Chromobacterium aquaticum sp. nov., isolated from spring water samples

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Strain CC-SEYA-1^T, a motile, Gram-negative, non-violet-pigmented bacterium, was isolated on nutrient agar from spring-water samples collected from Yang-Ming Mountain, Taipei County, Taiwan. 16S rRNA gene sequence studies showed that the strain clustered with *Chromobacterium violaceum* (96.8 % similarity) and *Chromobacterium subtsugae* (96.5 % similarity), followed by *Aquitalea magnusonii* (95.8 % similarity). The fatty acid profile was slightly different from those reported for *C. violaceum*, *C. subtsugae* and *A. magnusonii*. The results of DNA–DNA hybridization, and physiological and biochemical tests allowed both genotypic and phenotypic differentiation of the isolate from the described *Chromobacterium* species. It is evident from the data obtained that the strain should be classified as a novel species in the genus *Chromobacterium*. The name proposed for this taxon is *Chromobacterium aquaticum* sp. nov.; the type strain is CC-SEYA-1^T (=CCUG 55175^T=BCRC 17769^T).

During the characterization of micro-organisms from spring-water samples collected from Yang-Ming Mountain, Taipei County, Taiwan, strain CC-SEYA-1^T was isolated and maintained on nutrient agar (HI-MEDIA) after incubation at 32 °C for 48 h. Subcultivation was done on nutrient agar at 30 °C for 2–3 days. On this medium, CC-SEYA-1^T was able to grow at 25–40 °C, but not at 45 or 15 °C. The organism was also able to grow on tryptone soy agar (HI-MEDIA), R2A agar (Oxoid) and MacConkey agar. The pH range for growth was determined by measuring the optical density at 595 nm of cultures grown in nutrient broth (Difco) which was adjusted prior to sterilization to various pH values (pH 3−11 at intervals of 0.5 pH units) using appropriate biological buffers (Chung *et al.*, 1995). Growth at different temperatures (10–50 °C) was measured in nutrient broth. Growth under anaerobic conditions was determined after incubation in nutrient broth in an Oxoid AnaeroGen system. Growth was recorded by measuring the optical density at 595 nm of the culture.

Cell morphology was observed under a Zeiss light microscope at ×1000 magnification using cells that had been grown for 3 days at 30 °C on nutrient agar (Oxoid). Gram-staining was performed as described by Gerhardt *et al.* (1994). Poly-β-hydroxybutyrate granule accumulation was observed by light microscopy after staining the cells with Sudan black.

TBAB (Difco) with 5 % sheep blood was used for haemolysis testing; a clear or semi-clear zone around the colony indicated a positive test. Modified egg yolk agar (Difco) was used for testing lecithinase, lipase and proteolytic activity. Phenotypic characteristics, biochemical tests, carbon source utilization (Biolog-GN2), API ZYM enzyme profiles (bioMérieux), and API 20E (bioMérieux) and API 20NE (bioMérieux) profiles were investigated. Additionally, antibiotic susceptibility testing was carried out using ATB STAPH 5 strips (bioMérieux), according to the manufacturer's recommendations. Fluorescence was tested after plating on King's B medium after 48 h.

Results of morphological and physiological characterization are given in the species description and in Table 1. Strain CC-SEYA-1^T was non-fluorescent and was capable of producing acid from various carbohydrates. Carbon substrate utilization tests with organic acids as substrates showed several positive results. In the API 20E (bioMérieux) test, CC-SEYA-1^T was positive for arginine dihydrolase, citrate utilization, gelatinase, glucose oxidation and nitrate reduction, while in the API 20NE test, CC-SEYA-1^T was positive for reduction of nitrate to nitrite, glucose fermentation, arginine dihydrolase, aesculin hydrolysis, gelatinase, and assimilation of glucose, *N*-acetylglucosamine, potassium

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of CC-SEYA-1^T is EU109734.

Table 1. Some genotypic, phenotypic and nutritional characteristics that distinguish strain CC-SEYA-1^T from other members of the genera *Chromobacterium* and *Aquitalea*

Taxa: 1, CC-SEYA-1^T; 2, *C. violaceum* ATCC 12472^T (Biolog-GN2 data were obtained in this study for these two strains; all other data were obtained from Lau *et al.*, 2006); 3, *C. subtsugae* DSM 17043^T (Biolog-GN2 data were obtained in this study; all other data were obtained from: Martin *et al.*, 2007); 4, *Aquitalea magnusonii* ATCC BAA-1216^T (all the data were obtained from Lau *et al.*, 2006). −, Not detected.

Characteristic	1	2	3	4
Colony colour	Tan	Violet	Violet	Tan
DNA G+C content (mol%)	62.3	64	64.5	59.2
Isolation source	Spring water	Soil/water*	Soil	Humic lake
Catalase	−	+	+	+
Aesculin hydrolysis	+	−	−	−
Fermentation of ribose	−	+	−	−
Indole production	−	−	−	+
Gelatinase	+	+	−	−
3 % (w/v) NaCl	+	−	−	−
Assimilation of (Biolog-GN2):				
α-D-Glucose 1-phosphate	+	+	+	−
2,3-Butanediol	+	+	−	−
2-Aminoethanol	+	+	−	−
DL-α-Glycerol phosphate	+	+	−	−
D-Glucose 6-phosphate	+	+	+	−
D-Mannose	+	+	−	−
D-Serine	+	+	+	−
Trehalose	+	+	+	−
Inosine	+	+	+	−
L-Alaninamide	+	+	−	−
L-Phenylalanine	+	+	−	−
L-Threonine	+	+	+	−
Thymidine	+	+	−	−
Uridine	+	+	−	−
γ-Aminobutyric acid	+	−	−	+
p-Hydroxyphenylacetic acid	+	−	−	+
α-Ketoglutaric acid	+	−	−	+
cis-Aconitic acid	+	−	−	+
Citric acid	+	−	−	+
L-Leucine	+	−	+	+

*From Gillis & Logan (2005).

gluconate, capric acid, malic acid, trisodium citrate and phenylacetic acid. In API-ZYM enzyme profiling, CC-SEYA-1^T was positive for alkaline and acid phosphatase, butyrate esterase, caprylate esterase (C8), myristate lipase (C14), leucine arylamidase, α-chymotrypsin, naphthol-AS-B-1-phosphohydrolase and *N*-acetyl-β-glucosaminidase.

The 16S rRNA gene was analysed as described by Young *et al.* (2005). Analysis of the sequence data was performed by using the software package MEGA (Molecular Evolutionary Genetics Analysis), version 2.1 (Kumar *et al.*, 2001), after multiple alignments of the data by CLUSTAL_X (Thompson *et al.*, 1997). A distance matrix method (distance options according to the Kimura two-parameter model), including clustering by neighbour-joining (Fig. 1), and a discrete character-based maximum-parsimony method, were used. In each case bootstrap values were calculated based on 1000 replications. The 16S rRNA gene sequence of strain CC-SEYA-1^T was a continuous stretch of 1476 bp. Sequence similarity calculations indicated that strain CC-SEYA-1^T showed the greatest degree of similarity to *Chromobacterium violaceum* (96.8 %) (GenBank accession no. M22510), *Chromobacterium subtsugae* (96.5 %) (AY344056) and *Aquitalea magnusonii* (95.8 %) (DQ018117). Lower sequence similarities (<94.0 %) were found with all other genera shown in Fig. 1 in the family *Neisseriaceae*.

For G+C content calculations, a DNA sample was prepared and degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The obtained nucleoside mixture was then separated by HPLC. The G+C content of strain CC-SEYA-1^T was 62.31 mol%. DNA–DNA hybridization experiments were performed with the type strain of *C. violaceum* NCTC 9757^T and *C. subtsugae* DSM

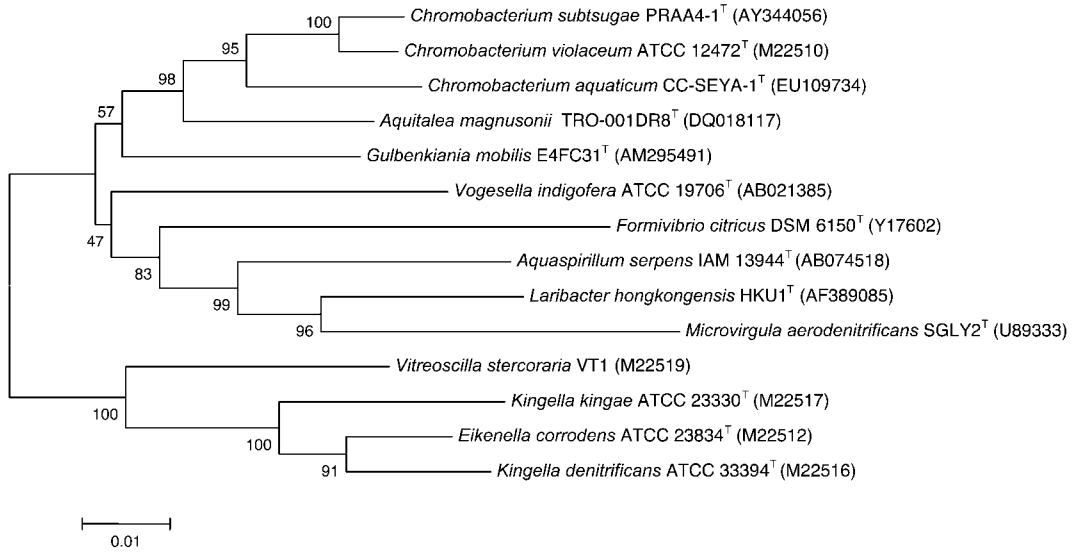

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences available from the EMBL database (accession numbers are given in parentheses) constructed after multiple alignments of the data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method was performed by using the software package MEGA version 2.1 (Kumar *et al.*, 2001). Bootstrap values based on 1000 replications are listed as percentages at the branching points. Bar, 0.01 substitutions per nucleotide position.

10743^T using the method described by Ziemke *et al.* (1998), except that for nick translation, 2 μg DNA was labelled during a 3 h incubation at 15 °C. Strain CC-SEYA-1^T showed relatively low DNA–DNA similarities to *C. violaceum* NCTC 9757^T (37 %, reciprocal analysis 49 %) and *C. subtsugae* DSM 10743^T (38 %, reciprocal analysis 44 %).

Fatty acid methyl esters were prepared, separated and identified according to the instructions of the Microbial Identification System (MIDI; Microbial ID). The fatty acid profile of strain CC-SEYA-1^T (given in the species description) was similar to those given for *Chromobacterium* and *Aquitalea* species, but also showed some differences (Table 2).

From the sequence analysis of the 16S rRNA gene, it is obvious that the isolate belongs to the genus *Chromobacterium*. A moderate relationship to *Aquitalea* is also clear (Fig. 1, Tables 1 and 2). It should be mentioned here, that non-pigmented isolates of *C. violaceum* are well known (Gillis & Logan, 2005). In our opinion, the differences in phenotype do not justify the proposal of a novel genus, separate from *Chromobacterium*. Hence, on basis of our results, we propose that strain CC-SEYA-1^T warrants status as a new member of the genus *Chromobacterium* for which the name *Chromobacterium aquaticum* sp. nov. is proposed.

Description of *Chromobacterium aquaticum* sp. nov.

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

Cells are Gram-negative, aerobic, rod-shaped (0.3–0.5 μm long and 1.5–2 μm wide) and motile by means of a single polar flagellum. Good growth occurs after 48 h incubation on tryptone soy agar and nutrient agar at 32 °C. Colonies on complex standard medium at 32 °C are tan-coloured, smooth, shiny and convex with a spreading edge, 1.0–2.0 mm in diameter and are non-fluorescent. No violet coloured pigmentation. Positive for haemolytic activity (5 % sheep blood), lecithinase and lipase production. The

Table 2. Major fatty acid compositions (%) of the genera *Chromobacterium* and *Aquitalea*

Taxa: 1, CC-SEYA-1^T; 2, *C. violaceum* ATCC 12472^T; 3, *C. subtsugae* DSM 17043^T (data from Martin *et al.*, 2007); 4, *A. magnusonii* ATCC BAA-1216^T (Lau *et al.*, 2006).

Fatty acid	1	2	3	4
$C_{10:0}$ 3-OH	4.6	5.2	4.3	1.5
$C_{12:0}$	8.8	5.6	4.9	5.1
$C_{12:0}$ 2-OH	0.2	3.0	2.8	0.0
$C_{12:0}$ 3-OH	4.4	5.0	3.9	1.2
$C_{14:0}$	2.6	1.7	3.2	2.8
$C_{15:0}$	0.6	–	1.2	2.0
$C_{16:1}\omega7c$ + $C_{16:1}\omega7c$ 2-OH iso*	33.4	35.8	41.9	52.5
$C_{16:0}$	25.8	23.9	24.9	21.7
$C_{17:0}$ cyclo	–	1.7	–	–
$C_{17:1}\omega6c$	–	–	–	1.2
$C_{18:1}\omega7c$	18.8	15.0	10.6	8.0

*These fatty acids cannot be separated by the MIDI system.

optimal temperature for growth is 32 °C. The optimal pH for growth is 6.0; growth occurs at pH 5 and 9.5, but not at pH 4.0 and 10. Growth occurs in the presence of 0–3.5 % (w/v) NaCl; optimal growth occurs in the presence of 0–0.1 % (w/v) NaCl. No anaerobic growth on plain nutrient agar or tryptone soy agar supplemented with nitrate was observed. No poly-β-hydroxybutyrate granule accumulation. Shows aerobic metabolism. Oxidase-, gelatinase- and aesculin-positive, catalase-negative. Nitrate is reduced to nitrite. The fatty acid profile of strain CC-SEYA-1^T is shown in Table 2. Dextrin, glycogen, Tween 40, Tween 80, *N*-acetyl-D-glucosamine, i-erythritol (weak), cellobiose, D-fructose, α-D-glucose, *m*-inositol, D-mannose, D-psicose, β-methyl-D-glucoside (weak), trehalose, turanose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, *cis*-aconitic acid, formic acid, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid (weak), α-hydroxybutyric acid, β-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid (weak), DL-lactic acid, propionic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, DL-carnitine (weak), urocanic acid, inosine, uridine, thymidine, phenylethylamine (weak), 2-aminoethanol, 2,3-butanediol, DL-α-glycerol phosphate, α-D-glucose 6-phosphate and D-glucose 6-phosphate were utilized (Biolog GN-II). α-Cyclodextrin, *N*-acetyl-D-galactosamine, adonitol, D-arabitol, L-fucose, D-galactose, gentiobiose, L-arabinose, DL-lactose, lactulose, maltose, D-mannitol, melibiose, raffinose, L-rhamnose, D-sorbitol, sucrose, xylitol, D-galactonic acid lactone, D-glucuronic acid, γ-hydroxybutyric acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, γ-aminobutyric acid and glycerol were not utilized as carbon sources (Biolog GN-II). Positive (API 20E and 20NE) for arginine dihydrolase, citrate utilization, gelatinase, glucose oxidation, reduction of nitrate to nitrite, glucose fermentation, aesculin hydrolysis, gelatinase, assimilation of glucose, *N*-acetylglucosamine, potassium gluconate, capric acid, malic acid, trisodium citrate and phenylacetic acid, but negative for β-galactosidase, lysine decarboxylase, H_2S production, urease, tryptophan deaminase, indole production, acetoin production, mannitol oxidation, inositol oxidation, sorbitol oxidation, rhamnose oxidation, sucrose oxidation, melibiose, amygdalin oxidation, arabinose oxidation, arabinose assimilation, mannose assimilation, mannitol assimilation, maltose assimilation and adipic acid assimilation. Positive for alkaline and acid phosphatase, butyrate esterase (C4), caprylate esterase (C8), myristate lipase (C14), leucine arylamidase, α-chymotrypsin, naphthol-AS-B-1-phosphohydrolase and *N*-acetyl-β-glucosaminidase, but negative for valine arylamidase, cystine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase in API ZYM enzyme reactions. Sensitive to cotrimoxazole, tetracycline, minocycline, nor/quinolones 2G, levofloxacin and nitrofurantonin; resistant to penicillin, gentamicin, erythromycin, clindamycin, vancomycin, teicoplanin, rifampicin, fusidic acid, quinupristine-dalfo, coagoxacillin and oxacillin. Further physiological features are given in Table 1.

The type strain, isolated from spring-water samples from Yang-Ming Mountain, Taipei County, Taiwan, is CC-SEYA-1^T (=CCUG 55175^T=BCRC 17769^T).

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