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ORIGINAL PAPER

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Abstract A novel thermophilic, alkali-tolerant, and CO-tolerant strain JW/WZ-YB58^T was isolated from green mat samples obtained from the Zarvarzin II hot spring in the Uzon Caldera, Kamchatka (Far East Russia). Cells were Gram-type and Gram stain-positive, strictly aerobic, 0.7–0.8 μm in width and 5.5–12 μm in length and produced terminal spherical spores of 1.2–1.6 μm in diameter with the mother cell swelling around 2 μm in diameter (drumstick-type morphology). Cells grew optimally at pH$^{25°C}$ 8.2–8.4 and temperature 50–52°C and tolerated maximally 6% (w/v) NaCl. They were strict heterotrophs and could not use either CO or CO_2 (both with or without H_2) as sole carbon source, but tolerated up to 90% (v/v) CO in the headspace. The isolate grew on various complex substrates such as yeast extract, on carbohydrates, and organic acids, which included starch, D-galactose, D-mannose, glutamate, fumarate and acetate. Catalase reaction was negative. The membrane polar lipids were dominated by branched saturated fatty acids, which included iso-15:0 (24.5%), anteiso-15:0 (18.3%), iso-16:0 (9.9%), iso-17:0 (17.5%) and anteiso-17:0 (9.7%) as major constituents. The DNA G+C content of the strain is 45 mol%. Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain JW/WZ-YB58^T is distantly (<93% similarity) related to members of *Bacillaceae*. On the basis of 16S rRNA gene sequence, physiological and phenotypic characteristics, the isolate JW/WZ-YB58^T (ATCC BAA-1258; DSM 17740) is proposed to be the type strain for the type species of the new taxa within the family *Bacillaceae*, *Thermalkalibacillus uzoniensis* gen. nov. sp. nov. The Genbank accession number for the 16S rRNA gene sequence is DQ221694.

Keywords *Bacillales* · *Bacillaceae* · Thermophilic · Alkaliphilic · Carbon monoxide-tolerant · Fatty acids · *Firmicutes*

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Introduction

Alkali-tolerant bacteria and alkaliphiles have been investigated for decades for their potential in industrial application and biotechnology (Horikoshi 1999, 2004). Many of these organisms are mesophilic and halophilic (Nielsen et al. 1995; Horikoshi 2004). Thermophilic representatives, however, are less well known. Many previously described aerobic alkaliphiles and alkali-tolerant bacteria belong to the *Firmicutes*, and are represented by species within the *Bacillaceae* (Nielsen et al. 1995; Blum et al. 1998; Yumoto et al. 1998, 2003), al-

though many thermophiles are not validly published taxa (Kevbrin et al. 2004).

The family *Bacillaceae* is presently one of the taxonomically and phylogenetically diverse taxa undergoing revaluation. Based on the 16S rRNA gene sequences and G + C contents, many members of *Bacillaceae* have been re-classed from the genus *Bacillus* to novel genera, or even to a novel family, for example, *Alicyclobacillaceae* (Garrity et al. 2004). Currently (January 2006), the family *Bacillaceae* contains 24 genera (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi). Most bacteria within these genera share the features of being aerobic or microaerophilic, Gram-type and Gram stain-positive and spore-forming rods. However, diversity within *Bacillaceae* species may result in creation of more novel genera and more separation from the genus Bacillus.

During the course of enrichments for carboxydotrophic bacteria from CO-oxidizing hot spring microbial mats in the Uzon Caldera, Kamchatka, we isolated a novel member of the *Bacillaceae* (strain JW/WZ-YB58) that tolerates high CO concentrations (up to 90%), but appears unable to utilize CO as a carbon or energy source. CO tolerance has been reported previously for several members of the *Proteobacteria* (Cypionka and Meyer 1982), but has not been documented for *Firmicutes*. King (2003) has shown that some CO-oxidizing bacteria only consume CO at low (< about 1%) concentrations. To verify the potential for CO-utilization, King (2003) used a polymerase chain reaction (PCR) assay to amplify the large sub-unit gene (*coxL*) of CO dehydrogenase. Primers for this assay (King 2003) have been used to successfully amplify *coxL* from another member of the *Bacillaceae*, the carboxydotroph, *Bacillus schlegelii* (Dunfield and King 2004).

Methods

Sampling and Enrichment

Enrichments were carried out using a suspended mat sample obtained from the Zarvarzin II hot spring in the Uzon Caldera, Kamchatka, Russia, which previously oxidized CO when incubated with concentrations from 3 to 5% (Wiegel and Weber, unpublished data). The mat was greenish in color, located at the edges of the pool and frequently, totally submerged by the splashing pool water but intermittently exposed to air. Water temperature varied from 55 to 72°C at the sampling location; the temperature below the mat surface (0.5–2 cm thick) was 55 to 65°C. The pH of the spring varied from 6.0 to 7.5 within the mats and at the locations where mats were collected.

Mineral media supplemented with 0.05% (w/w) yeast extract and 20–27 mM pyruvate were used for enrichment and subsequent isolations. The mineral media contained (L^{-1} distilled water): 1g NH_4Cl, 3.6g NaCl, 1g KCl, 0.5g Na_2SO_4, 0.04g $CaCl_2$, 0.04g $MgCl_2{\cdot}6H_2O$, 0.5g NaH_2PO_4 and 1g K_2HPO_4, 1-mL vitamin solution, 1-mL trace-element solution and 1-mL Na_2SeO_3-Na_2WO_3 solution (Widdel and Bak 1992). The pH of the media was adjusted to 8.5 by titration with 2-M NaOH. Serum bottles (125-mL) for tenfold-dilution series contained 15-mL media and 2–4% (v/v) CO in air as the headspace. Cultures were incubated at 60°C for up to 4 weeks. Positive-growth cultures from the highest dilution were used as inocula for three successive transfers on agar (2% w/v) plates. Agar plates were incubated in anaerobic containers (Difco) having approximately 4% (v/v) of CO in the air headspace. Colonies from the third batch of plates were transferred to liquid media. Cells in the culture were checked for uniformity by phase-contrast light microscopy (Olympus VANOX) and later by 16S rRNA sequence analysis.

Utilization of electron donors and acceptors

The ability of the bacterium to utilize different substrates was studied in mineral media containing 0.02% (w/v) yeast extract (Difco) supplemented with filter-sterilized substrate stock solutions (final substrate concentrations 20 mM). Autotrophic growth of the bacterium was checked using mineral media containing 0.02% (w/v) yeast extract under a gas phase of 5% (v/v) CO or 10% CO_2 plus and minus 20% H_2 (v/v) and balanced by air.

The CO utilization of enrichments and pure cultures was assessed by gas chromatography (Carle Analytical Gas Chromatograph Series 400 by Chandler Engineering LLC) equipped with a thermal conductivity detector, using helium as carrier gas, and an oven temperature of 70°C. The CO-tolerant range for the isolate was determined in mineral media supplemented with 0.05% (w/v) yeast extract, 30 mM lactate and air as head gas containing 10 to 90% (v/v) of CO. To assess the oxidation of CO at low concentration (100 ppm), the isolate was grown at 50°C in stoppered 60-ml serum bottles containing 5 ml of basal salts medium with 25 mM pyruvate and 0.05% yeast extract. CO was added to the headspace at a final concentration of 100 ppm. Using a needle and syringe headspace samples were obtained at intervals over a 57-h period. Samples were assayed using a Trace Analytical reduced gas detector (model RGA3) (Hardy and King 2001).

The use of electron acceptors was determined in anaerobic nutrient-broth media under N_2 atmosphere and supplemented with autoclaved stock solutions of 15 mM electron acceptors, that is, fumarate, nitrate, nitrite, sulfate, thiosulfate, Fe(III) hydroxide and Fe(III) citrate. Freshly grown aerobic cultures to be used as inoculum were pre-flushed with oxygen-free N_2 gas and supplemented with $Na_2S \cdot 9H_2O$ (0.5 g L^{-1}) to remove trace amounts of oxygen. The cultures were incubated for up to 2 weeks at 52°C. Utilization of a particular substrate or an electron acceptor by the culture was determined by both direct cell counts using a phase-contrast microscope and by measuring the increase in optical density at 600 nm. Controls containing mineral

medium with 0.02% (w/v) yeast extract were performed in triplicate. Positive growth of a culture was based on an optimal density at 600 nm (Spectronic 21; Baush & Lomb) that was at least two times higher than that of the controls.

Temperature, pH and NaCl concentration

The ranges and optima of temperature, pH and NaCl concentration for growth were determined using mineral media supplemented with 0.05% (w/v) yeast extract and 20 mM fumarate. Temperature optimum was determined using media with a $pH^{25°C}$ 8.0 in a temperature-gradient shaking incubator (Scientific Industries) set between 30 and 80°C with a 2°C interval. Increase in cell numbers was followed for up to 2 weeks. The pH range for growth was determined at 52°C using the mineral media with 0.05 % (v/w) yeast extract and supplemented with a mixture of 10 mM each of MES, HEPES, CAPS and TAPS as buffers for the different pH values. The $pH^{25°C}$ values were adjusted by titration with HCl (5 M) and NaOH (5 M) before autoclaving and measured at room temperature again after 30 min of incubation. Salt requirement and tolerance was determined at $pH^{25°C}$ 8.2 and 55°C over a range from 0 to15% NaCl (w/v) by measuring increase of optical density (600 nm) over time and calculating the doubling time of each individual culture.

Microscopy

Light microscopy: Routine examinations and cell counts were performed with an Olympus microscope (VANOX). Phase-contrast micrographs of bacteria were taken using agar-coated slides.

Scanning electron microscopy (SEM): Cells were fixed in 2% glutaraldehyde/0.1 M cacodylate, post-fixed in osmium tetroxide/0.1 M cacodylate, dehydrated through graded ethanol washes, critical point dried and coated with gold by an SPI-Module Sputter Coater. SEM images were gathered on LEO 982 SEM/FEG using an accelerating voltage of 5 kV.

Transmission electron microscopy (TEM): Cultured cells were fixed in 2% glutaraldehyde/0.1 M cacodylate buffer for 1 h (RT), rinsed, and post-fixed in 1% osmium tetroxide (OsO_4) for 1 h (4°C). Samples were then washed in deionized water, dehydrated stepwise with increased percentage of ethanol washes and embedded in Epon 812 resin. Cells were infiltrated in fresh resin and polymerized for 24–48 h at 60°C. Thick sections of 50 nm thickness were cut using a diamond knife and post-stained with 4% uranyl acetate for 30 min followed by staining in 1% lead citrate for 5 min. TEM images were captured on a JEOL 100CX with an accelerating voltage of 80 kV.

Lipid analysis

Bacterial cell membrane lipids were extracted from lyophilized cells grown in 0.8% w/v nutrient broth (Difco) under optimal conditions (pH 8.3, temperature 52°C) and collected at the late exponential growth phase by centrifugation. Cells were kept under oxygen-free N_2 gas at −80°C until analysis. The total lipids were extracted, fractionated and transformed to obtain fatty acid methyl esters (FAME) of phospholipids as described previously (Guckert et al. 1985). FAMEs were analyzed by gas chromatography equipped with a flame ionization detector (Hewlett-Packard 6890) (Guckert et al. 1985). The commercial bacterial acid methyl ester (BAME) standard and GC-MS system were used for peak identification.

Biochemical characterization

Sensitivity to antibiotics was determined using Sensititre COMEQ2F plates (Trek Diagnostic Systems Ltd., UK). Biochemical characterization was performed following Claus and Berkeley (1986) API 20E strips (BioMerieux Inc.) and the BBL Crystal GP ID system (Becton Dickinson and Company, USA). Incubation temperature was 50°C.

DNA isolation and base composition

DNA was extracted using Qiagen DNeasy Tissue kit (Qiagen Inc.) according to the manufacture's instruction. The DNA G+C content was determined by high-performance liquid chromatography as described previously (Mesbah et al. 1989).

16S rRNA gene sequence

16S rRNA genes were amplified using the universal primer pairs 27f and 1492r (*Escherichia coli* numbering). PCR was conducted with 30 cycles of amplification at 94°C (1 min), 58°C (30 s) and 72°C (30 s), and a final elongation step of 72°C for 7 min. PCR products were purified using Qiaquick PCR purification Kit (Qiagen Inc.), and sequenced by Macrogen Inc. (Seoul, Korea). The assembled 16S rRNA gene sequence was aligned with a representative set of 16S rRNA sequences obtained from the GenBank database using ClustalX software (Higgins and Sharp 1988). Pairwise evolutionary distances were calculated by using the correction of Jukes and Cantor (1969). Phylogenetic trees were constructed with both neighbor-joining (Saitou and Nei 1987) and maximum-parsimony methods, with bootstrap analysis of 1,000 replications using the Mega 3.1 software (Kumar et al. 2004).

PCR amplification of *cox*L gene fragments

PCR amplification of fragments of *cox*L, which codes for the large subunit of CO dehydrogenase, was carried out using 50 µl reaction in 200 µl tubes. Reactions contained standard final concentrations of deoxynucleoside triphosphates and buffer and 0.5U of MasterTaq DNA Polymerase (Brinkmann Inc.); primers were present at 0.1 µM each and magnesium ion was present at 1.5 mM. The final DNA template concentration in the reaction was 3–4 ng. Two seperate reactions were carried out using primer sets that target authentic (OMP) and putative (BMS) *cox*L fragments. The forward primers for OMP and BMS fragments have the following nucleic acid sequences: OMPf (5'-GGCGGCTT[C/T]GG[C/G]AA[C/G] AAGGT-3') and BMSf (5'- GGCGGCT T[C/T]GG[C/G]TC[C/G]AAGAT-3'). The same reverse primer was used in both reactions: O/Br (5'-[C/T]TCGA[T/C]GATCATCGG[A/G]TTGA-3'). A DNA extract from *Stappia aggregata* (King 2003), a known CO-oxidizer was used for a positive control in both reactions. Amplification was completed using an Eppendorf Mastercycler thermocycler (Brinkmann Inc.). First, the templates were denatured for 3 min at 94°C, then 30 cycles of the following steps were completed: denaturation for 45 s at 94°C, annealing for 60 s at 58°C and extension for 90 s at 72°C. The 30 cycles were followed by a final 10 min extension at 72°C. PCR products were viewed under UV light after standard ethidium bromide gel electrophoresis.

Results

Colony and cell morphology

Four to ten-day-old colonies of strain JW/WZ-YB58^T, growing aerobically on solidified (agar, 2% w/v) mineral media containing 0.05 % (w/v) yeast extract, were circular with entire edges, 2–3 mm in diameter and transparent. After an additional week, swarming was observed leading to irregular spreading of the colonies. Cells of strain JW/WZ-YB58^T were motile rods of 0.7–0.8 µm in width and 5.5–12 µm in length with rounded ends (Fig. 1) and two to five peritrichously inserted flagella (Fig. 2a). Cells from early stationary phase tended to form chains of 3–12 cells. Spherical spores (1.2–1.6 µm in diameter) were located terminally with swelling (around 2 µm in diameter) of the mother cell (drumstick morphology) (Fig. 1). TEM revealed that the cells contain two types of unusual cytoplasmic inclusions of unknown nature and function and thus need further studies in the future.

Physiology

Strain JW/WZ-YB58^T was unable to use CO (1–5% v/v) or CO_2 (10% v/v) as the sole carbon source in the presence or absence of 10–20 % (v/v) H_2 within a 4-week incubation with or without shaking. No CO-oxidation was observed at 100 ppm CO, neither, by using a PCR assay, could a presence of either the authentic or the putative *cox*L fragments of a gene for CoxL be demonstrated, suggesting that the bacterium does not contain the ability of CO-utilization. However, while growing on 0.05% (w/v) yeast extract alone or with 10-mM pyruvate, the cultures were not inhibited by the presence of air containing 0–70% (v/v) CO and still grew under 90% (v/v) CO-containing air but the cell morphology became irregular. In the presence of 0.02% (w/v) yeast extract, strain JW/WZ-YB58^T grew on complex organic compounds as carbon and energy sources, such as beef extract, casamino acid, peptone, starch and tryptone. The following compounds served as carbon and energy source: dextrin, raffinose, lactose, sucrose, arabinose, fructose, D-galactose, D-glucose, D-mannose, trehalose, gluconate, glutamate, fumarate, lactate, pyruvate and acetate. Cells did not grow on alcohols and sugar alcohols. Cells were strict aerobes and unable to use nitrate, nitrite, fumarate, thiosulfate, Fe(III) hydroxide and Fe(III) citrate as electron acceptors for anaerobic growth while using 20-mM pyruvate as the carbon source. The substrate and electron acceptor spectrum of strain JW/WZ-YB58^T indicated an aerobic, organoheterotrophic and CO-tolerant physiology.

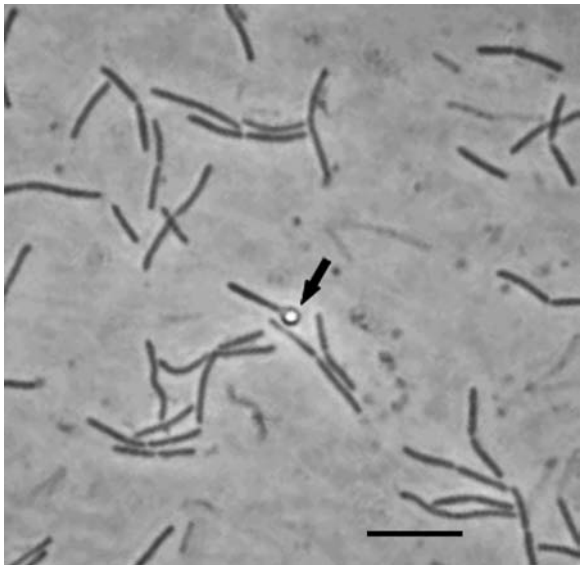

Fig. 1 Phase-contrast microphotograph of strain JW/WZ-YB58^T. Cells from the late exponential growth phase. *Arrows* indicate sporulated cell. *Bar* represents 10 µm

The temperature range for growth was between 42 and 64°C, with an optimum at 50–52°C at $pH^{25°C}$ 8.0. No growth occurred at 40°C or 66°C. At 52°C, the strain grew at $pH^{25°C}$ between 6.4 and 9.7, with an optimum of 8.2 to 8.4. No growth was observed at or below $pH^{25°C}$ 6.1 and at or above $pH^{25°C}$ 10.0. However, the growth of the strain at or below $pH^{25°C}$ 7 was very poor with doubling times longer than 14 h. The observed minimal

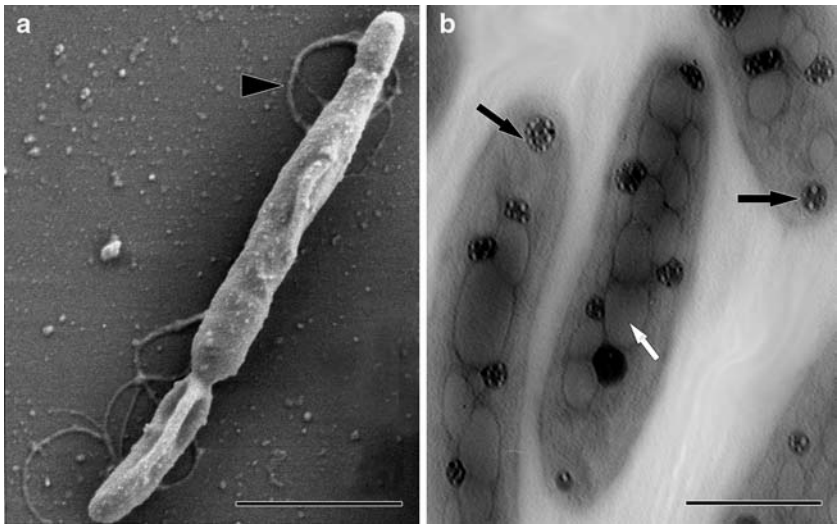

Fig. 2 Electron micrographs. **(a)** SEM revealed retarded perictrichous flagellation (*arrowhead*) and an uneven cell surface. Scale bar = 4 μm. **(b)** TEM revealed organized internal features (*white arrow*) and several circular electron dense bodies (*black arrow*) of unknown function and nature in each cell. *Scale bar* = 1 μm (courtesy of Rich Davis, UGA)

doubling time under optimal growth conditions (pH^{25C} 8.3, 52°C) was 2 h.

Biochemical characterization

Cultures were positive for Gram-staining reaction, oxidase reactions, ONPG hydrolysis and gelatin hydrolysis. Results were negative for catalase reaction, Voges-Proskauer reaction, indole production, urea hydrolysis and starch hydrolysis.

Cellular polar lipids of strain JW/WZ-YB58^T were dominated by branched saturated fatty acids including iso-14:0 (1%), iso-15:0 (24.5%), anteiso-15:0 (18.3%), iso-16:0 (9.9%), iso-17:0 (17.5%) and anteiso-17:0 (9.7%) accounting for about 81% of the total fatty acids (area %). Other important fatty acids were 14:0 (1.2%), 15:0 (5.1%), 16:1 (2.1%), 16:0 (7.6%), 17:0 (1.1%), 18:1 (0.9%) and an unknown fatty acid (1.1%).

The strain JW/WZ-YB58^T was sensitive to the following antibiotics (μg ml^{-1}): Amikacin, 4; Amoxicillin/clavulanic acid, 4/2; Ampicillin, 0.25; Cefazolin, 0.25; Cefoxitin, 2; Cefpodoxime, 2; Ceftiofur, 0.25; Cephalothin, 2; Chloramphenicol, 4; Clindamycin, 0.25; Enrofloxacin, 0.5; Erythromycin, 0.5; Imipenem, 1; Gentamicin, 1; Marbofloxacin, 0.25; Orbifloxacin, 1; Oxacillin,2; Penicillin, 0.06; Rifampin, 1; Tetracycline, 1; Ticarcillin, 8; Ticarcillin/clavulanic acid, 8/2; Trimethoprim/sulphamethoxazole, 0.5/9.5.

DNA composition and 16S rDNA sequences

The G+C content of genomic DNA of strain JW/WZ-YB58^T was 45 mol% (chromatographic method). The obtained 16S rRNA sequence (Genbank accession number DQ221694) contained 1,436 nucleotides corresponding to positions 33—1,470 of the *E. coli* 16S rRNA gene sequence. Using BLAST to compare with the currently available sequences in GenBank, no closely related sequence was found. The sequence suggest a distant relationship to the alkaliphilic isolate *Bacillus* sp. TA2.A1 (AF113512) (96.6% similarity) (Cook et al. 2003; Olsson et al. 2003; Peddie et al. 2000). A phylogenetic tree, based on neighbor-joining method, revealed that strain JW/WZ-YB58^T was phylogenetically related to the members of *Bacillaceae*. The closest neighbor was *Bacillus horti* DSM 12751 (93.1% similarity), a mesophilic and alkaliphilic bacterium isolated from the soil in Japan (Fig. 3). Similarities to other previously described *Bacillaceae* members were low (around 90.0%), and nearly equally distant to other major groups; for example, *B. clarkii* DSM8720^T 91.0% (X76444), *B. halodurans* DSM497^T 90.3% (AJ302709), *B. subtilis* W168/PY79 89.4% (K00637), *Geobacillus subterraneus* DSM13552^T 90.6% (AF276306), *G. stearothermophilus* DSM6285^T 90.3% (AY608989), *Aneurinibacillus thermoaerophilus* DSM10154^T 90% (X94186), *Brevibacillus thermoruber* DSM7064^T 89.2% (Z16921), and *B. thermocloacae* DSM5250^T 89.6% (Z26939).

Discussion

The pH and temperature profiles indicate that the strain JW/WZ-YB58^T is a facultative alkaliphilic, moderately thermophilic Gram staining and Gram-type positive (Wiegel 1981) rod-shaped bacterium, which differs from the closest phylogenetic neighbor *B. horti* in many aspects. Among the most distinguishable characteristics are the range of growth temperature and NaCl tolerance, spore morphology, O_2 requirement, Gram-staining and catalase reactions (Table 1). The 16S rRNA gene sequence analysis reveals that the other phylogenetic neighbors are mainly alkaliphilic or thermophilic members of the *Bacillaceae* family. However, the growth of strain JW/WZ-YB58^T occurs at higher temperatures and lower pH than other strictly alkaliphilic *Bacillus* species. In addition, strain JW/WZ-YB58^T is lower in tolerance to salt (0–6%, w/v, NaCl) and higher in DNA G+C content than major alkaliphilic *Bacillus* species (Nielsen

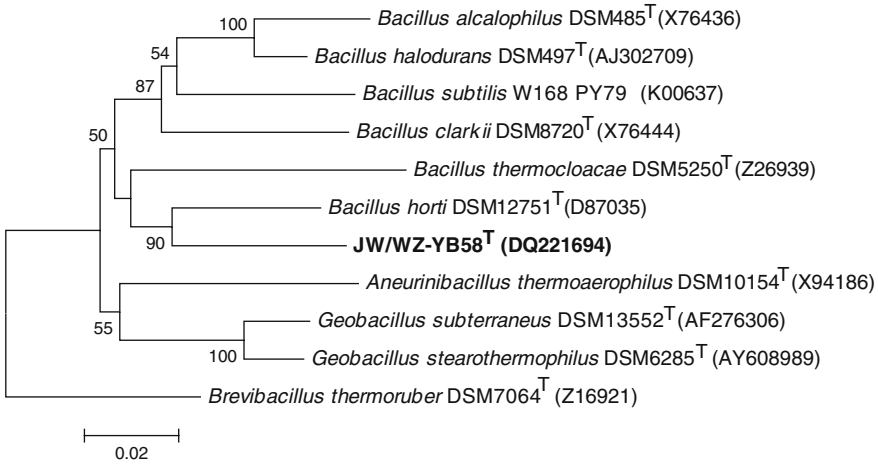

Fig. 3 Phylogenetic tree based on 16S rRNA gene sequence. Comparison between strain JW/WZ-YB58^T and other related, mainly alkaliphilic and thermophilic species was based on neighbor-joining method with bootstrap analysis of 1,000 replicates. Bootstrap values greater than 50% are shown. *Bar* equals 0.02 K_{nuc}

Table 1 Differentiating characteristics of strain JW/WZ-YB58^T and other related *Firmicutes* species

Characteristics	1	2	3	4	5	6	7	8	9	10
Sporangia										
Shape	S	E	ND	E	E	E	E	E	E	E
Position	T	S	ND	S	S	ST	ST	T	C	ST
Sporangia swollen	+	ND	ND	-	+	E	-	v	+	+
Catalase	-	+	ND	+	ND	+	+	+	-	-
Growth at pH 7	+	+	-	-	v	-	+	+	+	+
Growth at										
10°C	-	-	-	+	-	-	-	-	-	-
40°C	-	+	+	+	+	+	-	+	+	+
50°C	+	-	-	-	+	+	+	+	+	+
Growth in 10% (w/v) NaCl	-	+	+	-	v	-	-	-	-	-
Hydrolysis of starch	-	+	-	+	+	-	+	+	-	+
Reduction of nitrate	-	+	+	-	-	-	+	-	-	-
Mol% G+C content	45	40.2–40.9	42.4–43.0	36.2–38.4	42.1–43.9	41.7–42.1	49.7–52.3	51.9	46.7	56.2–57.8

Abbreviations: +, positive; -, negative; *v* variable; *ND* not determined; *S* spherical; *E* ellipsoidal; *T* terminal; *S* subterminal; *C* central. *Species:* 1, JW/WZ-YB58^T; 2, *B. horti* (Yumoto et al. 1998); *3*, *B. clarkii*; 4, *B. alcolophilus*; 5, *B. halodurans* (Nielsen et al. 1995); 6, *B. thermocloacae* (Demharter and Hensel 1989); 7, *Gb. subterraneus* (Nazina et al. 2001); 8, *Gb. stearothermophilus* (Kampfer 1994); 9, *Anb. thermoaerophilus* (Meier-Stauffer et al. 1996); 10, *Bb. thermoruber* (Manachini et al. 1985)

et al. 1995) (Table 1). Thermophilic members of the order *Bacillales* such as species belonging to the genera *Geobacillus, Aneurinibacillus* and *Brevibacillus*, on the other hand, usually grow optimally at neutral or slightly acidic pH (Nazina et al. 2001). The absence of a positive catalase reaction in strain JW/WZ-YB58^T distinguishes it from the majority of *Bacillus* species, which commonly show positive catalase reactions (Claus and Berkeley 1986). Terminally located spherical spores produced by strain JW/WZ-YB58^T rarely occur in its phylogenetic neighbors, which usually form centrally or sub-terminally located ellipsoidal spores (Table 1). The DNA G+C content for strain JW/WZ-YB58^T is 45 mol% and thus greater than that of most mesophilic–alkaliphilic *Bacillus* species but lower than that of neutral-thermophiles (Nazina et al. 2001; Manachini et al. 1985) (Table 1). Presently it is unclear how far the insensitivity toward CO is a taxonomically useful property because CO-utilization and/or inhibition by CO is not a common test for characterizing novel microorganisms, especially thermophiles. Gee and Brown (1980) and Cypionka and Meyer (1982) showed that different bacteria which includes *E. coli* (although at a slower growth rate), are not inhibited by concentrations of up to 30% v/v CO. Furthermore the question whether an organism can utilize CO needs to be carefully examined, since CO-utilization can be inhibited by elevated CO concentrations (King 2003; King et al., unpublished data; Moran et al. 2004) which includes e.g., CO-oxidizing members of the marine genus *Stappia*, which do not oxidize CO above 1000 ppm (King, unpublished data). However, the failure to demonstrate the presence of the *cox*L gene, which codes for the large subunit of CO dehydrogenase, suggests that this bacterium is not a CO-utilizer. At this time it is not clear how important the observed CO-insensitivity is for the isolate to function within the microbial mat from the hot pool Zarvarzin II and possibly in similar mats in the caldera. King and Weber (unpublished results) have measured in situ CO formation and CO utilization in these mats. The roles of CO-utilization and CO-insensitivity of bacteria in the hot spring mats need further investigations.

Cell membranes of *Bacillus* species are characterized by branched saturated fatty acids (Kaneda 1967; Claus and Berkeley 1986; Kampfer 1994; Nazina et al. 2001). The majority of alkaliphilic *Bacillus* species are dominated by anteiso-fatty acids with the ratio of total anteiso-/total iso- greater than 1, whereas strain JW/WZ-YB58^T has a total anteiso-/total iso- ratio of only 0.53 with the iso-15:0 as the most abundant component (Table 2). Thermophilic *Geobacillus* species and *Aneurinibacillus* species have similar cellular fatty acid compositions to strain JW/WZ-YB58^T with iso-15:0 being the most abundant fatty acid (Nazina et al. 2001; Heyndrickx et al. 1997; Meier-Stauffer et al. 1996). However, the total anteiso-/total iso- ratio is usually lower than 0.4 for *Geobacillus* species and even lower for *Aneurinibacillus* species (Nazina et al. 2001; Meier-Stauffer et al. 1996), which may be due to the lower growth pH of species in these genera than that of strain JW/WZ-YB58^T.

On the basis of physiological characteristics, phenotypic and phylogenetic properties, the isolate is placed according to the recent electronic Bergey' s Outline (Garrity et al. 2004) within the Phylum BXIII *Firmicutes*, Class *Bacilli*, Order I *Bacillales* and tentatively Family I *Bacillaceae* into a novel taxa, *Thermalkalibacillus uzonensis* gen. nov. sp. nov. with strain JW/WZ-YB58^T (ATCC BAA-1258 = DSM 17740) as the type strain for the type species *T. uzonensis*. Besides the type genus *Bacillus* with *B. subtilis* as type species, the Family I *Bacillaceae* contains also the genus *Geobacillus* (see Fig. 3). The genus *Geobacillus* contains several thermophilic and alkali-tolerant/alkaliphilic species formerly belonging to the genus *Bacillus*. However, the genus *Anaeurobacillus* (see Fig. 3) is assigned to Family V *Paenibacillaceae* (Garrison et al. 2004). Constructing several trees using the type species of the different genera and various algorithms (figures not shown), reveals that the phylogeny of the *Bacillales* is not clear at this time, as is evident from low (below 50%) boot strap values. Furthermore, defining the boundary for the novel genus has to await further isolations of species and strains belonging to this group, that is, to decide whether the closest neighbor in the phylogenetic trees we constructed, *B. horti* (Fig. 3), belongs to the novel genus or not.

Description of *Thermalkalibacillus* gen. nov

Thermalkalibacillus [therm.al.ka.li.ba.cil.lus. Gr. n. thermê, heat; Arabic word alkali (al-qaliy), the ashes of saltwort; L. masc. n. bacillus, a small staff, a wand; N.L. masc. n. *Thermalkalibacillus*, rod, loving combined growth conditions of elevated temperature and alkaline pH].

Cells are aerobic, spore-forming rods; thermophilic and facultative alkaliphilic or alkali-tolerant strict heterotrophs; utilize carbohydrate and organic acids for growth; DNA G + C mol% is about 45%.

Description of *T. uzonensis* sp. nov

T. uzonensis [u.zo.nen'sis. N.L. masc. adj. uzonensis pertaining to the isolation habitate Uzon Caldera, east of Mt Uzon in Kamchatka (Far East Russia)].

Table 2 Membrane phospholipid fatty acid composition of strain JW/WZ-YB58^T and other related species (shown in percentage of total fatty acid)

Fatty acids	1	2	3	4	5	6	7
a-13:0	-	-	-	-	5.1	-	-
i-14:0	1.0	-	0.6	1.8	0.1	2.9	0.2
14:0	1.2	2.4	0.5	0.3	1.5	-	0.2
i-15:0	24.5	38	32	29.1	40	37.8	54.3
a-15:0	18.3	30	43	37.9	6.4	2.3	0.6
15:0	5.1	-	0.3	-	0.5	1.4	0.6
i-16:0	9.9	4.4	1	4.9	6.2	29.2	2.3
16:1	2.1	5.3	1.5	0.2	-	1.7	3.5
16:0	7.6	1.1	2.1	3.2	9.2	-	-
i-17:0	17.5	1.4	4.3	11.2	17	18.5	32.8
a-17:0	9.7	7.9	11	9.6	13	5.8	0.8
i-17:1	-	-	1.7	0.3	-	-	-
a-17:1	-	-	0.9	0.2	-	-	-
C17:0	1.1	1.9	-	-	-	0.4	0.2
i-16:1	-	-	-	0.1	-	-	-
Others	2	8	0.6	1.3	0.8	-	4.5
Branched saturated	80.9	89	92	94.5	88	93.6	90.8
Total iso-	52.9	44	38	47	63	85.5	89.4
Total anteiso-	28	45	54	47.5	25	8.1	1.4
Anteiso-/iso-	0.53	1.02	1.44	1.01	0.39	0.09	0.02
Total	100	101	99	100	100	100	100

1, JW/WZ-YB58^T; 2, *B. horti* DSM12751^T (Yumoto et al. 1998); 3, *B. alcalophilus* DSM485^T; 4, *B. subtilus* (Yumoto et al. 2003); 5, *G. stearothermophilus* DSM6285^T (Kampfer 1994); 6, *G.subterraneus* DSM13552^T (Nazina et al. 2001); 7, *Aneurinibacillus thermoaerophilus* DSM10154^T (Meier-Stauffer et al. 1996)

Cells are Gram-type (Wiegel 1981) and Gram-staining positive, straight to slightly curved rods with 0.7–0.8 μm × 5.5–12 μm in dimension. Terminally located spherical spores (1.2–1.6 μm in diameter) cause swelling of the mother cells. Cells are motile with 2–5 peritrichous flagella. Growth only under aerobic conditions observed. Colonies on nutrient broth agar are circular with entire edges and transparent. Swarming occurs when colony ages. Oxidase reactions, ONPG hydrolysis and gelatin hydrolysis are positive. Catalase reaction, Voges-Proskauer reaction, indole production, urea hydrolysis and starch hydrolysis are negative. At $pH^{25°C}$ 8.0, the temperature range is 42–64°C with optimum at 50–52°C. The $pH^{25°C}$ range is 6.4–9.7 (pH optimum 8.2–8.4) when grown at 52°C. Tolerated NaCl concentration range is 0–6% (w/v). Cells grow on complex substrates (such as yeast extract), carbohydrates and acids but not on alcohols and sugar alcohols. Acid production occurs from trehalose, lactose, sucrose, arabinose and fructose. Major cellular fatty acids are iso-15:0 (24.5%), anteiso-15:0 (18.3%), iso-16:0 (9.9%), iso-17:0 (17.5%), anteiso-17:0 (9.7%) and 16:0 (7.6%). The G+C content of genomic DNA is 45 mol% (chromatographic method). Cells do not grow chemolithoautotrophically on 1–5% v/v CO or 10% CO_2 with or without H_2 and do not oxidize CO at low concentrations (100 ppm). Grown under shaking, CO is tolerated up to 90% (v/v) in air-containing gas headspace. The type strain is JW/WZ-YB58^T (ATCC BAA-1258; DSM 17740), which was isolated from a microbial mat sample collected from the edges of the hot spring Zarvarzin II in the Uzon Caldera, Kamchatka (Far East Russia). The GenBank accession number for the 16S rRNA gene sequence of the type strain is DQ221694.

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