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Oceanobacillus manasiensis sp. nov., a Moderately Halophilic Bacterium Isolated from the Salt Lakes of Xinjiang, China[§]

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Three Gram reaction positive, rod-shaped, moderately motile halophilic bacterial strains, designated YD3-56^T, YD16, and YH29, were isolated from the sediments of Manasi and Aiding salt lakes in the Xinjiang region of China, respectively. The strains grew optimally at 30-37°C, pH 8-11, in the presence of 5-10% (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the strains were closely related to members of the genus *Oceanobacillus*, exhibiting 99.1-99.2% similarity to *O. kapialis* KCTC 13177^T, 99.2-99.3% to *O. picturae* KCTC 3821^T, and 94.2-96% sequence similarity to other described *Oceanobacillus* species. SDS-PAGE of whole cell proteins preparations demonstrated that the strains exhibited high similarity to each other, but distinguished from *O. kapialis* KCTC 13177^T and *O. picturae* KCTC 3821^T (75%). DNA-DNA hybridization revealed that the similarity between the representative strain YD3-56^T and *O. kapialis* KCTC 13177^T was 35.3%, and the similarity between YD3-56^T and *O. picturae* KCTC 3821^T was 22.3%. Chemotaxonomic analysis of the strains showed menaquinone-7 was the predominant respiratory quinine. Major cellular fatty acids were anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The polar lipid pattern for strain YD3-56^T predominantly contained phosphatidylcholine, and trace to moderate amounts of phosphatidyl ethanolamine and hydroxy-phosphatidyl ethanolamine. The diamino acid in murein was *meso*-diaminopimelic acid. The DNA G+C content of the strains was 39.7-40.1 mol%. On the basis of these results, the three strains should be classified as a novel species of the genus *Oceanobacillus*, for which the name *Oceanobacillus manasiensis* sp. nov. has been proposed, with the type strain as YD3-56^T (=CGMCC 1.9105^T =NBRC 105903^T).

***Keywords*:** *Oceanobacillus manasiensis* sp. nov., moderate halophile, salt lake, Gram-positive

In recent years, extensive attention has been given to halophiles due to their special adoption mechanisms in high salt environments. The isolation of more unknown halophiles is a necessary prerequisite and key for the development of microorganism resources. There are many high-salt environments in China, including large areas of saline soil and many salt lakes in Xinjiang, Inner Mongolia, Shandong, Qinghai, and Gansu regions. These areas can provide unique conditions for studying the systematic taxonomy of moderately halophilic bacteria.

The genus *Oceanobacillus* was first described by Lu *et al.* (2001) with the type species *O. iheyensis* isolated from a deep-sea environment. Subsequently, 10 *Oceanobacillus* species, including two subspecies, have been reported. They are *O. caeni* from waste water (Nam *et al.*, 2008), *O. picturae* from a painting (Heyrman *et al.*, 2003; Lee *et al.*, 2006), *O. profundus* from deep sea sediment (Kim *et al.*, 2007), *O. chironomi* from a chironomid egg mass (Raats and Halpern, 2007), *O. oncorhynchi* subsp. *oncorhynchi* from the skin of a rainbow trout (Yumoto *et al.*, 2005), *O. oncorhynchi* subsp. *Incaldanensis* from an algal mat (Romano *et al.*, 2006), *O. kapialis* from fermented shrimp paste (Namwong *et al.*, 2009), *O. soja* from soy sauce production equipment (Tominaga *et al*., 2009), *O. neutriphilus* from activated sludge in a bioreactor (Yang *et al*., 2009) and *O. locisalsi* from a marine solar saltern of the Yellow Sea (Lee *et al.*, 2010).

In a previous study, we isolated 64 strains from the different salt lakes in Xinjiang, most of which were aerobic, rod-shaped, spore-forming, moderately halophilic bacteria. Furthermore, two novel species were identified (Liu *et al.*, 2005). Recently, the strains designated YD3-56^T and YD16 were isolated from sediments of Manasi (85°37′3″-86°16′20″E45°37′50″-45°55′47″N) and YH29 was isolated from Aiding salt lakes (89°10′32″-89°54′32″E42°32′10″-42°49′13″N) in Xinjiang. All of them demonstrated highest sequence similarity to members of the genus *Oceanobacillus.* The aim of the present study was to determine the taxonomic positions of a novel strain of *Oceanobacillus* using a polyphasic approach that included the analysis of phenotypic properties, phylogenetic analysis based on 16S rRNA gene sequences, DNA-DNA relatedness and SDS-PAGE of whole cell proteins.

Materials and Methods

Isolation, morphological, and physiological characterization

At the time of sampling, salinity was 12.1-15.7% (w/v) and pH was 7.1-

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Fig. 1. Transmission electron micrograph of strain YD3-56^T. Transmission electron micrograph of exponentially growing cells, showing the polar flagella of the rod. Bar=2 µm.

7.4. For isolation, the samples were suspended in 8% (w/v) NaCl, serially diluted and spread on 8% Gibbison medium (per L: tryptone 10 g, yeast extract 5 g, casein 5 g, KCl 2 g, sodium citrate 3 g, $MgSO_4.7H_2O$ 20 g, and NaCl 80 g). Unless indicated, the complex medium for the isolates was adjusted to pH 8.0, and the tests were carried out at 37°C. Colony morphology was observed after 2 days incubation at 37°C on Gibbson medium. The reference strains *O. kapialis* KCTC 13177^T and *O. picturae* KCTC 3821^T were obtained from the Korean Collection for Type Cultures (KCTC) and incubated as described by Heyrman *et al*. (2003) and Namwong *et al*. (2009). To characterize the strains phenotypically, standard tests were performed according to the proposed minimal standards for the description of aerobic, endospore-forming bacteria (Logan *et al*., 2009). The sea water medium described by Spring *et al*. (1996) was used for the investigation of spore formation. Photomicrographs of spores were obtained from cultures grown on sea water medium for 36 h. Morphological characteristics were examined using an optical microscope (BX40, Olympus) and a transmission electron microscope (JEM 1230) with cells grown at 37°C for 20 h on isolation medium (8% NaCl, pH 8.0). The pH range for growth was determined in WP medium (per L: tryptone 10 g, yeast extract 5 g), at various pH values (5, 6, 7, 8, 9, 10, and 11) by adding HCl or NaOH. The temperature range for detection was 4, 10, 15, 20, 25, 30, 35, 37, 40, and 42°C. Growth at different salinities was estimated by adding 0, 2.5, 5, 8, 10, 12.5, 15, 20, 25 or 30% (w/v) NaCl on Gibbson medium at pH 8.0. Anaerobic growth was prepared under nitrogen atmosphere. Activities of catalase, urease and oxidase, citrate utilization, nitrate reduction, Voges-Proskauer tests and H_2S production, hydrolysis of casein, starch, tyrosine, aesculin, Tween 20 and 80 were determined by previously described methods (Dong and Cai, 2001; Romano *et al.*, 2006). Acid production was tested using basal medium (per L: NaCl 80 g, $(NH_4)_2HPO_4$ 1 g, $MgSO_4{\cdot}7H_2O$ 0.2 g, KCl 0.2 g, yeast extract 0.2 g, 1.6% bromocresol purple 1-2 ml and with 1.0% sugars). The API 20E and API 50CHB microtest gallery systems (bioMérieux, France) were used to determine the physiological and biochemical characteristics. The strips were incubated for 24 h at 37°C. All suspension media were supplemented with 8% (w/v) NaCl. Susceptibility to the various antibiotics was investigated on Gibbson medium at pH 8.0 with 8% (w/v) NaCl by using antibiotic discs with the following concentrations (µg/ml): streptomycin (50, 200); ampicillin (50, 100); gentamicin (10, 40); chloramphenicol (10, 20); kanamycin (50); rifampicin (5, 10); erythromycin (30, 50); spectinomycin (50); and nalidixic acid (5, 20).

Molecular characterization

The 16S rRNA gene was amplified and sequenced for strains YD3-56^T, YD16, and YH29 using the primers P1 and P6 according to Tan *et al.* (1997). The sequences were aligned with those of related *Oceanobacillus* species using the CLUSTAL W program in the MEGA 4.0 software (Tamura *et al.*, 2007). Aligned sequences were analyzed using the same software to produce a Jukes-Cantor distance (Munro, 1969) and to construct an optimal unrooted tree using the neighbor joining (Saitou and Nei, 1987) and maximum-parsimony methods with their strains designations and accession numbers (Fig. 2 and Supplementary data Fig. 3). The robustness of the tree topologies was calculated by bootstrap analysis based on 1,000 replications of the sequences (Felsenstein, 1985).

Chemotaxonomic characterization

Cell biomass for the analysis of cell wall, polar lipid and isoprenoid quinones was harvested from cultures at 37°C after incubation on complex medium for 20 h. The analysis of the cell wall peptidoglycan was determined using TLC according to the method described by Schleifer and Kandler (1972, 1985), with *O. kapialis* (KCTC 13177^T) and *O. picturae* (KCTC 3821^T) used as references. Menaquinones were analyzed as described previously (Collins, 1985) using reverse-phase HPLC (HP-1050) with *O. picturae* (KCTC 3821^T) as the reference. For cellular fatty acid analysis, cell masses of strains YD3-56^T, YD16, YH29, and *O. picturae* (KCTC 3821^T) were identified under the same laboratory conditions in accordance with the manufacturer's instructions from the Microbial Identification System (MIDI). Extraction and analysis of polar lipids by two-dimensional TLC was performed according to Ventosa *et al*. (1993).

DNA G+C content and DNA-DNA hybridization

Total genomic DNA was extracted and purified in accordance with standard methods (Marmur, 1961). The DNA G+C content was determined using the thermal denaturation method (T_m) with *Escherichia coli* K-12 as a standard (Marmur and Doty, 1962). DNA-DNA hybridization was carried out with the spectrophotometric method (De Ley *et al.*, 1970). Each value given is the mean of at least two hybridization experiments. The optical density of DNA at 260 nm (OD_{260}) was 2.0, and DNAs were sheared by sonication at 40 W for three periods of 180 sec and the optimal renaturation temperature in 2× SSC (1× SSC is 0.15 M NaC1, 0.015 M sodium citrate, pH 7.0) was 65°C.

Whole-cell protein preparations of and SDS-PAGE

SDS-PAGE of whole cell proteins was performed as described by Tan *et al.* (1997). The tested strains were grown for 40 h at 37°C. The densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis were performed using the GelCompar II software package (Applied Maths). The similarity between each pair of samples (strains) was expressed by using the Dice coefficient and a UPGMA dendrogram was constructed (Vauterin and Vauterin, 1992). Reproducibility was checked by preparing protein extracts in duplicate. The correlation level between the patterns obtained with different extracts of the same strain was more than 97%.

Results and Discussion

The 16S rRNA gene sequence analysis indicated that strains YH3-56^T, YD16 and YH29 showed 99.1-99.2% similarity with respect to *O. kapialis* KCTC 13177^T, 99.2-99.3% to *O. picturae* KCTC 3821^T, 96-96.1% to *O. profundus* KCCM 42318^T, 95.5-95.6% to *O. caeni* KCTC 13061^T, 94.1-94.2% to *Oceanobacillus chironomi* DSM 18262^T, 95.1% to *O. iheyensis* JCM 11309^T, 95.3-95.4% to *O. oncorhynchi* subsp. *oncorhynchi* JCM 12661^T, 95.5-95.6% to *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T, 94.2-94.3% to *O. locisalsi* KCTC 13253^T, 94.5-94.6% to *O. neutriphilus* JCM 15776^T, and 95-95.1% to *O. soja* JCM 15792^T. The 16S rRNA gene sequence similarity values between strains YH3-56^T, YD16, and YH29 was 99.8-99.9%. The data revealed that these strains were on the same phylogenetic branch within the genus *Oceanobacillus* (Fig. 2). Strains YH3-56^T, YD16, YH29, *O. kapialis* KCTC 13177^T, and *O. picturae* KCTC 3821^T formed a monophyletic clade cluster, suggesting that we could compare the DNA relatedness between these strains. Strain YD3-56^T showed similarities to strains YD16 (88%) and YH29 (81%), respectively. The similarity between the representative strain YD3-56^T and *O. kapialis* KCTC 13177^T was 35.3%, and the similarity between YD3-56^T and *O. picturae* KCTC 3821^T was 22.3% at the genomic level. Since below 70% of the genomic relatedness is a key marker for the identification of a novel species (Wayne *et al.*, 1987), the three strains represented a novel species, sufficiently different from any current member of the genus *Oceanobacillus*. The DNA G+C content of strains YD3-56^T, YD16, and YH29 was 39.7, 39.3, and 40.1 mol%, respectively. The morphological, physiological, and biochemical characteristics of strains were given in the species description below. The strains YH3-56^T, YD16, and YH29 differentiated from the closely related species as shown in Table 1. They were able to hydrolyze aesculin, Tween 20 and 80, but failed to ferment L-arabinose, D-fructose, glycerol, D-mannitol, and D-maltose, when compared to *O. kapialis* KCTC13177^T and *O. picturae* KCTC3821^T. However, only the fermentation of L-rhamnose and D-ribose was different between the three strains. Few sugars were found to be fermented by the three strains.

Whole-cell protein preparations and SDS-PAGE analysis were also performed. After numerical analysis and visual

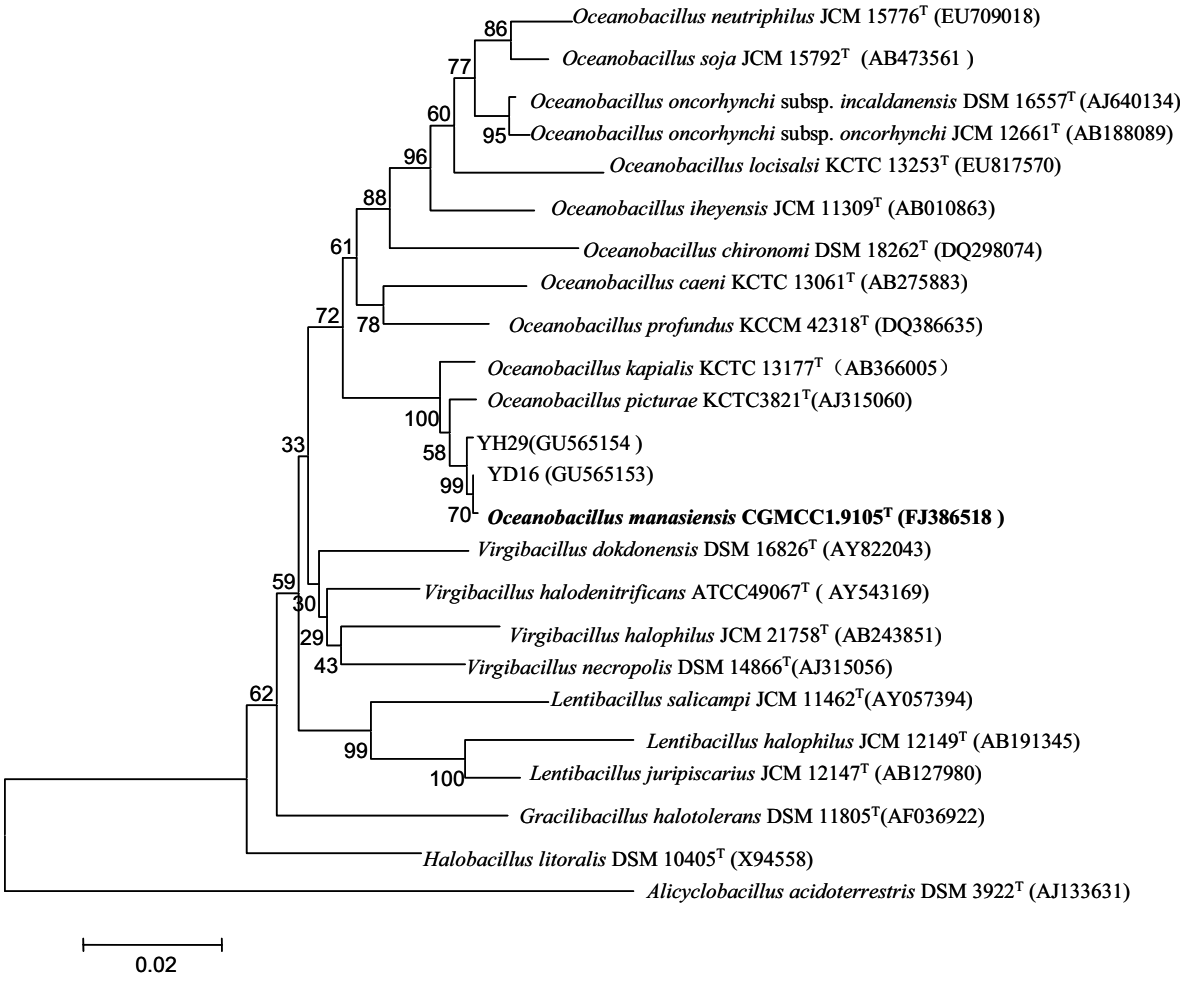

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of *O. manasiensis* YD3-56^T, other *Oceanobacillus* species, and some related taxa. Bootstrap values are shown at nodes as percentages of 1,000 replications. Bar=0.02 substitutions per site.

comparison of the profiles, the similarity between each strain was found to be 97.5-99%, but lower than 75% when compared with *O. kapialis* KCTC 13177^T and *O. picturae* KCTC 3821^T. The results of Chen (1995) and Tan (1997) indicated that if the similarity level between tested strains was under 80%, a unique genetic group should be formed, and hence the strains could be considered as a novel species which different from *O. kapialis* KCTC 13177^T and *O. picturae* KCTC 3821^T. Detailed information has been provided in Supplementary data Fig. 1. These results complement the DNA-DNA hybridization results, and could reflect intraspecies variation.

Table 1. Differential phenotypic characteristics of *O. manasiensis* YD3-56^T and the type strains of some phylogenetically related *Oceanobacillus* species. 1, strains YD3-56^T; YD16 and YH29; 2, *O. picturae* KCTC 3821^T; 3, *O. kapialis* KCTC13177^T. Data were obtained from this study.
Symbol: +, positive; -, negative; W, weak; E, ellipsoidal; ES, ellipsoidal or spherical endospores; C, Central; S, subterminal; T, terminal; PC, phosphatidylcholine; DPG, diphosphatidyl glycerol; PE, phosphatidyl ethanolamine; OH-PE, hydroxy-phosphatidyl ethanolamine

Characteristics	1	2	3
Colonies size (mm)	1.5-3	1-2	0.5-1
Cell size	0.3-0.5× 3-6 μm	0.5-0.7× 2-6 μm	0.4-0.5× 1-3 μm
Spore formation	E(T)	E(S)	E(T)
pH range for growth	6-11	6-10	6-10
NaCl concentration for growth (w/v, %)	2.5-15	2.5-15	2.5-20
Optimal NaCl (w/v, %)	5-10	5-10	5-15
Hydrolysis of			
Gelatin	-	-	+
Casein	-	-	+
Aesculin	+	-	-
Tween 80	+	-	-
Tween 20	+	-	-
Acid from			
Inulin	-	w	-
D-Maltose	-	-	+
Glucose	+	w	+
L-Arabinose	-	-	+
Glycerol	-	+	-
D-Mannitol	-	-	+
Methyl a-D-glucoside	w	+	-
L-Rhamnose	+[a]	-	-
D-Ribose	+[b]	+	-
Sensitive to			
Str200	-	+	+
Cm10	+	-	-
Gm40	w	+	+
Spc50	-	w	+
Nitrate reductase	+	+	-
Oxidase	-	-	+
Catalase	-	-	+
DNA G+C content (mol%)	39.7-40.1	37.3	39.3
Polar lipid	PC, PE, OH-PE	DPG, PG, PE	N.A

[a] YH29 (-)
[b] YD3-56 (-)

Chemotaxonomic analysis showed that the diamino acid in murein and the menaquinone in the isolated strains were the same as those of the reference strain *O. picturae* KCTC 3821^T, which were *m*-DAP and MK-7, respectively. The polar lipid pattern determined for the strain YD3-56^T, predominantly contained phosphatidylcholine, and trace to moderate amounts of phosphatidyl ethanolamine and hydroxy-phosphatidyl ethanolamine. The major cellular fatty acids of the strains detected in this study were anteiso-$C_{15:0}$ (52.1-56.8%) and anteiso-$C_{17:0}$ (23.8-24.7%). Detailed information on the cellular fatty acid composition of strains YD3-56^T, YD16, YH29, *O. picturae* KCTC 3821^T, and *O. kapialis* KCTC 13177^T has been provided in Supplementary data Table 1.

On the basis of morphological, physiological, chemotaxonomic characteristics, 16S rDNA sequence comparison data, SDS-PAGE of whole cell protein preparations and DNA-DNA hybridization, strains YD3-56^T, YD16, and YH29 should be treated as a new member of the genus *Oceanobacillus*, with the name *O. manasiensis* sp. nov. proposed and YD3-56^T as the type strain (=CGMCC 1.9105^T =NBRC105903^T).

Description of *Oceanobacillus manasiensis* sp. nov.

Oceanobacillus manasiensis (ma.na.si.en'sis N.L. masc. adj. manasiensis from Manasi salt lake, where the type strain was isolated)

Cells are rod-shaped with a size range of 0.3-0.5 μm×3-6 μm, Gram-positive and motile by means of polar flagella. Oval endospores are formed terminally in swollen sporangia. Colonies grown on solid complex medium used for maintenance are circular, smooth, low convex, cream and 1.5-2 mm in diameter after incubating for 2 days. Growth occurs at 2.5-15% (w/v) total salts, with the optimum at 5-10% (w/v) NaCl. The optimum temperature range for growth is 30-37°C, and growth occurs at 10-42°C. The optimal pH value for growth is 8-10, with a pH range of 6-11. According to the API 20E system and the conventional methods of Dong and Cai (2001), β-galactosidase is produced, and urease, oxidase, arginine dihydrolase, lysine decarboxylase, catalase, tryptophan deaminase, and ornithine decarboxylase are absent. Tween 80, Tween 20, and aesculin are hydrolyzed, but casein, gelatin, and tyrosine are not hydrolyzed. Nitrate reductase is positive. Citrate is not utilized and H_2S is not produced. The Voges-Proskauer reaction (acetoin) is negative. In assays with the API 50CHB system and traditional methods, acids are produced from D-fructose, D-glucose, D-mannose, and L-rhamnose, but not from glycerol, erythritol, methyl b-D-xyloside, D-methyl glucoside, D-ribose, L-sorbose, D-tagatose, D-turanose, dulcitol, methyl a-D-mannoside, melezitose, starch, glycogen, xylitol, D-lyxose, DL-fucose, inositol, gluconate, 2-ketogluconate, 5-ketogluconate, DL-arabinose, DL-xylose, inositol, D-sorbitol, D-maltose, D-adonitol, DL-arabitol, inulin, D-trehalose, D-mannitol, D-galactose, amygdalin, N-acetylglucosamine, raffinose, arbutine, lactose, gentiobiose, cellobiose, melibiose, and sucrose. The cells are susceptible to (μg/ml): rifampicin (5), streptomycin (200), erythromycin (30), spectinomycin (50), chloramphenicol (20),

and ampicillin (100), but not susceptible to kanamycin (50), nalidixic acid (20), gentamicin (40), streptomycin (50), chloramphenicol (10), and ampicillin (50). The diamino acid in murein, the menaquinone and polar lipid of strain YD3-56^T are *m*-DAP, MK-7 and phosphatidylcholine, respectively. The major cellular fatty acids are anteiso-$C_{15:0}$ (52.1%) and anteiso-$C_{17:0}$ (23.8%). The DNA G+C content of strain YD3-56^T is 39.7 mol%.

The type strain, YD3-56^T (=CGMCC 1.9105^T =NBRC 105903^T), was isolated from the Manasi salt lake in Xinjiang, China.

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