Chryseobacterium aquifrigidense sp. nov., isolated from a water-cooling system

Seong Chan Park,¹ Mi Sun Kim,¹ Keun Sik Baik,¹ Eun Mi Kim,^{1,2}
Moon Soo Rhee³ and Chi Nam Seong¹

Correspondence
Chi Nam Seong
scnu@scnu.ac.kr

¹Department of Biology, College of Natural Sciences, Suncheon National University, Suncheon 540-742, Republic of Korea

²Department of Dental Hygiene, Gwangyang Health College, Gwangyang 545-703, Republic of Korea

³Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-600, Republic of Korea

A non-motile, yellow-pigmented bacterium, designated strain CW9^T, was isolated from a water-cooling system in Gwangyang, Republic of Korea. The cells were Gram-negative, catalase- and oxidase-positive, short rods. The major fatty acids were iso-C_{15:0} (45.5%), iso-C_{17:1ω9c} (14.4%), iso-C_{17:0} 3-OH (13.0%) and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1ω7c}; 8.8%). The DNA G+C content was 35.6 mol%. A phylogenetic tree based on 16S rRNA gene sequences showed that strain CW9^T formed a lineage within the genus *Chryseobacterium* and was closely related to *Chryseobacterium gleum* ATCC 35910^T (98.4% sequence similarity) and *Chryseobacterium indologenes* ATCC 29897^T (97.8% sequence similarity). Phenotypic characteristics and DNA–DNA relatedness data served to distinguish strain CW9^T from these two species. On the basis of the evidence presented in this study, strain CW9^T represents a novel species of the genus *Chryseobacterium*, for which the name *Chryseobacterium aquifrigidense* sp. nov. is proposed. The type strain is CW9^T (=KCTC 12894^T=JCM 14756^T).

The genus *Chryseobacterium* (type species, *Chryseobacterium gleum*) was described by Vandamme *et al.* (1994) and, at the time of writing, comprises 20 species (<http://www.bacterio.cict.fr/c/chryseobacterium.html>). Strains belonging to this genus have been found in a wide variety of environments, such as fresh water (Kim *et al.*, 2005; Gallego *et al.*, 2006), sewage and wastewater (Kämpfer *et al.*, 2003; Yoon *et al.*, 2007), soil (Shen *et al.*, 2005; Tai *et al.*, 2006; Weon *et al.*, 2006), plant roots (Young *et al.*, 2005; Park *et al.*, 2006), clinical and veterinary samples (Yabuuchi *et al.*, 1983; Holmes *et al.*, 1984; Mudarris *et al.*, 1994), marine mud (Campbell & Williams, 1951), food products (Hugo *et al.*, 2003; de Beer *et al.*, 2005, 2006; Shimomura *et al.*, 2005) and bioreactor sludge (Quan *et al.*, 2007).

In the course of a study on the microbial diversity of a water-cooling system, a yellow-pigmented bacterium, designated CW9^T, was isolated and subjected to a polyphasic taxonomic investigation. This study demonstrated that strain

CW9^T represents a novel species within the genus *Chryseobacterium*.

Strain CW9^T was isolated from a cooled water sample from an oxygen-producing plant in the Republic of Korea, using the standard dilution plating technique. Isolation was achieved using plate count agar (Difco) incubated at 25 °C for 7 days. The isolate was routinely cultured on tryptic soy agar (TSA; Difco) and the culture was suspended in aqueous glycerol (20%, w/v) for storage at –80 °C.

Preparation of bacterial DNA and PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Chun & Goodfellow, 1995). The resulting sequence of strain CW9^T was aligned manually against sequences obtained from the GenBank database. Phylogenetic trees were inferred from the regions available in all sequences (positions 22–1450; *Escherichia coli* numbering system) using the Fitch–Margoliash (Fitch, 1971; Fitch & Margoliash, 1967) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices were generated according to Jukes & Cantor (1969). The topology of the resulting neighbour-joining tree was evaluated by means of bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. Alignment and phylogenetic analyses were carried out using the

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CW9^T is EF644913.

Biolog GN2 MicroPlate results for strain CW9^T are presented in a supplementary table available with the online version of this paper.

jPHYDIT program and PAUP 4.0 (Swofford, 1998) as described previously (Chun *et al.*, 2000).

Preliminary sequence comparisons with 16S rRNA gene sequences held in GenBank indicated that strain CW9^T was closely related to the genus *Chryseobacterium*. The newly determined sequence was then aligned manually against representatives of the genus *Chryseobacterium*. Strain CW9^T showed the highest 16S rRNA gene sequence similarity with respect to *C. gleum* ATCC 35910^T (98.4%) and *Chryseobacterium indologenes* ATCC 29897^T (97.8%); the sequence similarities with respect to all other species of the genus *Chryseobacterium* were below 96.0%. The neighbour-joining analysis confirmed that strain CW9^T was phylogenetically most closely related to members of the genus *Chryseobacterium* (Fig. 1). The tree based on the Fitch–Margoliash method showed essentially the same topology (data not shown).

Bacterial growth was tested on nutrient agar (NA; Difco), TSA, R2A agar (Difco), marine agar 2216 (Difco), cetrimide agar and MacConkey agar (Difco). Motility was examined in wet mounts using phase-contrast microscopy (TMS-F; Nikon) on cells grown in tryptic soy broth (Becton Dickinson) for 3 days at 25 °C. The growth temperature (4–42 °C), pH (pH 4–12) range and NaCl tolerance (0, 1, 2, 3, 5, 10 %, w/v) were determined on TSA after incubation for up to 1 week. Catalase and oxidase activities were determined using 3 % (v/v) hydrogen peroxide and Kovács reagent (Kovács, 1956), respectively. Acid production from sugars was tested as described by Yamaguchi & Yokoe (2000). Nitrate reduction was tested

in nitrate broth containing 0.2 % KNO₃ (Skerman, 1967). Citrate utilization was tested on Simmons' citrate agar (Sigma). Indole production was determined with Kovács indole reagent in 1 % tryptone broth. H₂S production was determined on Kligler's iron agar (Difco). L-Phenylalanine deamination was examined according to the method of Richard & Kiredjian (1995). Urease activity was tested on Christensen's medium (Oxoid). Hydrolysis of casein, starch and L-tyrosine was examined on NA containing 2 % skim milk, 0.2 % soluble starch and 0.5 % L-tyrosine (Barrow & Feltham, 1993), respectively. Precipitation on egg yolk was determined on NA containing 10 % egg yolk (Barrow & Feltham, 1993). The pigment absorption spectrum between 250 and 700 nm was analysed by using an Ultrospec 2100 pro UV/visible spectrophotometer (Amersham Biosciences) following ethanol extraction of the pigments (Gosink *et al.*, 1998) from cells grown on TSA for 3 days at 25 °C. The presence of flexirubin-type pigments was investigated using the KOH test as described by Bernardet *et al.* (2002). Antibiotic resistance was determined with the disc diffusion method (NCCLS, 2003) on TSA incubated for up to 3 days at 25 °C.

Additional physiological and biochemical tests were performed using the API 20E, API 20NE, API 50 CH and API ZYM systems (bioMérieux) and the GN2 MicroPlate (Biolog) according to the manufacturers' instructions.

Detailed results from the morphological, physiological and biochemical tests are given in the species description, in Table 1 and in Supplementary Table S1 (available with the online version of this paper). Pigment extracts from strain

Fig. 1. Neighbour-joining phylogenetic tree, based on almost-complete 16S rRNA gene sequences, showing the relationships between strain CW9^T, other members of the genus *Chryseobacterium* and representatives of related taxa. Bootstrap percentages (based on 1000 resampled datasets) are shown at the nodes (when >50 %). The sequence of *Ornithobacterium rhinotracheale* LMG 9086^T (GenBank accession no. L19156) was used as an outgroup (not shown). Bar, 0.01 nucleotide substitutions per position.

CW9^T showed an absorption maximum at 454 nm. The addition of alkali changed the colour of the pigment to red and also broadened the peak, confirming that it was a flexirubin-type pigment (Weeks, 1981).

Table 1 lists the phenotypic characteristics that clearly differentiate strain CW9^T from its phylogenetic neighbours, *C. gleum* and *C. indologenes*, and from other related *Chryseobacterium* species.

Cellular fatty acids from strain CW9^T grown on TSA for 2 days at 25 °C were prepared and analysed by GLC according to the instructions of the Microbial Identification System (MIDI, 1999). The detailed cellular fatty acid profile is given in Table 2. The DNA G+C content of strain CW9^T was 35.6 mol%, as determined by using the thermal denaturation method of Marmur & Doty (1962).

The taxonomic relationship between strain CW9^T, *C. gleum* ATCC 35910^T and *C. indologenes* ATCC 29897^T was further examined by using DNA–DNA hybridization with

a membrane filter technique, as described by Baik *et al.* (2006). Strain CW9^T shared 39 and 17 % DNA–DNA relatedness with *C. gleum* ATCC 35910^T and *C. indologenes* ATCC 29897^T, respectively, demonstrating that it represents a novel species of the genus *Chryseobacterium* (Wayne *et al.*, 1987), for which the name *Chryseobacterium aquifrigidense* sp. nov. is proposed. A number of phenotypic characteristics clearly distinguish the novel species from the other *Chryseobacterium* species (Tables 1 and 2).

Description of *Chryseobacterium aquifrigidense* sp. nov.

Chryseobacterium aquifrigidense (a.qui.fri.gi.den'se. L. fem. n. *aqua* water; L. adj. *frigidus* cold, cool, chilled; L. suff. *-ensis* *-is* *-e* suffixes used in the sense of 'belonging to' or 'coming from'; N.L. neut. adj. *aquifrigidense* pertaining to cold water, as the strains were isolated from a water-cooling system).

Cells are Gram-negative, non-motile, aerobic rods 0.7–0.8 µm in diameter and 1.5–2.0 µm in length. Good growth occurs on TSA, NA, plate count agar and R2A agar. No growth occurs on marine agar 2216, cetrimide agar or MacConkey agar. Colonies on TSA are convex, circular, smooth, opaque with entire margins, dark yellow in colour and approximately 3 mm in diameter after 3 days at 25 °C (pH 7). Growth occurs in the presence of 0–2 % (w/v) NaCl (optimum, 0 %), at pH 6–9 (optimum, pH 7) and at 15–37 °C (optimum, 25 °C). Oxidase and catalase activities are present, but arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, β-galactosidase and L-phenylalanine deaminase activities are absent. Casein and starch are hydrolysed, but tyrosine is not. Citrate is not utilized. Flexirubin-type pigments are produced. Acid is produced from 0.5 % (w/v) D-glucose and maltose. No precipitate is formed on egg-yolk agar. Aesculin and gelatin are hydrolysed (API 20NE). None of the substrates in the API 50 CH gallery, except glucose, maltose and aesculin, is utilized as a sole carbon and energy source. In the API ZYM gallery, alkaline phosphatase, acid phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase activities are present, but esterase (C4), lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase activities are absent. Cells are sensitive to (µg per disc, unless otherwise indicated) amikacin (30), chloramphenicol (30), nalidixic acid (30), streptomycin (10), tetracycline (30) and vancomycin (30), but resistant to ampicillin (10), erythromycin (15), kanamycin (30), penicillin (10 U) and polymyxin B (300 U). The major fatty acids are iso-C_{15:0} (45.5 %), iso-C_{17:1ω9c} (14.4 %), iso-C_{17:0} 3-OH (13.0 %) and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1ω7c}; 8.8 %). The complete fatty acid content is given in Table 2. The DNA G+C content is 35.6 mol%. Other physiological and biochemical characteristics are given in

Table 1. Characteristics that differentiate strain CW9^T from closely related *Chryseobacterium* species

Strains: 1, CW9^T; 2, *C. gleum* NCTC 11432^T; 3, *C. indologenes* GIFU 1347^T; 4, *Chryseobacterium joostei* LMG 18212^T; 5, *Chryseobacterium shigense* NCIMB 14047^T; 6, *Chryseobacterium vrystaatense* LMG 22846^T. Data are from Yabuuchi *et al.* (1983), Holmes *et al.* (1984), Hugo *et al.* (2003), de Beer *et al.* (2005) and Shimomura *et al.* (2005). +, Positive; –, negative; NA, data not available.

Characteristic	1	2	3	4	5	6
Growth at/on:						
5 °C	–	–	–	+	+	+
42 °C	–	–	+	–	–	–
Cetrimide agar	–	–	+	+	NA	+
MacConkey agar	–	+	+	+	–	–
Starch hydrolysis	+	+	+	+	+	–
Acid production from maltose	+	+	+	+	–	NA
Production of:						
Indole	–	+	+	+	+	+
H ₂ S	–	–	–	–	–	+
Reduction of:						
Nitrate	+	+	–	–	–	–
Nitrite	+	+	–	–	NA	+
Urease activity	–	–	–	+	–	+
Utilization of (API 50 CH):						
Trehalose	–	+	+	+	–	NA
D-Xylose	–	+	–	–	–	–
Enzyme activities (API ZYM):						
Cystine arylamidase	–	–	+	+	NA	NA
Trypsin	–	–	–	–	NA	NA
α-Chymotrypsin	–	–	+	–	NA	NA
α-Glucosidase	–	+	+	+	NA	NA
β-Glucosidase	–	+	–	–	NA	NA
DNA G+C content (mol%)	35.6	38.0	38.5	36.8	36.6	37.1

Table 2. Fatty acid content (%) of strain CW9^T and closely related *Chryseobacterium* species

Taxa: 1, strain CW9^T; 2, *C. gleum* (*n*=5); 3, *C. indologenes* (*n*=45); 4, *C. joostei* (*n*=11); 5, *C. shigense* NCIMB 14047^T; 6, *C. vrystaatense* (*n*=7). Data are from Yabuuchi *et al.* (1983), Hugo *et al.* (2003), de Beer *et al.* (2005) and Shimomura *et al.* (2005). Some of the species included were grown under different culture conditions. tr, <1.0%; NA, data not available.

Fatty acid	1	2	3	4	5	6
iso-C _{13:0}	1.1	tr	tr	tr	NA	1.1 ± 0.4
ECL 13.565*	tr	1.2 ± 0.4	2.1 ± 0.7	1.1 ± 0.2	NA	1.4 ± 0.3
anteiso-C _{15:0}	1.1	tr	tr	tr	NA	1.7 ± 0.7
iso-C _{15:0}	45.5	35.4 ± 2.9	34.3 ± 4.9	34.6 ± 2.0	39.7	41.8 ± 1.4
iso-C _{15:0} 3-OH	2.6	2.5 ± 0.1	2.6 ± 0.2	2.9 ± 0.3	NA	2.7 ± 0.3
C _{16:0}	1.8	1.3 ± 0.4	tr	tr	NA	1.1 ± 0.3
C _{16:0} 3-OH	1.6	1.1 ± 0.1	1.0 ± 0.2	1.2 ± 0.2	NA	1.3 ± 0.3
ECL 16.582*	tr	1.7 ± 0.1	1.7 ± 0.2	1.6 ± 0.1	NA	1.2 ± 0.2
iso-C _{17:0}	1.5	1.6 ± 0.6	tr	tr	NA	tr
iso-C _{17:0} 3-OH	12.9	21.8 ± 0.3	19.2 ± 1.8	20.1 ± 1.2	19.6	15.4 ± 1.8
iso-C _{17:1} ω9c	14.4	20.2 ± 3.9	24.2 ± 3.1	22.9 ± 1.9	NA	19.7 ± 2.3
Summed feature 3†	8.8	11.8 ± 0.8	11.1 ± 1.3	12.1 ± 1.3	NA	9.1 ± 0.9
Summed feature 4‡	3	tr	tr	tr	NA	tr

*ECL, Equivalent chain-length (i.e. the identity of the fatty acid is unknown).

†Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained iso-C_{15:0} 2-OH and/or C_{16:1} ω7c. Summed feature 4 contained iso-C_{17:1} I and/or anteiso-C_{17:1} B.

Table 1 and Supplementary Table S1 (available at IJSEM Online).

The type strain, CW9^T (=KCTC 12894^T=JCM 14756^T), was isolated from a water-cooling system in Gwangyang, Republic of Korea.

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