Arthrobacter defluvii sp. nov., 4-chlorophenol-degrading bacteria isolated from sewage

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Two 4-chlorophenol-degrading bacteria, strains 4C1-a^T and 4C1-b, were isolated from sewage flowing into Geumho River near the Daegu industrial complex in Korea. Cells of the strains were Gram-positive and non-motile, displayed a rod-coccus life cycle and formed creamy white colonies on R2A agar or peptone-carbohydrate agar. The strains had chemotaxonomic markers that were consistent with classification in the genus *Arthrobacter*, i.e. MK-9(H₂) as the major menaquinone, iso- and anteiso-branched components as the predominant fatty acids, galactose, glucose and rhamnose as cell-wall sugars, peptidoglycan-type A3 α , and DNA G + C content of 63.5–64.4 mol%. Phylogenetic analysis, based on 16S rRNA gene sequencing, showed that the strains were most similar to *Arthrobacter chlorophenolicus* DSM 12829^T, *Arthrobacter oxydans* DSM 20119^T and *Arthrobacter scleromae* JCM 12642^T (with 98.4, 97.8 and 97.8% similarity, respectively) and formed a separate lineage with *A. chlorophenolicus* in the genus *Arthrobacter*. Combined phenotypic data and DNA–DNA hybridization data supported the conclusion that strains 4C1-a^T and 4C1-b represent a novel species in the genus *Arthrobacter*, for which the name *Arthrobacter defluvii* sp. nov. is proposed. The type strain is 4C1-a^T (=KCTC 19209^T=DSM 18782^T).

The genus *Arthrobacter* within the family *Micrococcaceae* was first established by Conn & Dimmick (1947) and the description was emended by Koch *et al.* (1995) with the reclassification of *Micrococcus agilis* to this genus as *Arthrobacter agilis*. Members of the genus *Arthrobacter* comprise Gram-positive, catalase-positive, actinomycete-coryneform bacteria having a high DNA G + C content (Keddie *et al.*, 1986; Jones & Keddie, 1992). They have been divided into two groups on the basis of their peptidoglycan, which contains lysine as the diamino acid. Members of group I (including the type species, *Arthrobacter globiformis*) contain a peptidoglycan of A3 α type in which cross-linkage is made by interpeptide bridges consisting of monocarboxylic L-amino acids or glycine, or both, whereas members of group II contain a peptidoglycan of A4 α type in which cross-linkage is made by interpeptide bridges involving a dicarboxylic acid

(Schleifer & Kandler, 1972; Stackebrandt *et al.*, 1983). Two exceptions to this are *Arthrobacter duodecadis* and *Arthrobacter viscosus*, which have a peptidoglycan of A4 γ or A1 γ type, respectively. Consequently, *A. duodecadis* has been reclassified as *Tetrasphaera duodecadis* (Ishikawa & Yokota, 2006), but the position of *A. viscosus* remains uncertain. At the time of writing, the genus *Arthrobacter* contains 53 recognized species, representative strains of which have been isolated from a variety of environmental sources including soil, air, water, oil brine, plants, biofilms, cyanobacterial mats, sediment, poultry litter, cheese, human clinical specimens and animal specimens (e.g. cows' milk, fish, flies and seals). *Arthrobacter oryzae* and *Arthrobacter humicola* have been described recently (Kageyama *et al.*, 2008).

During the screening of 4-chlorophenol (4-CP)-degrading bacteria from sewage flowing into Geumho River near the Daegu industrial complex in Korea, strains 4C1-a^T and 4C1-b were recovered, showing creamy white colonies when grown on R2A agar (Difco) at 25 °C. On the basis of 16S rRNA gene sequence comparisons, the two strains were found to belong to the genus *Arthrobacter*. For further classification, they were subjected to a polyphasic investigation.

A sewage sample was initially stimulated with 50 p.p.m. (0.39 mM) 4-CP and the stimulated culture was then

Abbreviation: 4-CP, 4-chlorophenol.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 4C1-a^T and 4C1-b are AM409361 and AM409362, respectively.

A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strains 4C1-a^T and 4C1-b among all described *Arthrobacter* species (with the exclusion of *Arthrobacter viscosus*) is available as supplementary material with the online version of this paper.

diluted serially in 0.85 % saline solution. Aliquots of each serial dilution were spread on R2A agar and incubated at 25 °C for 14 days. Two creamy white colonies, designated strains 4C1-a^T and 4C1-b, respectively, were isolated and subcultivated onto R2A agar at 25 °C for 48 h. For most experiments, the strains were cultivated on R2A agar or broth at 30 °C for 48 h. For analysis of fatty acids, the strains were cultivated on trypticase soy agar (Difco) at 30 °C for 48 h. *Arthrobacter chlorophenolicus* DSM 12829^T, *Arthrobacter oxydans* DSM 20119^T, *Arthrobacter polychromogenes* DSM 20136^T, *Arthrobacter scleromae* JCM 12642^T and *Arthrobacter sulfonivorans* DSM 14002^T were used as reference strains under the same conditions.

The Gram reaction was performed as described by Gerhardt *et al.* (1994). Cell morphology and motility were observed under a phase-contrast microscope (Optiphot; Nikon), at ×1000 magnification, with cells grown for 1–7 days. Motility was tested by the hanging-drop technique (Skerman, 1967). Oxidase activity was tested by using 1 % tetramethyl-*p*-phenylenediamine (Tarrand & Groschel, 1982) and catalase activity was tested by using 3 % H₂O₂. Growth was investigated at different temperatures (5, 10, 15, 20, 25, 30, 37 and 42 °C), at different NaCl concentrations (1, 2, 3, 5 and 10 %) and at pH 5–11 (increments of 1 pH unit). For the pH experiments, appropriate biological buffers were used, as follows: Na₂HPO₄/NaH₂PO₄ buffer was used for pH 5–7, and Na₂CO₃/NaHCO₃ buffer was used for pH 8–11 (Bates & Bower, 1956; Gomori, 1955). Hydrolysis of casein and starch was tested on casein agar and starch agar (Difco). The H₂S production test was performed on triple-sugar-iron agar (BBL). Carbon source utilization tests, acid production tests and additional physiological tests were performed by using API 20NE, API 20E, API 32GN, API 50 CH and API ZYM galleries according to the manufacturer's instructions (bioMérieux).

For determining vitamin requirements, strains were grown in M9 medium (Miller, 1972) containing 0.2 % (w/v) glucose and/or 0.5 % (v/v) vitamin solution (DSM catalogue no. 461). To check for utilization of nicotine and production of pigment, strains were cultivated on nicotine agar containing 4 % nicotine (N2067; Sigma), 0.2 % KH₂HPO₄, 0.5 % KCl, 0.01 % yeast extract, 0.0025 % MgSO₄, 0.0025 % FeSO₄ and 1.5 % Bacto agar (Difco). Strains were grown on peptone-carbohydrate agar, containing 1 % peptone, 1 % glucose, 0.5 % NaCl and 2 % Noble agar (Difco), to determine production of blue pigments characteristic of *A. polychromogenes*.

The ability of strains 4C1-a^T and 4C1-b to degrade different concentrations of 4-CP was determined in broth cultures. Cells were pregrown in M9 medium, containing 50 p.p.m. 4-CP as a sole carbon source, to stationary phase. Pregrown cells were then inoculated into M9 medium containing 4-CP concentrations of 100–300 p.p.m. (at increments of 50 p.p.m.). Growth was measured by determining OD₆₀₀ with a Beckman DU 640 spectropho-

tometer. The 4-CP concentration in broth cultures was determined by measuring the absorbance of the supernatant at 280 nm with a Beckman DU 640 spectrophotometer and relating the value to a standard curve prepared from analysis of sterile M9 medium (Westerberg *et al.*, 2000).

Fatty acid methyl esters were prepared and analysed as described by Klatte *et al.* (1994) by using the standard Microbial Identification System (MIDI Inc.) for automated gas chromatographic analysis (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Isoprenoid quinones were extracted with chloroform/methanol (2 : 1, v/v) and were purified by using TLC on Kieselgel 60 F₂₅₄ plates (Merck; 20 × 20 cm, 0.5 mm thickness) with petroleum ether/diethyl ether (9 : 1, v/v) as the solvent. The identities of the quinones were determined by using reversed-phase HPLC analysis, as described by Shin *et al.* (1996). Purified cell-wall preparations were obtained as described by Schleifer & Kandler (1972). Amino acids and peptides in cell-wall hydrolysates were analysed by two-dimensional TLC on cellulose plates by using the solvent systems described by Schleifer & Kandler (1972). Cell-wall sugars were analysed according to the procedures of Stanek & Roberts (1974).

Extraction of genomic DNA, PCR-mediated amplification of the 16S rRNA genes and sequencing of purified PCR products were carried out according to Rainey *et al.* (1996). The 16S rRNA gene sequences were aligned with published sequences retrieved from EMBL by using CLUSTAL_X (Thompson *et al.*, 1997) and were edited by using BioEdit (Hall, 1999). Phylogenetic trees were constructed on the basis of the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods; distances were estimated according to the method of Jukes & Cantor (1969) by using MEGA version 2.1 (Kumar *et al.*, 2001). The resultant tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resampled datasets. The DNA G + C content was determined by HPLC after hydrolysis, as described by Tamaoka & Komagata (1984), and non-methylated λ DNA (Sigma) was used as a standard. DNA–DNA hybridization to determine genomic relatedness was performed fluorometrically according to the method of Ezaki *et al.* (1989) by using DNA probes labelled with photobiotin (A1935; Sigma) and 96-well microdilution plates (Greiner Bio-One) at 50 °C.

Strains 4C1-a^T and 4C1-b formed visible colonies (about 1 mm in diameter) within 48 h on R2A agar incubated at 30 °C. Growth occurred at temperatures ranging from 5 to 37 °C, but no growth was observed at 42 °C within 14 days. Growth occurred at pH 6–10. Strains 4C1-a^T and 4C1-b were able to grow with up to 100 p.p.m. 4-CP, removing it completely from the medium, and were resistant at up to 200 p.p.m. 4-CP for 4 weeks. Cells of the two strains were aerobic, Gram-positive, non-motile, non-spore-forming and displayed a rod-coccus life cycle. Colonies were creamy white, translucent and circular with entire edges.

Analysis of the almost-complete 16S rRNA gene sequences (approximately 1450 nt) of strains 4C1-a^T and 4C1-b indicated that they belonged to the genus *Arthrobacter*. Levels of 16S rRNA gene sequence similarity among the type strains of recognized species of the genus *Arthrobacter* ranged from 91.8 to 99.8%. Strains 4C1-a^T and 4C1-b, which shared 99.9% sequence similarity, showed highest levels of sequence similarity to *A. chlorophenolicus* DSM 12829^T, *A. oxydans* DSM 20119^T and *A. scleromae* JCM 12642^T (98.4, 97.8 and 97.8%, respectively). Levels of sequence similarity with respect to the type strains of other recognized *Arthrobacter* species (except *A. viscosus*) were in the range 93.1–97.7%. In the neighbour-joining phylogenetic tree, strains 4C1-a^T and 4C1-b occupied a distinct position, clustering only with *A. chlorophenolicus* (Fig. 1), but they were also closely related to *A. oxydans*, *A. polychromogenes*, *A. scleromae* and *A. sulfonivorans*. An extended version of the 16S rRNA gene phylogenetic tree, including all recognized *Arthrobacter* species except *A. viscosus*, is available as Supplementary Fig. S1 in IJSEM Online.

The chemotaxonomic properties of strains 4C1-a^T and 4C1-b were consistent with their classification within the genus *Arthrobacter* (Huang *et al.*, 2005; Keddie *et al.*, 1986; Reddy *et al.*, 2002). The major menaquinone was MK-9(H₂); small amounts of MK-8(H₂) and MK-7(H₂)

were also present. The predominant fatty acids were anteiso-C_{15:0} (56.4±0.4%; mean±SD of 2 determinations), iso-C_{16:0} (13.7±0.8%) and iso-C_{15:0} (12.3±2.1%). The cell-wall peptidoglycan was of A3 α type with an L-Lys-L-Ser-L-Thr-L-Ala interpeptide bridge, which is also found in closely related *Arthrobacter* species, i.e. *A. chlorophenolicus*, *A. oxydans*, *A. polychromogenes*, *A. scleromae* and *A. sulfonivorans*. The cell-wall sugars were galactose, glucose and rhamnose. Detailed chemotaxonomic characteristics of strains 4C1-a^T and 4C1-b are summarized in Table 1.

However, strains 4C1-a^T and 4C1-b showed significant differences from their closest related *Arthrobacter* species in terms of acid production, carbon source utilization and substrate hydrolysis, and also with regard to colony colour. The physiological and biochemical characteristics of strains 4C1-a^T and 4C1-b are summarized in Table 2 and in the species description below.

Levels of DNA–DNA hybridization between strain 4C1-a^T and the type strains of *A. chlorophenolicus*, *A. oxydans*, *A. scleromae*, *A. polychromogenes* and *A. sulfonivorans* were 38, 22, 15, 25, 18%, respectively, whereas the level between strains 4C1-a^T and 4C1-b was 93%. Consequently, the DNA–DNA hybridization results confirmed that strains 4C1-a^T and 4C1-b represent a distinct genomic species (Wayne *et al.*, 1987).

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, constructed according to the neighbour-joining method (Saitou & Nei, 1987), showing the positions of strains 4C1-a^T and 4C1-b among species of the genus *Arthrobacter*. Solid circles indicate generic branches of the tree that were also found with the maximum-parsimony treeing algorithm (Fitch, 1971). Numbers at branch points refer to bootstrap percentages (from 1000 resamplings; only values above 50% are shown). Bar, 1 substitution per 100 nucleotide positions. An extended version of this tree is available as Supplementary Fig. S1 in IJSEM Online.

Table 1. Chemotaxonomic characteristics of strains 4C1-a^T and 4C1-b (*Arthrobacter defluvii* sp. nov.) and the type strains of closely related *Arthrobacter* species

Strains: 1, 4C1-a^T; 2, 4C1-b; 3, *A. chlorophenolicus* DSM 12829^T; 4, *A. oxydans* DSM 20119^T; 5, *A. polychromogenes* DSM 20136^T; 6, *A. scleromae* JCM 12642^T; 7, *A. sulfonivorans* DSM 14002^T. Data are from Borodina *et al.* (2002), Huang *et al.* (2005), Kodama *et al.* (1992), Reddy *et al.* (2002), Westerberg *et al.* (2000) and the present study. Fatty acids are listed using standard abbreviations (number of carbon atoms: number of double bonds). ND, Not detected.

Characteristic	1	2	3	4	5	6	7
DNA G + C content (mol%)	64.4	63.5	65.1	63.1	62.9	64.7	61.0
Cell-wall sugars*	Gal, Glc, Rha	Gal, Glc, Rha	Gal, Glc, Rha	Gal, Glc	Gal	Gal, Glc	Gal, Glc
Major menaquinone	MK-9(H ₂)	MK-9(H ₂)	MK-9(H ₂)	MK-9(H ₂)	MK-9(H ₂)	MK-8(H ₂)	MK-9(H ₂)
Fatty acid composition (%)							
iso-C _{14:0}	3.3	3.4	2.0	1.8	2.3	2.3	3.1
C _{14:0}	1.7	1.8	1.8	2.0	2.9	1.1	2.4
iso-C _{15:0}	14.3	10.2	7.7	18.1	11.7	12.3	16.0
anteiso-C _{15:0}	56.0	56.7	62.1	51.7	54.1	51.2	49.0
C _{15:0}	ND	ND	0.6	ND	0.8	ND	ND
iso-C _{16:1}	0.6	ND	ND	ND	1.1	3.4	0.9
iso-C _{16:0}	12.8	14.5	10.2	8.6	6.7	6.2	12.6
C _{16:1} ω7c	ND	ND	ND	2.2	4.3	4.0	3.2
C _{16:0}	3.6	4.5	6.5	5.1	5.6	2.8	5.9
iso-C _{17:1} ω9c	ND	ND	ND	ND	0.9	1.1	ND
anteiso-C _{17:1} ω9c	0.6	ND	ND	1.0	2.3	6.2	0.9
iso-C _{17:0}	1.0	0.8	0.6	1.6	0.9	1.2	1.1
anteiso-C _{17:0}	6.2	8.0	8.4	7.9	6.4	8.2	4.9

*Gal, Galactose; Glc, glucose; Rha, rhamnose.

On the basis of the genotypic and phenotypic data, strains 4C1-a^T and 4C1-b are considered to represent a novel species of the genus *Arthrobacter*, for which the name *Arthrobacter defluvii* sp. nov. is proposed.

Description of *Arthrobacter defluvii* sp. nov.

Arthrobacter defluvii (de.flu'vi.i. L. gen. n. *defluvii* of sewage).

Cells are aerobic, Gram-positive, non-motile, non-spore-forming and display a rod-coccus life cycle. The cocci are approximately 0.6 µm in diameter. The rods are approximately 0.4–0.6 × 1.0–2.0 µm in size. Catalase-positive but oxidase-negative. Growth occurs at 5–37 °C (optimum 25–30 °C) and at pH 6–10 (optimum pH 7–8). Growth occurs in the presence of up to 5 % NaCl but not with 10 % NaCl. Colonies are creamy white, translucent and circular with entire edges. Indole and H₂S are not produced. Voges–Proskauer reaction is positive. Nitrate is reduced but nitrite is not. Aesculin, casein and starch (weakly) are hydrolysed, but gelatin and urea are not. Acid is produced from ribose, D-xylose, inositol, mannitol and aesculin, but not from glycerol, erythritol, D-arabinose, L-arabinose, L-xylose, adonitol, methyl β-D-xylose, galactose, glucose, fructose, mannose, sorbose, rhamnose, dulcitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose,

starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. The following compounds are utilized as sole carbon sources: mannitol, rhamnose, D-glucose, salicin, D-ribose, melibiose, L-fucose, sucrose, maltose, mannose, L-arabinose, propionate, malonate, valerate, acetate, citrate, gluconate, DL-lactate, malate, phenylacetate, histidine, L-alanine, 2-ketogluconate, 5-ketogluconate, 3-hydroxybutyrate, glycogen, 3-hydroxybenzoate, 4-hydroxybenzoate, L-proline and L-serine. The following carbon sources are not utilized: N-acetylglucosamine, inositol, D-sorbitol, adipate, itaconate, suberate and caprate. According to the results from the API ZYM tests, 2-naphthyl butyrate, L-leucyl 2-naphthylamide, 2-naphthyl phosphate (pH 5.4), 6-bromo-2-naphthyl α-D-galactopyranoside and 2-naphthyl α-D-glucopyranoside are hydrolysed, but 2-naphthyl phosphate (pH 8.5), 2-naphthyl caprylate, 2-naphthyl myristate, L-valyl 2-naphthylamide, L-cystyl 2-naphthylamide, N-benzoyl-DL-arginine 2-naphthylamide, N-glutaryl-phenylalanine 2-naphthylamide, naphthol-AS-BI-phosphate, 2-naphthyl β-D-galactopyranoside, naphthol-AS-BI-β-D-glucuronide, 6-bromo-2-naphthyl β-D-glucopyranoside, 1-naphthyl N-acetyl-β-D-glucosaminide, 6-bromo-2-naphthyl β-D-mannopyranoside and 2-naphthyl α-L-fucopyranoside are not hydrolysed. The major menaquinone is MK-9(H₂); small amounts of MK-8(H₂) and MK-7(H₂) are also present. Predominant fatty acids are anteiso-C_{15:0} (56.4 ± 0.4 %),

Table 2. Differential physiological and biochemical characteristics between strain 4C1-a^T (*Arthrobacter defluvii* sp. nov.) and the type strains of closely related *Arthrobacter* species

Strains: 1, 4C1-a^T (identical results were obtained for strain 4C1-b); 2, *A. chlorophenolicus* DSM 12829^T; 3, *A. oxydans* DSM 20119^T; 4, *A. polychromogenes* DSM 20136^T; 5, *A. scleromae* JCM 12642^T; 6, *A. sulfonivorans* DSM 14002^T. Data are from Borodina *et al.* (2002), Huang *et al.* (2005), Kodama *et al.* (1992), Reddy *et al.* (2002), Westerberg *et al.* (2000) and the present study. +, Positive; (+), weakly positive; –, negative; NA, not applicable.

Characteristic	1	2	3	4	5	6
Colony colour on peptone-carbohydrate medium	Creamy white	Pearl grey	Pearl grey to yellow	Blue	White	Creamy yellow
Motility	–	+	–	–	–	+
Nitrate reduction	+	–	+	+	(+)	–
Growth at/on/in:						
5 °C	+	+	–	–	–	+
37 °C	+	+	+	+	+	–
42 °C	–	–	–	+	–	–
5 % NaCl	+	+	+	+	+	–
10 % NaCl	–	–	+	–	–	–
Nicotine	–	+	+	–	–	–
Mineral salts medium without biotin	+	+	–	–	+	+
Colony colour on nicotine-yeast extract medium	NA	Pearl grey	Blue	NA	NA	NA
Acid production from:						
L-Arabinose	–	+	+	–	–	–
Galactose	–	+	+	+	–	+
Glucose	–	+	+	+	–	+
Rhamnose	–	+	+	–	–	–
Inositol	+	–	+	–	–	+
Mannitol	+	–	+	–	+	+
Maltose	–	+	+	–	–	–
Lactose	–	+	+	+	–	+
Trehalose	–	+	+	+	–	–
Utilization of:						
Salicin	+	–	–	+	+	+
Inositol	–	+	–	–	(+)	+
L-Fucose	+	–	–	–	–	–
D-Sorbitol	–	+	+	+	+	+
Hydrolysis of:						
Gelatin	–	–	(+)	+	–	+
Starch	(+)	–	+	(+)	+	–
2-Naphthyl butyrate*	+	–	–	+	–	–
2-Naphthyl phosphate (pH 5.4)*	+	–	–	–	–	–
2-Naphthyl β-D-galactopyranoside*	–	+	+	+	–	+
6-Bromo-2-naphthyl β-D-glucopyranoside*	–	+	–	+	–	–

*Data from API ZYM tests. The intensity of the colour was measured on a scale from 0 to 5 and was interpreted as negative at values of 0 or 1 and positive at values of 2–5 (Mudarris *et al.*, 1994).

iso-C_{16:0} (13.7 ± 0.8 %) and iso-C_{15:0} (12.3 ± 2.1 %). Cell-wall peptidoglycan is of A3α type with an L-Lys–L-Ser–L-Thr–L-Ala interpeptide bridge. Cell-wall sugars are galactose, glucose and rhamnose. The G + C content of the DNA is 63.5–64.4 mol% (64.4 mol% for the type strain).

The type strain, 4C1-a^T (=KCTC 19209^T=DSM 18782^T), was isolated from sewage flowing into Geumho River in

Daegue, Korea. 4C1-b, isolated from the same source, is a second strain of the species.

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