Bacillus macauensis sp. nov., a long-chain bacterium isolated from a drinking water supply

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A novel long-chain, Gram-positive, rod-shaped, endospore-forming strain was isolated from the influent of a drinking water treatment plant in Macau, China. This facultatively anaerobic isolate (2·0–5·0 μm in length and 0·8–1·2 μm in diameter) formed long chains over 100 μm in length in both liquid and solid media. It had a growth temperature range of 20–40 °C, with an optimum at about 30 °C, and a growth pH range of 6·0–10·0, with an optimum at pH 8·5. The G+C content of the DNA extracted was 40·8 mol%. The main fatty acid components were anteiso-$C_{15:0}$ (67·2 %) and iso-$C_{15:0}$ (21·7 %), with small quantities of iso-$C_{14:0}$, $C_{14:0}$, iso-$C_{16:0}$ and iso-$C_{17:0}$. The main quinone component was MK-7. Phylogenetic analyses based on its 16S rRNA gene sequence revealed that this isolate is a member of the genus *Bacillus*, with no close relatives at the species level (sequence similarity $<$96·1 %). These phenotypic and genetic properties suggested that this strain represents a novel species, for which the name *Bacillus macauensis* sp. nov. is proposed. The type strain is ZFHKF-1^T (=JCM 13285^T=DSM 17262^T).

At the time of writing, the genus *Bacillus* consists of 226 aerobic or facultatively anaerobic spore-forming species that are ubiquitous in nature. Their presence has been found in soil, water and even airborne dust (Berkeley *et al.*, 2002). Cells of *Bacillus* species are straight-sided with rounded or square ends. Most of them grow as single cells, but a few may form chains and filaments. The filamentous *Bacillus* species may be either pathogenic, such as *Bacillus anthracis* (Berkeley *et al.*, 2002), or non-pathogenic, such as *Bacillus funiculus* (Ajithkumar *et al.*, 2002) and *Bacillus mycoides* (von Klopotek, 1969). Long-chain *Bacillus* species have been isolated from wastewater-treatment systems (Ajithkumar *et al.*, 2002) and soil (von Klopotek, 1969), but (so far) not from drinking water supplies. In this study, we report on the taxonomic characterization of a novel long-chain *Bacillus* bacterium, strain ZFHKF-1^T, which was isolated from the drinking water supply of Macau, a city of subtropical climate located at the mouth of the Pearl River in China.

Strain ZFHKF-1^T was isolated, using Luria–Bertani agar medium, from water conveyed to the Macao Water Supply Company from the West River, upstream of Pearl River. The Gram-staining and endospore-forming features of this isolate were examined using a light microscope (E600; Nikon). The morphology was investigated using a confocal laser scanning microscope (PASCAL 5; Zeiss) after staining of the cells using SYTO9 (Molecular Probe). The ultrastructure was examined using a transmission electron microscope (100SX; JEOL) and a scanning electron microscope (Cambridge-360; Leica Cambridge). The cells of ZFHKF-1^T were rod-shaped, 0·8–1·2 μm in diameter and 2·0–5·0 μm in length (Fig. 1a, b) and formed long chains in both liquid and solid media. In solid medium, the cells formed large, white, opaque, irregularly shaped, flatly spread colonies with fuzzy boundaries. The long chains were intertwined within the colony-forming clusters (Fig. 1c), which did not appear to have any regular pattern. The ZFHKF-1^T cells in the colony grew into long unbranched chains over 100 μm in length (Fig. 1d). In liquid medium, the cells formed flocs with a net-like structure.

Strain ZFHKF-1^T was facultatively anaerobic and mostly Gram-positive. It degraded glucose under anaerobic conditions without producing gas. It was catalase-positive and capable of hydrolysing gelatin and forming swollen endospores. Tests to determine the optimum temperature and pH for cell growth were conducted in 150 ml flasks containing Luria–Bertani medium and with shaking at 60 r.p.m. Cell concentrations were based on OD_{660} measurements. Glass

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ZFHKF-1^T is AY373018.

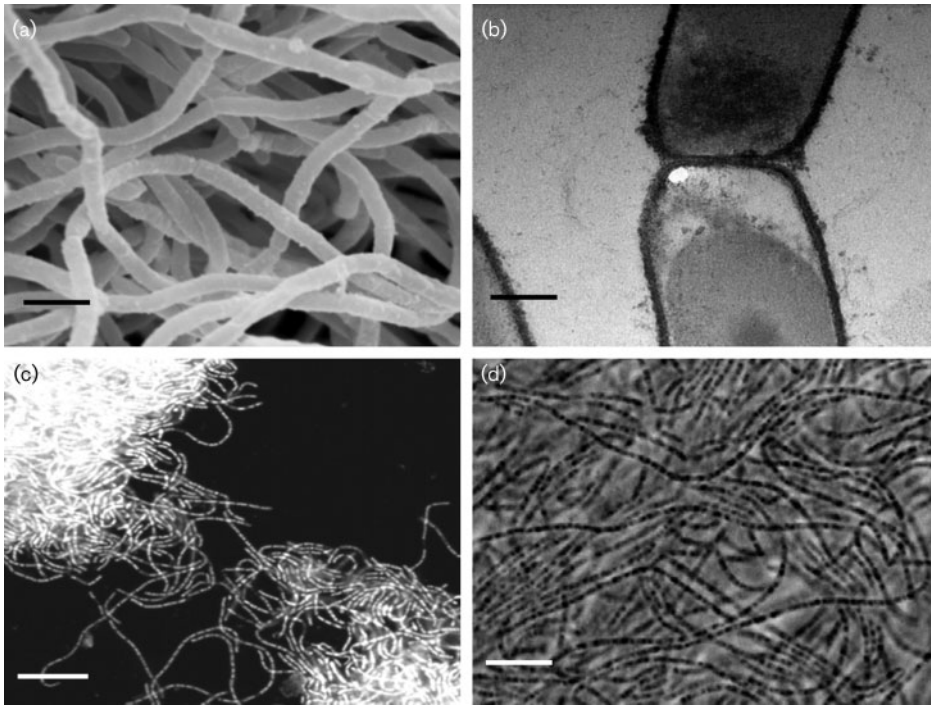

Fig. 1. Images of ZFHKF-1^T. (a) Scanning electron microscope image showing the long chains. (b) Transmission electron microscope image showing the connection between two adjacent cells. (c) Confocal laser scanning microscope image of cell clusters in the solid Luria–Bertani medium. (d) Phase-contrast microscope image of a cell cluster in Luria–Bertani medium. Bars: (a) 2 μm; (b) 0·5 μm; (c) 10 μm; (d) 8 μm.

beads were added to the incubation flasks for the dispersal of cells. Strain ZFHKF-1^T grew at pH 6·0–10·0 at 25 °C, with an optimum at pH 8·5, indicating its tolerance of slightly alkaline conditions. It grew at 20–40 °C at pH 8·5, with an optimum growth temperature of 30 °C. No growth was observed above 45 °C after 40 days incubation. The cells survived heat treatment at 80 °C for 10 min. Growth was slightly inhibited by NaCl at 20 g l^{-1}, and was completely inhibited by NaCl at 50–100 g l^{-1}.

The metabolic profile of strain ZFHKF-1^T was examined using the Biolog system, which included an array of 96 wells for the oxidation of 95 carbon sources. Sample preparation and analysis was performed according to the directions of the manufacturer (Biolog). The triplicate microplates were read, after 4 and 24 h incubation, using the Microstation hardware (Biolog), and the data were analysed using MICROLOG 3 software (Biolog). Of the 95 carbon sources tested, 22 could be oxidized by the isolate: β-cyclodextrin, cellobiose, D-galactose, gentiobiose, α-D-glucose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, 3-methyl glucose, L-rhamnose, D-ribose, sedoheptulosan, α-ketoglutaric acid, L-malic acid, monomethyl succinic acid, L-glutamic acid, glycyl L-glutamic acid, uridine, uridine 5′-monophosphate and glucose 1-phosphate. Oxidation of 55 of the remaining carbon sources varied among the three replicate microplates: inulin, mannan, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, amygdalin, D-arabitol, arbutin, D-fructose, L-fucose, D-gluconic acid, *myo*-inositol, α-D-lactose, D-melezitose, D-melibiose, methyl α-D-galactoside, methyl β-D-galactoside, methyl β-D-glucoside, methyl α-D-mannoside, palatinose, D-psicose, D-raffinose, salicin, D-sorbitol, stachyose, sucrose, turanose, D-xylose, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenyl acetic acid, α-ketovaleric acid, L-lactic acid, D-malic acid, methyl pyruvate, propionic acid, pyruvic acid, succinamic acid, succinic acid, *N*-acetyl-L-glutamic acid, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-serine, putrescine, 2,3-butanediol, glycerol, inosine, thymidine, adenosine 5′-monophosphate, thymidine 5′-monophosphate, fructose 6-phosphate, glucose 6-phosphate and α-DL-glycerol phosphate. The remaining 18 carbon sources tested could not be oxidized by the isolate.

DNA was extracted and purified as described previously (Kamagata & Mikami, 1991). The DNA G+C content of ZFHKF-1^T was 40·8 mol%, as determined by HPLC using LC-6A apparatus (Shimadzu) equipped with a UV detector (Shintani *et al.*, 2000). For analysis of quinones, fatty acids and cell walls, cells of ZFHKF-1^T were harvested from cultures grown in Luria–Bertani medium. Quinones were extracted from freeze-dried cells by using chloroform/methanol (2 : 1, v/v) and n-hexane. Extracts were purified using Sep-Pak Plus (Waters) (a cartridge type of silica gel containing spin purification column) and analysed by reverse-phase HPLC for identification (Tamaoka *et al.*,

1983). Whole-cell fatty acids were first converted, using anhydrous methanolic HCl (Komagata & Suzuki, 1987), to methyl esters, which were then extracted by n-hexane for analysis by GC/MS (M7200A GC/3DQMS equipment; Hitachi). The capillary used was DB-5ms coated with 5 % phenylmethylpolysiloxane at a thickness of 250 nm (Hanada *et al.*, 2002). The presence of diaminopimelic acid isomers in the cell-wall peptidoglycan was determined by TLC (no. 5716; Merck) after hydrolysis with 6 M HCl at 100 °C for 18 h (Komagata & Suzuki, 1987). Fatty acid methyl ester analysis showed that ZFHKF-1^T contained anteiso-$C_{15:0}$ (67·2 %), iso-$C_{15:0}$ (21·7 %), with small quantities of iso-$C_{14:0}$ (4·7 %), iso-$C_{17:0}$ (2·7 %), $C_{14:0}$ (2·2 %) and iso-$C_{16:0}$ (1·6 %). No unsaturated fatty acids were detected. MK-7 was the major quinone. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid.

The 16S rRNA gene fragment was amplified from the suspended cells of ZFHKF-1^T by means of a whole-cell PCR using the *Eubacteria*-specific primer set of 8F (5′-AGAGTTTGATCCTGGCTCAG-3′; positions 8–27, *Escherichia coli* numbering) and the prokaryote universal primer 1490R (5′-GGTTACCTTGTTACGACTT-3′; positions 1491–1509, *E. coli* numbering) (Weisburg *et al.*, 1991) in an automated thermal cycler (GeneAmp PCR 9700; Perkin-Elmer) (Zhang & Fang, 2001). After purification using the Wizard PCR Preps DNA purification kit (Promega), the 16S rRNA gene fragment was sequenced using an autosequencer (ABI model 377A; Perkin-Elmer) and the dRhodamine Terminator Cycle Sequencing FS Ready Reaction kit (Perkin-Elmer) with the primer set of EUB8F, 1490R and 1055R (5′-CACGAGCTGACGACAGCCAT-3′). The 16S rRNA gene sequence was then manually edited using BioEdit (Hall, 1999) and compared, using a BLAST search, with the sequences of reference micro-organisms available in GenBank. The 16S rRNA gene sequence was then aligned with the most similar 16S rRNA gene sequences of reference micro-organisms retrieved from GenBank, and checked manually using BioEdit. A phylogenetic tree was subsequently constructed using MEGA 2.1 software (Kumar *et al.*, 1993).

On the basis of analysis of 1355 bp of the 16S rRNA gene sequence, strain ZFHKF-1^T was found to be most closely related to three unidentified *Bacillus* species, including two deep-sea isolates, i.e. strain HTA437 (97 % similarity) and strain HTA506 (97 % similarity) (Takami *et al.*, 1997), and the isolated bacterium strain 47083 (96 %) (Drancourt *et al.*, 2000), as well as to two known species, i.e. *Bacillus barbaricus* (96 % similarity) (Täubel *et al.*, 2003) and *Bacillus megaterium* (95 % similarity) (Suzuki & Yamasato, 1994). The phylogenetic tree shown in Fig. 2 demonstrates that strain ZFHKF-1^T is a member of the genus *Bacillus*. On the basis of the widely recognized criterion in current bacteriology that bacteria with more than a 3 % difference in 16S rRNA gene sequences are of different species (Stackebrandt & Goebel, 1994; Rossello-Mora & Amann, 2001), ZFHKF-1^T is therefore a novel species of the genus *Bacillus*.

The phenotypic properties and fatty acid profiles of strain ZFHKF-1^T and its closest known species, *B. barbaricus* V2-BIII-A2 and *B. megaterium* DSM 32, as well as *B. funiculus* NAF001, which has a similar long-chain morphology, are summarized in Tables 1 and 2. Although all of the closest relatives of *Bacillus* species together with strain ZFHKF-1^T share common fatty acid components, having anteiso-$C_{15:0}$ and iso-$C_{15:0}$ as the main constituents, there were clear differences among them in terms of physiological and biochemical traits. In particular, the isolate could be differentiated from the others in utilizing salicin and not utilizing D-trehalose. On the basis of these data, strain ZFHKF-1^T should be placed in the genus *Bacillus* as a novel species, for which the name *Bacillus macauensis* sp. nov. is proposed (in acknowledgement of the city in which it was first found).

Description of *Bacillus macauensis* sp. nov.

Bacillus macauensis (ma.cau.en′sis. N.L. masc. adj. *macauensis* pertaining to Macau, the city where the type strain was isolated).

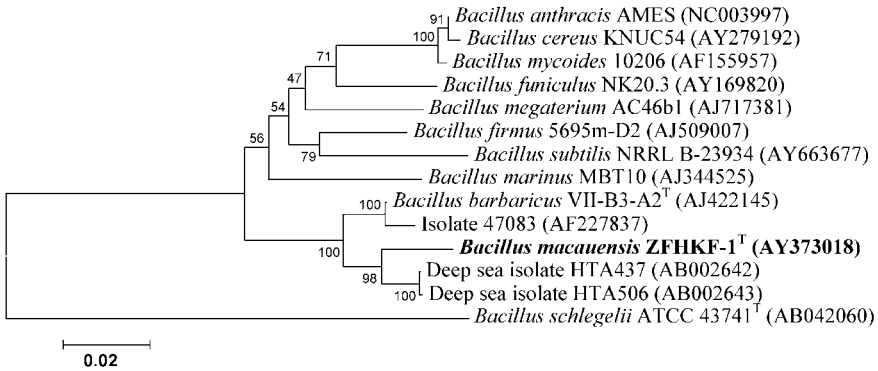

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences, using Jukes–Cantor distance, showing the phylogenetic relationships of ZFHKF-1^T. Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points. *Bacillus schlegelii* ATCC 43741^T was used as the outgroup species. Bar, 0·02 substitution per nucleotide position.

Table 1. Differential morphological, physiological and biochemical properties of ZFHKF-1^T and related species

Strains: 1, ZFHKF-1^T; 2, *B. barbaricus* V2-BIII-A2, data from Täubel *et al.* (2003); 3, *B. megaterium* DSM 32, data from Täubel *et al.* (2003); 4, *B. funiculus* NAF001, data from Ajithkumar *et al.* (2002). +, Positive; −, negative; ND, not determined.

Characteristic	1	2	3	4
Long-chain morphology	+	−	−	+
Budding cells	−	−	−	+
Growth at pH 9·5	+	+	−	−
Growth with 2 % NaCl	+	+	ND	−
Hydrolysis of:				
Gelatin	+	ND	+	−
Tween 80	−	−	+	−
Utilization of:				
p-Arbutin	+	−	−	ND
D-Cellobiose	+	−	−	ND
D-Galactose	+	−	−	+
D-Mannose	+	+	−	ND
L-Rhamnose	+	−	+	ND
D-Ribose	+	+	−	+
Salicin	+	−	−	−
Sucrose	+	−	−	+
D-Trehalose	−	+	+	+
i-Inositol	+	−	+	−
D-Mannitol	+	−	+	ND
DL-Lactate	+	−	+	ND
L-Malate	+	+	−	−
Pyruvate	+	+	−	ND
DNA G+C content (mol%)	40·8	ND	37·5	37·2

ZFHKF-1^T cells (rod-shaped, 2·0–5·0 μm × 0·8–1·2 μm) form long unbranched chains over 100 μm in length on agar as well as in broth. Gram-positive, catalase-positive, gelatin-hydrolysing and endospore-forming. Grows at 20–40 °C (optimum 30 °C) and pH 6·0–10·0 (optimum pH 8·5). NaCl at 5–10 % concentration inhibits its growth. Cellular fatty acids mainly comprise anteiso-$C_{15:0}$ (67·2 %) and iso-$C_{15:0}$ (21·7 %), with a little iso-$C_{14:0}$, $C_{14:0}$, iso-$C_{16:0}$ and iso-$C_{17:0}$. MK-7 is the major quinone. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. May oxidize β-cyclodextrin, cellobiose, D-galactose, gentiobiose, α-D-glucose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, 3-methyl glucose, L-rhamnose, D-ribose, sedoheptulosan, α-ketoglutaric acid, L-malic acid, monomethyl succinate, L-glutamic acid, glycyl L-glutamic acid, uridine, uridine 5′-monophosphate and glucose 1-phosphate. The DNA G+C content of the type strain is 40·8 mol%.

Table 2. Fatty acid content (%) of ZFHKF-1^T and related species

Strains: 1, ZFHKF-1^T; 2, *B. barbaricus* V2-BIII-A2, data from Täubel *et al.* (2003); 3, *B. megaterium* DSM 32, data from Täubel *et al.* (2003); 4, *B. funiculus* NAF001, data from Ajithkumar *et al.* (2002).

Fatty acid	1	2	3	4
iso-$C_{14:0}$	4·6	9·4	6·7	0·0
$C_{14:0}$	2·2	0·9	0·0	0·0
iso-$C_{15:0}$	21·7	19·0	13·9	18·0
anteiso-$C_{15:0}$	67·2	42·8	70·3	44·3
iso-$C_{16:0}$	1·6	6·0	0·0	3·84
iso-$C_{17:0}$	2·7	0·0	0·0	1·24

The type strain, ZFHKF-1^T (=JCM 13285^T=DSM 17262^T) was isolated from the drinking water supply of Macau, a city of subtropical climate in China.

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