Cohnella fontinalis sp. nov., a xylanolytic bacterium isolated from fresh water

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A novel xylan-degrading bacterium, YT-1101^T, was isolated from fresh water. The isolate was a Gram-reaction-negative, aerobic, motile, endospore-forming and rod-shaped bacterium. Phylogenetic analysis of the 16S rRNA gene sequence indicated that strain YT-1101^T belonged to the genus *Cohnella*, sharing sequence similarities of less than 94 % with the type species. The genomic G + C content was 58.6 mol%. The predominant menaquinone was MK-7. The major fatty acids were anteiso-C_{15:0}, iso-C_{16:0} and iso-C_{15:0}. Major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. On the basis of morphological, physiological and phylogenetic properties, strain YT-1101^T represents a novel species of the genus *Cohnella*, for which the name *Cohnella fontinalis* sp. nov. is proposed. The type strain is YT-1101^T (=NBRC 104957^T =DSM 21753^T).

The genus *Cohnella* was proposed by Kämpfer *et al.* (2006) as a homogeneous group within the family *Paenibacillaceae*. The members of the genus are aerobic, spore-forming and contain MK-7 as the main menaquinone and some kinds of phosphatidylglycerol as the predominant polar lipid. The genus is also characterized by the presence of two unknown phospholipids and four unknown aminophospholipids. García-Fraile *et al.* (2008) emended the description of the genus *Cohnella* as follows: cells are non-motile or motile, and most species are thermotolerant; predominant polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. At the time of writing, the genus *Cohnella* consisted of four species with validly published names (<http://www.bacterio.cict.fr/c/cohnella.html>): *Cohnella thermotolerans* (type strain isolated from industrial starch production), *Cohnella hongkongensis* (human blood), *Cohnella laeviribosi* (a volcanic area) and *Cohnella phaseoli* (a root nodule of *Phaseolus coccineus*).

Recently, we isolated bacteria that required a high-CO₂ atmosphere and studied their 16S rRNA gene sequences (Ueda *et al.*, 2008). The results highlighted the potential diversity of such bacteria and showed that the use of high-CO₂ air helps in effective mining of uncultured microorganisms. In this study, we characterized one of the isolates taxonomically, strain YT-1101^T, which belonged to the genus *Cohnella*. This is the first report of the isolation of a member of this group of bacteria from the hydrosphere.

In the original isolation process, YT-1101^T was one of the strains that exhibited marked dependence on a high-CO₂ atmosphere; however, after successive cultivation, the isolate became culturable even under a normal atmosphere, as did several other isolates (Ueda *et al.*, 2008). Here, to characterize its physiological properties, strain YT-1101^T was grown aerobically under a normal atmosphere for 1–2 days at 40 °C on tryptic soy agar (TSA; Difco) adjusted to pH 7.0. The bacterium was maintained as a glycerol suspension (20 %, w/v) or by using Microbank (Pro-Lab Diagnostics) at –80 °C. The reference strains *C. thermotolerans* DSM 17683^T, *C. hongkongensis* DSM 17642^T, *C. phaseoli* DSM 19269^T and *C. laeviribosi* CCUG 52217^T were obtained from the DSMZ or CCUG.

Cell morphology was observed under a BZ-9000 phase-fluorescence microscope (Keyence), a VE-8800 scanning electron microscope (Keyence) and a JEM-1200EX electron microscope (JEOL), using cells grown for 24 h at 40 °C. For transmission electron microscopy, cells were fixed with 2.0 % (v/v) glutaraldehyde and 2.0 % (v/v) osmium tetroxide. Samples were embedded in Epoxy resin (Epon 812). Ultrathin sections were prepared using an LKB-8800 ultramicrotome. Specimens were stained with uranyl acetate solution (containing 2.0 % uranyl acetate and lead citrate) and exposed to carbon vapour prior to observation. Motility was investigated in wet mounts under a Zeiss Axioskop 2 optical microscope (Carl Zeiss Microimaging) at 12 and 48 h. Flagella were stained by the method of Toda (1928). The Gram reaction was performed following the method described by Bartholomew & Mittwer (1952). Oxidase was tested using oxidase reagent (bioMérieux) according to the instructions of the manufacturer. Catalase activity was determined by treating cells with 3.0 % (w/v) H₂O₂ and observing formation

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene of strain YT-1101^T is AB362828.

Micrographs of strain YT-1101^T and results of 2D TLC of its polar lipids are available as supplementary material with the online version of this paper.

of bubbles. To test biochemical characteristics, the methods of Tindall *et al.* (2007) were used for the following tests: hydrolysis of agar (1.0 %, w/v), casein (5.0 %, w/v), starch (1.0 %, w/v), DNA (0.2 %, w/v), chitin (2.0 %, w/v), tyrosine (0.5 %, w/v), xylan (1.0 %, w/v) and powdered cellulose (type D; Advantec) (1.0 %, w/v); nitrate reduction; indole production; gelatin liquefaction; and activities of urease, lecithinase (using egg yolk agar) and lipase (using Tweens 20, 40 and 80 at 1.0 %, w/v). Carbon source utilization tests and additional physiological tests were performed by using API 50CH (with API CHB/E medium), API 20E and API 20NE galleries according to the instructions of the manufacturer (bioMérieux). The range of temperature (20, 28, 30, 35, 37, 40, 45, 50 and 60 °C) and salinity [0, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 8, 10, 15 and 20 % NaCl (w/v)] for growth were determined after 24 h cultivation in tryptic soy broth (TSB; Difco). The pH range for growth was assessed in TSB at 0.5 pH unit intervals between pH 5.0 and 12.0 using 100 mM citrate phosphate buffer (pH 5.0–5.5), 100 mM sodium phosphate buffer (pH 6.0–8.5), 100 mM sodium carbonate buffer (pH 9.0–10.0) and 100 mM sodium sesquicarbonate/NaOH buffer (pH 10.5–12.0). Growth was judged by the increase of optical density or decrease of pH after 24 h of cultivation. Growth on nutrient agar (Difco), R2A (Difco) and MacConkey agar (Oxoid) was also studied at 40 °C. Growth under anaerobic conditions was checked using an Anoxomat Mark II system (Mart Microbiology) after 10 days incubation at 40 °C on TSA.

Cells of YT-1101^T were aerobic, spore-forming, motile rods. The cells were peritrichously flagellated (Supplementary Fig. S1a, b, available in IJSEM Online). Although the Gram-staining reaction was negative, the cell-wall ultrastructure resembled that of Gram-positive bacteria (Supplementary Fig. S1c, d). Central or subterminal ellipsoidal endospores were observed in swollen sporangia. Observation using transmission electron microscopy revealed that the surface of the spores was covered with spikes, and that the spores exhibited a star-shape morphology in cross-section (Supplementary Fig. S1e, f). Colonies on TSA were translucent, cream-coloured, low-convex and circular with irregular edges, 1.0–1.5 mm in diameter. Strain YT-1101^T grew on nutrient agar and R2A, but not on MacConkey agar. Strain YT-1101^T and all members of *Cohnella* except *C. hongkongensis* showed xylanolytic activity (Table 1). Other physiological and biochemical characteristics of YT-1101^T are summarized in Table 1 and the species description.

Chemotaxonomic characterization of YT-1101^T was carried out using cells cultured in TSB for 24 h at 40 °C. DNA was extracted by using a bacterial genomic DNA purification kit (Edge Biosystems). The DNA G + C content of YT-1101^T, determined by an HPLC method (Mesbah & Whitman, 1989), was 58.6 mol% (mean of three measurements). The fatty acid methyl esters of YT-1101^T were extracted and analysed according to the standard protocol of the Sherlock Microbial Identification System (version 5.0; MIDI). As shown in Table 2, the major fatty acids of YT-1101^T were anteiso-C_{15:0}, iso-C_{16:0} and iso-C_{15:0}, which accounted for 68.1 % of the total fatty acids in

Table 1. Differential characteristics of strain YT-1101^T and type strains of *Cohnella* species

Strains: 1, *Cohnella fontinalis* sp. nov. YT1101^T; 2, *C. thermotolerans* CCUG 47242^T (data from Kämpfer *et al.*, 2006; Cho *et al.*, 2007); 3, *C. hongkongensis* HKU3^T (Teng *et al.*, 2003; Kämpfer *et al.*, 2006; Cho *et al.*, 2007); 4, *C. laeviribosi* RI-39^T (Cho *et al.*, 2007); 5, *C. phaseoli* GSPC1^T (García-Fraile *et al.*, 2008). Data for reference strains were based on the previous studies indicated except that xylanase activity of all species and colony colour of *C. thermotolerans* were observed in this study using the strains mentioned in the text. +, Positive; –, negative; w, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5
Colony colour*	C	WH	G	WH	WH–C
Motility	+	–	–	–	+
Temperature range (°C)	25–55	20–55	37–65	30–60	10–45
Growth at pH 8.5	+	ND	ND	–	–
Growth in 2 % NaCl	+	ND	ND	–	+
Gram-staining reaction	–	+	–	+	+
Catalase/oxidase	+/+	ND/+	w/+	+/–	+/w
Nitrate reduction	+	ND	+	ND	–
Xylanase	+	+	–	+	+
Assimilation of carbon sources					
Mannitol	+	–	–	+	–
N-Acetyl-D-glucosamine	–	–	+	–	+
Gluconate	+	+	+	–	–
DNA G + C content (mol%)	58.6	59	60.9	51	60.3

*C, Cream; G, grey; WH, white.

TSB; this feature is common to members of the genus *Cohnella*. However, the fatty acid profile of YT-1101^T differed from those of the other *Cohnella* species in terms of the absence of unsaturated fatty acids and the greater proportion of iso-C_{17:0}. The major respiratory quinone was determined to be MK-7 (99.6 %); in addition, a small amount of MK-8 was detected (0.4 %) by an HPLC method (Nishijima *et al.*, 1997). This quinone profile supports the affiliation of YT-1101^T to the genus *Cohnella* (Kämpfer *et al.*, 2006).

Polar lipid extraction and identification were performed according to Komagata & Suzuki (1987). Sugar- and choline-containing lipids were detected by using *p*-anisaldehyde and Dragendorff reagent, respectively. The spots were identified by using a phospholipid kit (Funakoshi) as a standard and referring to previous descriptions regarding polar lipid profiles of *Cohnella* species (Kämpfer *et al.*, 2006; Cho *et al.*, 2007; García-Fraile *et al.*, 2008). The dried lipid fraction was dissolved in chloroform/methanol (2:1, v/v) and subjected to HPTLC (Merck silica gel 60, 10 × 10 cm). Solvent systems used for two-dimensional development were chloroform/methanol/water (65:25:4 by vol.) in the first dimension and chloroform/acetic acid/methanol/water (80:15:12:4 by

Table 2. Cellular fatty acid compositions of strain YT-1101^T and type strains of the genus *Cohnella*

Strains: 1, *C. fontinalis* sp. nov. YT-1101^T; 2, *C. thermotolerans* CCUG 47242^T; 3, *C. hongkongensis* HKU3^T; 4, *C. laeviribosi* RI-39^T; 5, *C. phaseoli* GSPC1^T. The three major fatty acids for each taxon are shown in bold. Data for reference type strains are from Cho *et al.* (2007) and García-Fraile *et al.* (2008). Values are percentages of total fatty acids. tr, Trace (<1%); ND, not detected. C_{13:0} and C_{18:0} were not detected in any strain.

Fatty acid	1	2	3	4	5
Saturated					
C _{14:0}	1.0	1.0	5.0	1.9	1.8
C _{15:0}	2.3	1.4	8.0	1.3	5.3
C _{16:0}	12.5	6.6	25.3	9.2	8.9
C _{17:0}	tr	ND	1.2	tr	tr
Unsaturated					
C _{14:1ω5c}	ND	ND	ND	tr	ND
C _{16:1ω7c}	ND	ND	ND	ND	1.9
C _{16:1ω11c}	ND	ND	tr	tr	1.2
C _{17:1ω6c}	ND	1.0	ND	tr	ND
iso-C _{17:1}	ND	1.1	1.9	ND	ND
C _{18:1ω7c}	ND	4.0	ND	ND	ND
Branched					
iso-C _{12:0}	ND	ND	ND	ND	tr
iso-C _{13:0}	tr	ND	tr	tr	tr
anteiso-C _{13:0}	ND	ND	ND	tr	1.4
iso-C _{14:0}	4.0	2.1	2.3	3.9	2.6
iso-C _{15:0}	14.3	3.2	8.1	11.7	14.3
anteiso-C _{15:0}	33.2	28.4	31.2	22.0	44.5
iso-C _{16:0}	20.6	45.5	11.9	40.5	14.1
iso-C _{17:0}	7.2	ND	ND	2.8	3.1
anteiso-C _{17:0}	4.3	6.7	2.6	5.8	2.3

vol.) in the second dimension. Spots were visualized using 50% aqueous sulfuric acid.

Strain YT-1101^T contained diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine as the predominant polar lipids (Supplementary Fig. S2). This was consistent with the polar lipids profiles of *Cohnella* species (Kämpfer *et al.*, 2006; Cho *et al.*, 2007; García-Fraile *et al.*, 2008). An unknown phospholipid (PL2) and two unknown aminophospholipids (PN1 and PN2) were also detected. Lysyl phosphatidylglycerol and glycolipid were not detected. No spots were observed with Dragendorff reagent. The polar lipid pattern of YT-1101^T was distinct from those of *C. thermotolerans*, *C. hongkongensis* and *C. laeviribosi* in the absence of lysyl phosphatidylglycerol and that of *C. phaseoli* in the absence of glycolipids. In addition, the absence of unknown phospholipids and aminophospholipids with low *R_f* values (PL3, PL4, PN3 and PN4) distinguished YT-1101^T from the other *Cohnella* species that contain either type of phospholipid.

The 16S rRNA gene of YT-1101^T was obtained by PCR amplification using a bacterial domain-specific primer set, 27F/1492R (Wang *et al.*, 2007). PCR was performed on a

T1 Thermocycler (Biometra) using Ex *Taq* polymerase (Takara-shuzo). The PCR protocol included an initial denaturation period of 4 min at 94 °C, 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, a final elongation step at 72 °C for 3 min and incubation at 4 °C until further processing. The nucleotide sequence of the 16S rRNA gene fragment was determined directly by using the BigDye terminator version 3.1 cycle sequencing kit on an ABI 3130 Genetic Analyzer (Applied Biosystems). Phylogenetic analyses were performed using CLUSTAL W (Thompson *et al.*, 1994) and MEGA 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were constructed by neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods. The evolutionary distance matrix for the neighbour-joining method was generated according to Kimura's two-parameter model (Kimura, 1983). To evaluate the topology of the resultant tree, bootstrap analysis (Felsenstein, 1985) with 1000 resamplings was used. The 16S rRNA gene sequence of YT-1101^T (1518 bp) was compared with those from the GenBank/EMBL/DDBJ nucleotide sequence database, using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

The 16S rRNA gene sequence identity of YT-1101^T to the type strains of *C. thermotolerans*, *C. hongkongensis*, *C. laeviribosi* and *C. phaseoli* was 92.6, 93.8, 93.2 and 93.4%, respectively. These identity scores were low enough, according to Stackebrandt & Goebel (1994), to justify the placement of YT-1101^T within a novel species of the genus *Cohnella*. In the neighbour-joining phylogenetic tree (Fig. 1), YT-1101^T was affiliated to the genus *Cohnella*, and formed a distinct lineage with respect to closely related species. The topology of the phylogenetic tree constructed by using the maximum-parsimony algorithm was similar to that of the neighbour-joining tree (not shown).

Strain YT-1101^T differed from *C. laeviribosi*, its closest relative, in terms of the Gram-staining reaction, motility, DNA G+C content and temperature and pH ranges for growth. Strain YT-1101^T, but not *C. laeviribosi*, could be grown in the presence of 2% NaCl. Fatty acid and polar lipid profiling clearly differentiated YT-1101^T from the remaining species of *Cohnella*. In addition, strain YT-1101^T could be distinguished from members of the genus *Cohnella* by substrate-utilization patterns (Table 1).

On the basis of the data presented, we propose that strain YT-1101^T represents a novel species of the genus *Cohnella*, for which the name *Cohnella fontinalis* sp. nov. is proposed.

Description of *Cohnella fontinalis* sp. nov.

Cohnella fontinalis (fon.ti.na'lis. L. fem. adj. *fontinalis* of or from a fountain, referring to the isolation of the type strain from fresh water from a fountain).

Cells are Gram-type-positive, aerobic, endospore-forming rods, 0.5–0.7 × 1.5–6.5 µm. Motile by means of peritrichous flagella. Colonies are irregular, translucent, cream-coloured and usually 1.0–1.5 mm in diameter within 48 h

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain YT-1101^T with representatives from related genera. Bootstrap values (expressed as percentages of 1000 resampled datasets) are shown where >50%. The sequence of *Lactobacillus delbrueckii* subsp. *lactis* DSM 20072^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

at 40 °C on TSA. Growth occurs at 25–55 °C (optimum 40 °C) and pH 5.5–8.5 (optimum pH 6.0–7.0). Growth occurs at NaCl concentrations of up to 2.0% (w/v). Oxidase- and catalase-positive. Hydrolysis of xylan and aesculin is positive, while that of DNA, starch, agar, chitin, cellulose, casein, gelatin and tyrosine is negative. Positive for nitrate reduction. Negative for indole production, urease, lecithinase and lipase. β -Galactosidase is produced, but arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are not produced (API 20E). Acetoin and hydrogen sulfide are not produced. The following compounds in the API 20NE and API 50CH system are assimilated: D-glucose, L-arabinose, D-mannose, D-mannitol, maltose and potassium gluconate. The following compounds are not utilized as carbon and energy sources: N-acetyl-D-glucosamine, capric acid, adipic acid, malic acid, trisodium citrate, phenylacetic acid, glycerol, erythritol, ribose, xylose, adonitol, methyl β -D-xylopyranoside, galactose, fructose, sorbose, rhamnose, dulcitol, sorbitol, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, lactose,

melibiose, sucrose, trehalose, inulin, melezitose, raffinose, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, fucose, arabinol, 2-ketogluconate and 5-ketogluconate. The major isoprenoid quinone is MK-7. The major fatty acids are anteiso-C_{15:0}, iso-C_{16:0} and iso-C_{15:0}. Predominant polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The genomic DNA G+C content of the type strain is 58.6 mol%.

The type strain, YT-1101^T (=NBRC 104957^T =DSM 21753^T), was isolated from fresh water of a fountain in Japan.

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