Chryseobacterium aquaticum sp. nov., isolated from a water reservoir

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Strains 10-46^T and 10-106 were isolated from a water reservoir in Korea. They were Gram-negative, non-motile, non-spore-forming rods, produced yellow-pigmented colonies on nutrient agar, contained MK-6 as the predominant menaquinone and 15:0 iso and 17:0 iso 3-OH as the major fatty acids and had polar lipid profiles consisting of phosphatidylethanolamine and several unknown aminolipids. Phylogenetic analysis, based on 16S rRNA gene sequencing, showed that the strains were closely related to *Chryseobacterium indoltheticum* ATCC 27950^T, *Chryseobacterium scophthalmum* LMG 13028^T and *Chryseobacterium soldanellicola* KCTC 12382^T (96.9, 96.8 and 96.7 % sequence similarity, respectively) and formed a separate lineage within the genus *Chryseobacterium*. The combined genotypic and phenotypic data supported the conclusion that the strains represent a novel species of the genus *Chryseobacterium*, for which the name *Chryseobacterium aquaticum* sp. nov. is proposed. The type strain is 10-46^T (=KCTC 12483^T =CECT 7302^T).

The genus *Chryseobacterium* (type species, *Chryseobacterium gleum*), within the family *Flavobacteriaceae*, was erected by Vandamme *et al.* (1994) to accommodate six species formerly classified within the genus *Flavobacterium*. A number of novel *Chryseobacterium* species have been described since then, while two former *Chryseobacterium* species, namely *Chryseobacterium meningosepticum* and *Chryseobacterium miricola*, have been transferred to the newly erected genus *Elizabethkingia* (Kim *et al.*, 2005). At the time of writing, the genus *Chryseobacterium* comprised 22 recognized species isolated from a variety of environmental sources (including soil, water, sludge and plants), food products (such as fish, meat, milk, poultry and lactic acid beverages) and human clinical specimens (Bernardet *et al.*, 2006).

During investigation of the bacterial community present in a water reservoir in Buyeo, Korea, strains 10-46^T and 10-106 were recovered, showing yellow-pigmented colonies when grown on nutrient agar (Difco) at 30 °C. On the basis of preliminary 16S rRNA gene sequence comparisons, the strains were found to belong to the genus *Chryseobacterium*, but they showed low levels of 16S rRNA gene sequence similarity (<97 %) to all recognized species of this genus. To elucidate the taxonomic status of the two isolates further, they were subjected to a polyphasic investigation.

For most experiments, strains were cultivated on nutrient agar or broth (Difco) at 30 °C for 48 h. For analysis of fatty

acids, strains were cultivated on trypticase soy agar (Difco) at 30 °C for 48 h. *Chryseobacterium indoltheticum* KCTC 2920^T, *Chryseobacterium scophthalmum* KCTC 2907^T, *Chryseobacterium soldanellicola* KCTC 12382^T and *Chryseobacterium balustinum* KCTC 2903^T, grown under identical conditions, were used as reference strains for fatty acid analysis.

The Gram reaction was performed as described by Gerhardt *et al.* (1994). Cell morphology and motility were observed under a light microscope (Nikon Optiphot-2, ×1000 magnification) with cells grown for 1–3 days. Motility was tested by the hanging-drop technique (Skerman, 1967). Flexirubin-type pigments were detected with 20 % KOH according to the method of Fautz & Reichenbach (1980). Oxidase activity was tested by using Bactident-Oxidase strips (Merck) and catalase activity was tested by using 3 % H₂O₂. Growth was investigated at 5, 10, 15, 20, 25, 30, 37 and 42 °C, in the presence of 1, 2, 3 and 5 % NaCl and at pH 5–10 (in increments of 1 pH unit). The following biological buffers were used to adjust the pH: Na₂HPO₄/NaH₂PO₄ for pH 5–7 and Na₂CO₃/NaHCO₃ for pH 8–10 (Gomori, 1955; Bates & Bower, 1956). Growth on MacConkey agar (Difco) was investigated. Hydrolysis of casein and starch was tested on casein agar and starch agar (Difco). Carbon source utilization tests were performed by using GN2 microplates (Biolog) according to the manufacturer's instructions. Acid production tests and additional physiological tests were performed by using API 20E, API 20NE, API 50CH and API ZYM galleries according to the instructions of the manufacturer (bioMérieux).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 10-46^T and 10-106 are AM748690 and AM748691, respectively.

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, constructed according to the neighbour-joining method, showing the positions of strains 10-46^T and 10-106, other *Chryseobacterium* species and related members of the family *Flavobacteriaceae*. Dots indicate branches that were also found with the maximum-parsimony treeing algorithm. Numbers at branch points refer to bootstrap percentages (from 1000 resamplings; only values ≥ 50 % are shown). Bar, 1 substitution per 100 nucleotide positions.

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 GC analysis (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v) and 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. Quinones were identified by using reversed-phase HPLC, as described by Shin *et al.* (1996). Polar lipids were extracted, examined by two-dimensional TLC and identified according to Komagata & Suzuki (1987).

Extraction of genomic DNA, PCR-mediated amplification of 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 edited via the BioEdit program (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 by 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. DNA G + C contents were determined by HPLC, following hydrolysis, as described by Tamaoka & Komagata (1984), and non-methylated λ DNA (Sigma) was used as a standard. DNA–DNA hybridization was performed fluorometrically (Ezaki *et al.*, 1989) by using DNA probes labelled with photobiotin (A1935; Sigma) and 96-well microdilution plates (Greiner Bio-One) at 37 °C.

Analysis of the almost-complete 16S rRNA gene sequences (approximately 1420 nt) of strains 10-46^T and 10-106 indicated that they belonged to the genus *Chryseobacterium*; sequence similarity ranges from 91.8 to 99.1 % among the type strains of recognized species of the genus *Chryseobacterium*. Strains 10-46^T and 10-106 shared 99.9 % 16S rRNA gene sequence similarity. The novel strains showed highest levels of 16S rRNA gene sequence similarity to *C. indoltheticum* ATCC 27950^T, *C. scophthalmum* LMG 13028^T and *C. soldanellicola* KCTC 12382^T, with values of 96.9, 96.8 and 96.7 %, respectively, which are below the level indicative of relatedness at the species level (Stackebrandt & Goebel, 1994). The levels of 16S rRNA gene sequence similarity with other recognized

Table 1. Characteristics that differentiate strains 10-46^T and 10-106 from their closest phylogenetic neighbours in the genus *Chryseobacterium*

Strains: 1, 10-46^T; 2, 10-106; 3, *C. indoltheticum* KCTC 2920^T; 4, *C. scopthalmum* KCTC 2907^T; 5, *C. soldanellicola* KCTC 12382^T; 6, *C. balustinum* KCTC 2903^T; 7, *C. piscium* LMG 23089^T. Data are from Kim *et al.* (2005), Park *et al.* (2006), de Beer *et al.* (2006) and the present study. +, Positive; –, negative; NA, no data available.

Characteristic	1	2	3	4	5	6	7
DNA G + C content (mol%)	38.5	38.3	33.8	34.1	28.8	34.7	33.6
Indole production	–	–	+	–	–	+	+
H ₂ S production	–	–	+	–	–	–	–
Nitrate reduction	–	–	–	–	–	+	+
Growth at/on:							
37 °C	+	+	+	–	+	–	–
MacConkey agar	–	–	+	–	–	+	–
Acid production from:							
Fructose	+	+	–	–	–	+	NA
Maltose	+	+	+	–	–	–	NA
Sucrose	+	+	–	–	–	–	NA
Trehalose	+	+	–	+	–	–	NA
Utilization of:							
Cellobiose	+	+	+	+	–	+	NA
Xylitol	+	+	–	+	–	–	NA
Methyl pyruvate	+	+	+	+	–	+	NA
Formic acid	+	+	–	–	–	–	NA
α -Ketobutyric acid	+	–	–	+	+	–	NA
Malonic acid	+	–	–	–	–	–	NA
Propionic acid	+	+	–	+	+	–	NA
Succinic acid	+	+	+	+	–	+	+
Glycyl L-aspartic acid	+	+	+	+	–	+	NA
L-Leucine	+	+	+	+	–	–	NA
DL-Carnitine	+	–	–	–	–	–	NA
Inosine	–	–	+	+	–	+	NA
Uridine	+	+	+	+	–	+	NA
Thymidine	–	–	–	+	–	+	NA
Glucose 1-phosphate	+	+	+	+	–	+	NA
Glucose 6-phosphate	+	+	+	+	–	+	NA
Hydrolysis of:							
Starch	+	+	–	–	+	–	–
Urea	–	–	–	+	–	+	+
2-Naphthyl butyrate*	+	+	–	+	+	–	NA
L-Cystyl 2-naphthylamide*	+	+	+	+	–	–	NA
N-Benzoyl-DL-arginine 2-naphthylamide*	+	+	–	+	–	–	NA
2-Naphthyl α -D-glucopyranoside*	–	–	+	+	+	+	NA
6-Bromo-2-naphthyl β -D-glucopyranoside*	–	–	–	–	–	+	NA
1-Naphthyl N-acetyl- β -D-glucosaminide*	–	–	+	+	–	+	NA

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

Chryseobacterium species were 92.8–96.6 %. In the phylogenetic tree, strains 10-46^T and 10-106 occupied a distinct position and formed a relatively stable branch with *C. indoltheticum*, *C. scopthalmum*, *C. soldanellicola*, *C. balustinum* and *Chryseobacterium piscium* (Fig. 1).

DNA–DNA hybridization experiments were performed to confirm the taxonomic status of the novel strains. Strains 10-46^T and 10-106 shared 90 % DNA–DNA relatedness,

and showed levels of relatedness of 15–39 % with *C. indoltheticum* KCTC 2920^T, *C. scopthalmum* KCTC 2907^T and *C. soldanellicola* KCTC 12382^T. Consequently, the DNA–DNA hybridization results confirmed that strains 10-46^T and 10-106 belong to a distinct genomic species (Wayne *et al.*, 1987).

The morphological, biochemical and chemotaxonomic characteristics of strains 10-46^T and 10-106 are given in

Table 2. Cellular fatty acid profiles of strains 10-46^T and 10-106 and their closest phylogenetic neighbours in the genus *Chryseobacterium*

Strains: 1, 10-46^T; 2, 10-106; 3, *C. indoltheticum* KCTC 2920^T; 4, *C. scopthalmum* KCTC 2907^T; 5, *C. soldanellicola* KCTC 12382^T; 6, *C. balustinum* KCTC 2903^T; 7, *C. piscium* LMG 23089^T. Data for *C. piscium* were taken from de Beer *et al.* (2006). All other data are from the present study. Growth conditions for *C. piscium* differed from those used herein. Fatty acids are listed using standard abbreviations (number of carbon atoms: number of double bonds). Fatty acids that amounted to less than 1.0% of the total fatty acids in all strains studied are not shown. Therefore, percentages do not add to 100%. tr, Trace (<1.0%); ND, not detected; ECL, equivalent chain-length (i.e. the identity of the fatty acid is unknown).

Fatty acid	1	2	3	4	5	6	7
13:0 iso	2.6	2.6	1.2	tr	1.4	1.1	tr
Unknown 13.566	1.3	1.5	1.3	3.0	2.1	2.6	tr
14:0	1.2	1.3	tr	ND	tr	ND	ND
15:0 iso	37.8	38.0	35.3	37.5	42.9	36.4	38.3
15:0 iso 3-OH	2.8	2.1	2.0	2.7	2.8	2.2	2.4
15:0 anteiso	2.9	2.6	6.8	tr	2.3	2.2	2.7
16:0	7.2	7.6	1.0	1.6	1.3	2.8	1.1
16:0 3-OH	4.8	4.3	1.1	1.9	1.5	1.0	1.3
16:0 iso 3-OH	tr	tr	tr	tr	tr	tr	tr
Unknown 16.580	tr	1.1	1.3	1.0	1.1	1.4	1.2
17:0 2-OH	1.1	tr	2.5	ND	tr	tr	ND
17:0 iso	1.3	1.6	tr	1.2	tr	1.9	1.2
17:0 iso 3-OH	16.2	15.9	14.4	15.7	17.4	13.2	16.2
17:1 iso ω 9c	6.6	6.5	20.7	23.1	14.8	24.4	18.7
Summed feature 4*	12.8	13.3	10.6	9.9	10.5	9.4	10.8

*Summed feature 4 comprises 16:1 ω 7c and/or 15:0 iso 2-OH, which could not be separated by the MIDI system.

the species description below and those that distinguish them from their closest phylogenetic neighbours are listed in Table 1. Strains 10-46^T and 10-106 had fatty acid profiles similar to those of their closest phylogenetic neighbours, but they had a larger proportion of 16:0 and a smaller proportion of 17:1 iso ω 9c (Table 2).

On the basis of the genotypic and phenotypic data, strains 10-46^T and 10-106 are considered to represent a novel species of the genus *Chryseobacterium*, for which the name *Chryseobacterium aquaticum* sp. nov. is proposed.

Description of *Chryseobacterium aquaticum* sp. nov.

Chryseobacterium aquaticum (a.qua'ti.cum. L. neut. adj. *aquaticum* living, growing or found in or by water, aquatic).

Cells are Gram-negative, non-motile, non-spore-forming rods, 0.4–0.5 μ m in diameter and 0.8–2.0 μ m in length. Catalase- and oxidase-positive. Growth occurs at 5–37 °C

(optimum, 25–30 °C) and at pH 6–8 (optimum, pH 6–7). Growth occurs in the presence of 1, 2 and 3 % NaCl, but not with 5 %. No growth is observed on MacConkey agar. Colonies are yellowish, translucent and shiny with entire edges, becoming mucoid after 14 days incubation. Non-diffusible, non-fluorescent, bright-yellow, flexirubin-type pigments are produced on nutrient agar; these pigments turn reddish brown upon addition of 20 % KOH. Indole and H₂S are not produced. Nitrate and nitrite are not reduced. Aesculin, casein, gelatin and starch are hydrolysed, but urea is not. Arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase and β -galactosidase are not produced. Methyl-red test is negative, but Voges-Proskauer test is positive. In the API 50CH gallery, acid is produced from glucose, fructose, mannose (weakly), rhamnose (weakly), amygdalin (weakly), aesculin, cellobiose (weakly), maltose, melibiose (weakly), sucrose, trehalose, starch, glycogen and gentiobiose, but not from glycerol, erythritol, D- or L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β -D-xylose, galactose, sorbose, dulcitol, inositol, mannitol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, N-acetylglucosamine, arbutin, salicin, lactose, inulin, melezitose, raffinose, xylitol, turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. The following compounds are utilized as sole carbon sources in GN2 microplates: α -cyclodextrin, dextrin, glycogen, Tweens 40 and 80, N-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, cellobiose, D-fructose, D-galactose, gentiobiose, α -D-glucose, myo-inositol, lactulose, maltose, D-mannose, melibiose, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, methyl pyruvate, monomethyl succinate, acetic acid, formic acid, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, propionic acid, succinic acid, alaninamide, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglytamic acid, L-serine, L-threonine, uridine, 2,3-butanediol, DL- α -glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. The following carbon sources are not utilized: N-acetyl-D-glucosamine, i-erythritol, L-fucose, methyl β -D-glucoside, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, D-glucuronic acid, α -, β - and γ -hydroxybutyric acids, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketoglutaric acid, DL-lactic acid, D-saccharic acid, sebacic acid, bromosuccinic acid, succinamic acid, glucuronamide, D-alanine, L-histidine, hydroxy-L-proline, D-serine, γ -aminobutyric acid, urocanic acid, inosine, thymidine, phenylethylamine, putrescine, 2-aminoethanol and glycerol. In the API ZYM gallery, 2-naphthyl phosphate (pH 8.5), 2-naphthyl butyrate, 2-naphthyl caprylate, L-leucyl 2-naphthylamide, L-valyl 2-naphthylamide, L-cystyl 2-naphthylamide, N-benzoyl-DL-arginine 2-naphthylamide, 2-naphthyl phosphate (pH 5.4) and naphthol-AS-BI-phosphate are hydrolysed, but 2-naphthyl myristate, N-glutaryl-phenylalanine 2-naphthylamide, 6-bromo-2-naphthyl α -D-galactopyranoside, 2-naphthyl β -D-galactopyranoside,

naphthol-AS-BI- β -D-glucuronide, 2-naphthyl α -D-glucopyranoside, 6-bromo-2-naphthyl β -D-glucopyranoside, 1-naphthyl N-acetyl- β -D-glucosaminide, 6-bromo-2-naphthyl α -D-mannopyranoside and 2-naphthyl α -L-fucopyranoside are not hydrolysed. The only isoprenoid quinone is menaquinone MK-6. The polar lipids comprise phosphatidylethanolamine and several unknown aminolipids. The predominant fatty acids are 15:0 iso ($37.9 \pm 0.1\%$), 17:0 iso 3-OH ($16.1 \pm 0.1\%$) and summed feature 4 (16:1 ω 7c and/or 15:0 iso 2-OH, $13.1 \pm 0.2\%$). The G+C content of the DNA is 38.3–38.5 mol% (38.5 mol% for the type strain).

The type strain, 10-46^T (=KCTC 12483^T =CECT 7302^T), was isolated from a water reservoir in Buyeo, Korea. Strain 10-106, isolated from the same source, is a second strain of the species.

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