similarity between strains CA15-8^T and CA15-9 and other *Arthrobacter* species ranged from 87.4 to 98.9 %.

DNA-DNA hybridization was done by the membrane filter technique using DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Molecular Biochemicals). Genomic DNA (200 ng) was denatured by the alkaline method, then immobilized on a nylon membrane (Hybond-N⁺; Amersham) by applying a small vacuum. DNA preparations were labelled with DIG High Prime DNA Labelling and Detection Starter Kit II. The denatured DNA samples (1 µg) were labelled according to the manufacturer's protocol. The membranes were prehybridized in a hybridization solution at 52 °C for 30 min. The actual hybridization was carried out in a hybridization solution containing labelled DNA (25 ng ml⁻¹) at 52 °C for 16 h. After hybridization, the membranes were washed twice in primary washing solution (2 × SSC, 0.1 % SDS) and subsequently washed twice in a secondary solution (0.5 × SSC, 0.1 % SDS) at 68 °C. Detection reagents were added to the membranes for 5 min at room temperature, then the excess liquid was squeezed out. The membranes were exposed to autoradiography film (Hyperfilm-ECL; Amersham) for 10 min, and the signal intensities were determined using the TINA 2.0 program. The signal produced by self-hybridization was taken as 100 %, and percentage homology values were calculated from duplicate samples.

DNA-DNA hybridization values between the two novel isolates, CA15-8^T and CA15-9, and the reference strain *A. luteolus* KCTC 9907^T were below 8 %. DNA-DNA hybridization values between strains CA15-8^T and CA15-9 showed high levels of homology (90–104 %) between the two strains.

The phylogenetic definition of a species generally involves 'strains with approximately 70 % or greater DNA–DNA relatedness' (Wayne *et al.*, 1987). According to the available compiled data, organisms that have less than 97·0 % sequence homology will not reassociate to more than 60 %, no matter which hybridization method is applied (Stackebrandt & Goebel, 1994). The results of the phylogenetic and DNA–DNA hybridization analyses demonstrated that strains CA15-8^T and CA15-9 were not related to any of the recognized members of the genus *Arthrobacter* at the species level.

On the basis of morphological, physiological and chemotaxonomic characteristics, DNA–DNA hybridization data and 16S rDNA sequence comparisons, we propose a novel species of the genus *Arthrobacter*, namely *Arthrobacter koreensis*, to accommodate strains CA15-8^T and CA15-9 (= KCTC 9923 = IFO 16788), two organisms isolated from soil from Daejeon City, Korea.

Description of *Arthrobacter koreensis* sp. nov.

Arthrobacter koreensis (ko.re.en'sis. N.L. adj. *koreensis* pertaining to Korea, where the organisms were isolated).

Gram-positive and coryneform. Cells are motile. Colonies on trypticase/soy agar are round, smooth and yellow. Tests for the reduction of nitrate, the production of catalase, indole, acetoin, pyrazinamidase, alkaline phosphatase and α -glucosidase, and the assimilation of glucose, mannose, mannitol, maltose, gluconate and malate are all positive. Oxidizes Tween 40, Tween 80, D-arabitol, D-ribose, xylitol, acetic acid, *p*-hydroxyphenylacetic acid, methyl pyruvate, pyruvic acid, adenosine, 2'-deoxyadenosine, inosine, thymidine, uridine and thymidine 5'-monophosphate. Cells are alkalitolerant and do not grow at pH 6·0, but they do grow at pH 7·0–12·0, with an optimum at pH 7·0–8·0. G+C content of the DNA is 63 $\pm$ 2 mol%. Major isoprenoid quinones are menaquinones MK-8(H₂) and MK-9(H₂). Major cellular fatty acids are anteiso-C_{15:0} and iso-C_{15:0}. Cell-wall peptidoglycan contains Lys-Thr-Ala₂, and the whole-cell sugar is rhamnose. Isolated from soil from Daejeon City in Korea.

The type strain is CA15-8^T (= KCTC 9922^T = IFO 16787^T).

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