Schlegelella aquatica sp. nov., a novel thermophilic bacterium isolated from a hot spring

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A moderately thermophilic bacterial strain designated wcf1^T, isolated from a hot spring located in the Tainan area, southern Taiwan, was characterized using a polyphasic approach. The cells were Gram-negative, non-pigmented, rod-shaped, non-spore-forming and motile. Phylogenetic analysis using 16S rRNA gene sequences showed that the strain formed a monophyletic branch towards the periphery of the evolutionary radiation occupied by the genus *Schlegelella*; its only close neighbour was the type strain of *Schlegelella thermodepolymerans*, K14^T (97·8 %). The isolate was clearly distinguishable from other strains using phylogenetic analysis, DNA–DNA hybridization, fatty acid composition data and a range of physiological and biochemical characteristics. It was evident from the genotypic and phenotypic data that strain wcf1^T represents a novel species in the genus *Schlegelella*, for which the name *Schlegelella aquatica* sp. nov. is proposed, with the type strain wcf1^T (=BCRC 17557^T=LMG 23380^T).

The genus *Schlegelella* was proposed by Elbanna *et al.* (2003) and belongs to the subgroup *Rubrivivax* of the *Betaproteobacteria*. The genus *Schlegelella* currently comprises a single species with a validly published name, *Schlegelella thermodepolymerans*. The two strains of *S. thermodepolymerans* (K14^T and DhA-71) (Elbanna *et al.*, 2003) and an independently isolated bacterium, *Caenibacterium thermophilum* (N2-680^T) (Manaia *et al.*, 2003), were described simultaneously as gen. nov., sp. nov. However, after confirming their closer relationship, Lütke-Eversloh *et al.* (2004) reclassified *C. thermophilum* N2-680^T as a later synonym of *S. thermodepolymerans*. All of the strains of *S. thermodepolymerans* are moderately thermophilic (optimum growth at 45–50 °C) and have been isolated from man-made habitats. The type strain of *S. thermodepolymerans* was isolated from activated sludge, whilst *C. thermophilum* N2-680^T was isolated from an aerobic digester of municipal sludge.

The aim of the present study was to determine the taxonomic position of the isolate wcf1^T, which was isolated from a water sample (natural habitat) collected from a hot spring located in the Tainan area, southern Taiwan.

The water sample was collected in July 2005 from a hot spring in Guanzingling located in southern Taiwan. The temperature of the spring water ranged from 60 to 65 °C and the pH value was approximately 8. The chemical composition of the Guanzingling hot spring water sample is listed in Supplementary Table S1 in IJSEM Online. After diluting with sterile distilled water, the water sample was plated on 10 % Luria–Bertani (LB) agar plates (1·0 g tryptone, 0·5 g yeast extract, 1·0 g NaCl and 15 g agar in 1 l distilled water) and incubated at 55 °C. A bacterial strain, designated wcf1^T, was isolated, maintained and subcultivated further on 10 % LB medium. The organism was the subject of a polyphasic taxonomic study, which showed that it represented a novel species of *Schlegelella*.

Cell morphology was observed under a light microscope. The motility of cells was tested by the hanging-drop method. A Gram stain Set S (Difco) kit was used for testing the Gram reaction, and poly-β-hydroxybutyrate granule accumulation was observed by light microscopy after staining the cells with Sudan black. Flagellar staining was performed using a spot test flagella stain (Difco). The pH range for growth was determined by measuring OD_{595} of the culture grown in 10 % LB broth that had been 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 various temperatures (15–70 °C) was measured on 10 % LB medium. Growth under anaerobic conditions was determined after incubation in an Oxoid AnaeroGen system. The performance of growth was recorded by measuring the OD of the culture with respect to time.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain wcf1^T is DQ417336.

A table detailing the chemical composition of the Guanzingling hot spring water sample is available as supplementary material in IJSEM Online.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Chen *et al.*, 2001). Sequence analysis was achieved using a DNA sequencer (ABI PRISM 310; Applied Biosystems) and sequence assembly by using the Fragment Assembly System program from the Wisconsin Package 9.1 supplied by the National Health Research Institute of Taiwan. The resultant sequence was compared with available 16S rRNA gene sequences in GenBank. Multiple-sequence alignment including strain wcf1^T and its closest relatives was performed using BIOEDIT software (Hall, 1999). Phylogenetic trees were inferred using the maximum-parsimony and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms. An evolutionary distance matrix was generated for the neighbour-joining algorithm using the Jukes & Cantor (1969) distance model and bootstrap analysis (1000 resamplings).

For determination of G+C content, 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. DNA–DNA hybridization experiments were performed with photobiotin-labelled probes as described by Ezaki *et al.* (1989). The degree of hybridization was determined from two replicate experiments. Further differentiation of strain wcf1^T from its closest phylogenetic neighbour was determined by cellular fatty acid profiling using gas chromatography according to the instructions of the Microbial Identification System (MIDI; Microbial ID).

Strain wcf1^T was examined for a broad range of phenotypic properties. Additional biochemical tests were performed at 50 °C using the Biolog GN2 (Biolog), API ZYM (bioMérieux) and API 20NE (bioMérieux) microtest systems according to the methods outlined by the manufacturers. Sensitivity to antibiotics was examined after spreading cells (0·5 McFarland) on 10 % LB medium and incubating at 50 °C. Antibiotic discs (Difco) contained the following antibiotics: ampicillin (10 μg), cefotaxime (30 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), novobiocin (30 μg), rifampicin (5 μg), penicillin G (10 μg), streptomycin (10 μg) or tetracycline (30 μg). The effect of antibiotics on cell growth was assessed after 3 days and susceptibility was scored based on the distance from the edge of clear zone to that of the disc. If the distance was larger than 3 mm the strain was classified as susceptible, between 1 and 3 mm it was classified as moderately susceptible and if the clear zone was less than 1 mm it was considered resistant.

Strain wcf1^T grew well aerobically in complex media with a reduced concentration of nutrients, such as 10 % LB medium. When higher concentrations of nutrients (such as trypticase soy, nutrient and LB medium) were provided, growth was comparatively poor. Strain wcf1^T formed visible, cream or white colonies of circular and convex shape with entire edges. The colony size was approximately 1·0–1·5 mm in diameter on 10 % LB agar plates after incubation for 48 h at 55 °C. Strain wcf1^T grew well at temperatures ranging from 30 to 60 °C and from pH 6 to 8. Optimal growth occurred at 50 °C and pH 7·0. Strain wcf1^T did not grow after 120 h of incubation at 50 °C under anaerobic conditions.

Cells of strain wcf1^T were Gram-negative rods, motile by means of polar flagellum, non-spore-forming, 0·4–0·5 μm in diameter and 0·8–2·0 μm in length. Poly-β-hydroxybutyrate granules were observed after staining with Sudan black.

A nearly complete 16S rRNA gene sequence (1419 nt) was obtained for strain wcf1^T. A comparison of the sequence with those of representatives of genera classified in the *Betaproteobacteria* showed that the organism fell within the evolutionary radiation occupied by the genus *Schlegelella* (Fig. 1). According to the sequence similarity calculations, the organism was most closely related to *S. thermodepolymerans* strain K14^T (97·8 % similarity) (Elbanna *et al.*, 2003), *S. thermodepolymerans* strain N2-680 (97·9 % similarity) (Manaia *et al.*, 2003), *S. thermodepolymerans* strain SA8 (97·9 % similarity), *S. thermodepolymerans* strain SA1 (97·9 % similarity) (Romen *et al.*, 2004) and *S. thermodepolymerans* strain DhA-71 (97·8 % similarity) (Yu & Mohn, 1999) (Fig. 1). The similarity levels of strain wcf1^T with other bacterial species with validly published names within the *Betaproteobacteria* were less than 95 %.

In the whole-genome DNA–DNA hybridization experiments, strain wcf1^T showed a relatively low DNA–DNA relatedness value (45·0 %) with *S. thermodepolymerans* K14^T, clearly indicating that strain wcf1^T represents a novel species.

The G+C content of strain wcf1^T was 69·2 mol%, which is lower than that of *S. thermodepolymerans* (70·0 mol%).

The cellular fatty acid composition of strain wcf1^T is shown in Table 1 in comparison with *S. thermodepolymerans* K14^T. The fatty acid profile of strain wcf1^T was similar to that of *S. thermodepolymerans* K14^T, although the amounts of 16 : 0 and 17 : 0 cyclo differed. However, strain wcf1^T could clearly be distinguished from *S. thermodepolymerans* K14^T by the presence of a significant amount of summed feature 3 (19·5 %).

In API 20NE tests, strain wcf1^T was positive for oxidase (weak), nitrate reduction, aesculin hydrolysis, gelatin hydrolysis and assimilation of glucose, maltose, gluconate, adipate and phenyl acetate and negative for catalase, indole production, glucose fermentation, arginine dihydrolase, urease, β-galactosidase and assimilation of arabinose, mannose, mannitol, *N*-acetylglucosamine, caprate, malate and citrate. In API ZYM tests, wcf1^T was positive for alkaline phosphatase, C4 esterase, C8 lipase, C14 lipase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and α-glucosidase and negative for valine arylamidase, cystine

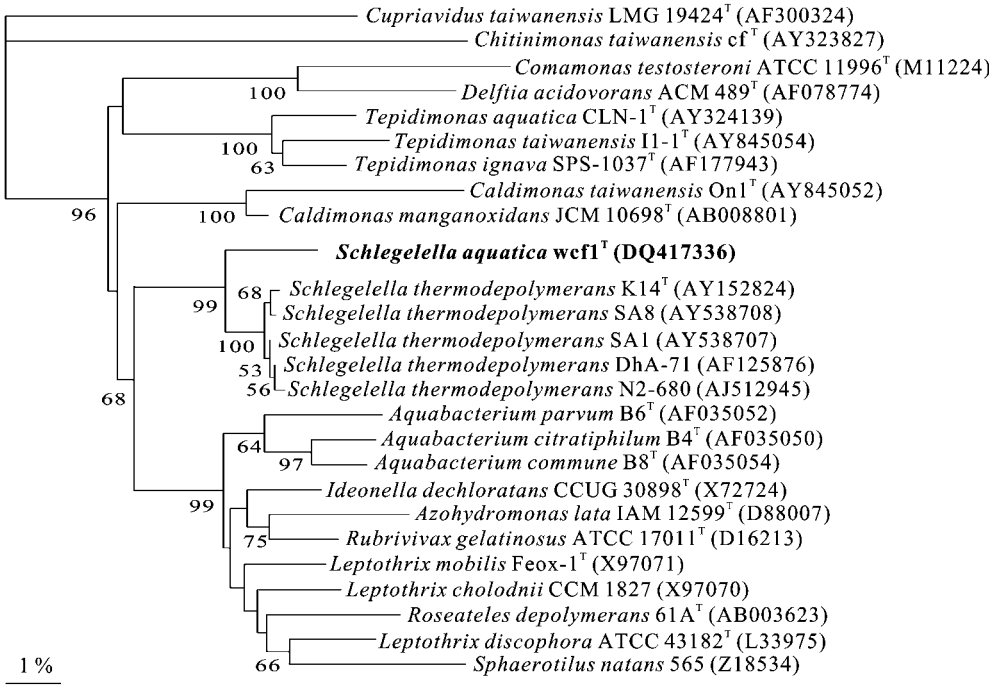

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences available from GenBank/EMBL/DDBJ (accession numbers are given in parentheses) constructed after multiple alignments of data showing the position of strain wcf1^T in the genus *Schlegelella*. Distances and clustering with the neighbour-joining method were performed using BioEdit. Numbers at nodes are percentage bootstrap values based on 1000 resampled datasets; only values above 50 % are given. The sequence of *Cupriavidus taiwanensis* was used as an outgroup. Bar, 1 % sequence dissimilarity per nucleotide position.

Table 1. Comparison of long-chain fatty acid compositions (%) of *S. aquatica* sp. nov. strain wcf1^T and *S. thermodepolymerans* K14^T

Data for *S. thermodepolymerans* K14^T are from Elbanna *et al.* (2003). Strain wcf1^T was cultivated for 48 h on TSA medium (Difco) at 50 °C, similar to the conditions described for *S. thermodepolymerans* K14^T by Elbanna *et al.* (2003). –, Fatty acid values of less than 1 %; ND, not detected.

Fatty acid	Strain wcf1^T	*S. thermodepolymerans* K14^T
10 : 0	1·2	1·3
10 : 0 3-OH	5·1	3·3
12 : 0	2·3	1·6
12 : 0 3-OH	ND	2·0
16 : 0	48·7	43·1
16 : 0 iso	ND	–
17 : 0 cyclo	16·5	32·6
18 : 1ω7*c*	4·3	4·4
18 : 1	–	–
18 : 0	1·0	–
Summed feature 3*	19·5	–

*Summed feature 3 comprises 16 : 1ω7*c* and/or 15 : 0 iso 2-OH.

arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The following compounds were utilized as sole carbon sources in the Biolog GN2 test system: glycogen, methyl pyruvate, monomethyl succinate, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, ρ-hydroxyphenylacetic acid, α-ketobutyric acid, α-ketoglutaric acid, DL-lactate, propionic acid, succinic acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-asparagine, L-proline, L-pyroglutamic acid and urocanic acid. However, strain wcf1^T could not oxidize α-cyclodextrin, dextrin, Tween 40, Tween 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, arabinose, arabitol, cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, *myo*-inositol, α-D-lactose, lactulose, D-mannitol, D-mannose, melibiose, methyl β-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, *cis*-aconitic acid, citrate, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, γ-hydroxybutyric acid, itaconic acid, α-ketovaleric acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, bromosuccinic acid, glucuronamide, L-alanyl glycine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl

L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, D-serine, L-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, DL-α-glycerol phosphate, D-glucose 6-phosphate and α-D-glucose 1-phosphate.

Strain wcf1^T was susceptible to ampicillin, cefotaxime, chloramphenicol, gentamicin, kanamycin, nalidixic acid, novobiocin, penicillin G, rifampicin, streptomycin and tetracycline.

Further comparisons of physiological and biochemical characteristics between strain wcf1^T and *S. thermodepolymerans* K14^T are presented in Table 2. It is apparent from Table 2 that strain wcf1^T could be distinguished from *S. thermodepolymerans* K14^T using a combination of phenotypic attributes (nitrate reduction, assimilation of carbon substrates such as arabinose, adipate, citrate, maltose and phenyl acetate and some enzyme activities such as catalase, cystine arylamidase and α-glucosidase). Despite physiological and biochemical attributes, the results of fatty acid composition and DNA–DNA hybridization clearly distinguished strain wcf1^T from *S. thermodepolymerans* K14^T. On the basis of the results of this polyphasic taxonomic study, strain wcf1^T represents a novel species of the genus *Schlegelella* for which the name *Schlegelella aquatica* sp. nov. is proposed.

Description of *Schlegelella aquatica* sp. nov.

Schlegelella aquatica (a.qua′ti.ca. L. fem. adj. *aquatica* living in water).

Cells are Gram-negative, non-spore-forming, rod-shaped, 0·4–0·5 μm in diameter and 0·8–2·0 μm in length. Cells occur singly and are motile with a single polar flagellum. Growth is evident at temperatures of 30–60 °C and pH 6·0–8·0. Optimum growth is at 50 °C and pH 7·0. Positive for nitrate reduction, gelatin hydrolysis and aesculin hydrolysis and weakly positive for oxidase activity. Assimilates glucose, maltose, gluconate, adipate and phenyl acetate. Major fatty acids are 16 : 0, 17 : 0 cyclo and summed feature 3 (16 : 1ω7*c* and/or 15 : 0 iso 2-OH). Additional biochemical characteristics are listed above and in Table 2.

Table 2. Phenotypic characteristics that differentiate *S. aquatica* sp. nov. strain wcf1^T from *S. thermodepolymerans* K14^T

Data for *S. thermodepolymerans* K14^T were acquired in the present study, except for the data on DNA G+C content (Elbanna *et al.*, 2003). +, Positive; −, negative; W, weak reaction; R, resistant; S, sensitive. Both strains are positive for C4 esterase, C8 esterase lipase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and assimilation of gluconate, lactate, acetate, succinate and pyruvate. The following characteristics are negative for both strains: trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-fucosidase, α-mannosidase and assimilation of mannose, galactose, fructose, rhamnose, sucrose, trehalose, mannitol, inositol, erythritol, adonitol and xylitol.

Characteristic	Strain wcf1^T	*S. thermodepolymerans* K14^T
Oxidase	W	+
Catalase	−	+
Cystine arylamidase	−	+
α-Glucosidase	+	−
Nitrate reduction	+	−
Assimilation of:		
Arabinose	−	+
Maltose	+	−
Adipate	+	−
Citrate	−	+
Phenyl acetate	+	−
L-Glutamic acid	−	+
Susceptibility to:		
Penicillin	S	R
Ampicillin	S	R
Streptomycin	S	R
Rifampicin	S	R
Maximum temperature for growth (°C)*	60	65
Optimum growth temperature (°C)*	50	50–55
Isolation source	Hot spring	Sludge
DNA G+C content (mol%)	69·2	70·0

*Strains K14^T and wcf1^T were inoculated into 50 ml 10 % LB broth in a 250 ml flask and agitated at 125 r.p.m. for 48 h.

The DNA G+C content of the type strain is 69·2 mol%. The type strain, wcf1^{T} (=BCRC 17557^{T}=LMG 23380^{T}), was isolated from a water sample from a hot spring in Guanzingling located in the Tainan area of southern Taiwan.

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