Oceanobacillus neutriphilus sp. nov., isolated from activated sludge in a bioreactor

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A Gram-stain-positive, neutrophilic, rod-shaped bacterium, strain A1g^T, was isolated from activated sludge of a bioreactor and was subjected to a polyphasic taxonomic characterization. The isolate grew in the presence of 0–17.0 % (w/v) NaCl and at pH 6.0–9.0; optimum growth was observed in the presence of 3.0–5.0 % (w/v) NaCl and at pH 7.0. Strain A1g^T was motile, formed cream-coloured colonies, was catalase- and oxidase-positive and was able to hydrolyse aesculin, Tween 40 and Tween 60. Chemotaxonomic analysis revealed menaquinone-7 as the predominant respiratory quinone and anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{16:0}$ and iso-$C_{15:0}$ as major fatty acids. The genomic DNA G+C content of strain A1g^T was 36.3 mol%. Comparative 16S rRNA gene sequence analysis revealed that the new isolate belonged to the genus *Oceanobacillus* and exhibited closest phylogenetic affinity to the type strains of *Oceanobacillus oncorhynchi* subsp. *incaldanensis* (97.9 % similarity) and *O. oncorhynchi* subsp. *oncorhynchi* (97.5 %), but less than 97 % sequence similarity with respect to the type strains of other recognized *Oceanobacillus* species. Levels of DNA–DNA relatedness between strain A1g^T and reference strains *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T, *O. oncorhynchi* subsp. *oncorhynchi* JCM 12661^T and *Oceanobacillus iheyensis* DSM 14371^T were 29, 45 and 38 %, respectively. On the basis of phenotypic and genotypic data, strain A1g^T is considered to represent a novel species of the genus *Oceanobacillus*, for which the name *Oceanobacillus neutriphilus* sp. nov. is proposed. The type strain is A1g^T (=CGMCC 1.7693^T =JCM 15776^T).

The genus *Oceanobacillus* was proposed by Lu *et al.* (2001, 2002) with the description of *Oceanobacillus iheyensis* as the type species; the description of the genus was later emended by Yumoto *et al.* (2005) and Lee *et al.* (2006). The genus *Oceanobacillus* comprises aerobic, Gram-positive, motile, rod-shaped bacteria that are characterized chemotaxonomically by the presence of menaquinone-7 as the major isoprenoid quinone and anteiso-$C_{15:0}$ as the predominant cellular fatty acid (Lee *et al.*, 2006).

At the time of writing, the genus comprises seven recognized species, including two subspecies. Members of the genus have been isolated from diverse sources such as deep-sea sediment (*O. iheyensis* Lu *et al.* 2002; *O. profundus* Kim *et al.* 2007), mural paintings [*O. picturae* (Heyrman *et al.* 2003) Lee *et al.* 2006], freshwater fish (*O. oncorhynchi* subsp. *oncorhynchi* Yumoto *et al.* 2005), algae (*O. oncorhynchi* subsp. *incaldanensis* Romano *et al.* 2006), an insect (*O. chironomi* Raats and Halpern 2007), activated sludge (*O. caeni* Nam *et al.* 2008) and food (*O. kapialis* Namwong *et al.* 2009). The type strains of *O. oncorhynchi* subsp. *oncorhynchi* and *O. oncorhynchi* subsp. *incaldanensis* share high 16S rRNA gene sequence similarity (99.5 %) but can be differentiated on the basis of phenotypic traits. The former is sporulating, facultatively aerobic and obligately alkaliphilic (pH 9–10), whereas the latter is non-sporulating, obligately aerobic and alkalitolerant (pH 6.5–9.5) (Romano *et al.*, 2006).

The aim of the present study was to determine the exact taxonomic position of a novel *Oceanobacillus*-like strain by using a polyphasic approach that included analysis of phenotypic properties and phylogenetic analysis based on 16S rRNA gene sequences and DNA–DNA relatedness data.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A1g^T is EU709018.

Detailed fatty acid profiles of strain A1g^T and related type strains and maximum-parsimony and maximum-likelihood 16S rRNA gene sequence-based phylogenetic trees are available as supplementary material with the online version of this paper.

Strain A1g^T was isolated from activated sludge of a sequential batch reactor treating salt-containing wastewater taken from a preserved Szechuan pickle factory. The reactor had been operational for 9 months at the time when the sludge was sampled. The average *in situ* temperature was 30 °C and the pH was approximately 7.4. The sludge sample was suspended in sterile wastewater and vortexed for 15 min. A portion of the suspension was spread directly on sterile wastewater agar plates, which contained (per litre wastewater) 1.0 g glucose, 1.0 g peptone (BD) and 15.0 g agar (pH 7.0). The plates were incubated at 30 °C for several days. Single colonies on the plates were picked out and strain A1g^T was obtained by repeated restreaking. This isolate was routinely cultured on marine agar 2216 (MA; BD) and was maintained as a glycerol suspension (30 % v/v) at −80 °C.

The optimal conditions for growth were determined in PY broth (Lu *et al.*, 2001) with different NaCl concentrations (0, 0.5, 1, 3, 5, 7.5, 10, 12.5, 15, 16, 17, 18, 19, 20, 22.5 and 25 %, w/v). The pH range for growth was determined by adding MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5), CAPSO (pH 9.0–10.0) or CAPS (pH 10.5) to PY broth supplemented with 3 % (w/v) NaCl. The temperature range for growth was determined in PY broth (pH 7.0) at 4, 10, 15, 20, 25, 30, 35, 37, 40, 42, 45 and 48 °C. Cell morphology and motility were examined by optical microscopy (Olympus BX40) and electron microscopy (Hitachi H-7650 and JEOL JEM-1230).

Oxidase activity was determined based on oxidation of 1 % *p*-aminodimethylaniline oxalate and catalase activity was determined based on bubble production in 3 % (v/v) H_2O_2 solution (Dong & Cai, 2001). Biochemical characteristics were determined according to the methods described by Dong & Cai (2001) and Romano *et al.* (2006). Single carbon source assimilation tests were performed by using medium 2 supplemented with 3 % (w/v) NaCl (Romano *et al.*, 2006). The corresponding filter-sterilized sugar (0.2 %), alcohol (0.2 %), organic acid (0.1 %) or amino acid (0.1 %) was added to liquid medium. Acid production was tested by using MOF medium supplemented with 1.0 % sugars or alcohols (Leifson, 1963; Xu *et al.*, 2008). Susceptibility to antibiotics was determined on agar plates by using antibiotic discs with the following compounds (amounts in μg unless otherwise stated): amoxicillin (10), ampicillin (10), carbenicillin (100), cefotaxime (30), cefoxitin (30), chloramphenicol (30), erythromycin (15), kanamycin (30), neomycin (30), nitrofurantoin (300), novobiocin (30), nystatin (100), penicillin (10), polymyxin B (300 IU), rifampicin (5), streptomycin (10) and tetracycline (30). Additional enzyme activities and biochemical characteristics were determined by using API 20E, API 20 NE, API 50 CH and API ZYM kits as recommended by the manufacturer (bioMérieux). *O. iheyensis* DSM 14371^T, *O. oncorhynchi* subsp. *oncorhynchi* JCM 12661^T and *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T were used as controls in these tests.

Fatty acid methyl esters were obtained from cells grown on MA for 2 days at 35 °C and were analysed by using GC/MS (Kuykendall *et al.*, 1988). Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987) by using reversed-phase HPLC. Cell-wall peptidoglycan was prepared and hydrolysed according to the methods given by Kawamoto *et al.* (1981) and the amino acid composition was analysed with an automatic amino acid analyser (Hitachi L-8900). Genomic DNA was obtained by using the method described by Marmur (1961). Purified DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with calf intestine alkaline phosphatase; the G+C content of the resulting deoxyribonucleosides was determined by reversed-phase HPLC and was calculated from the ratio of deoxyguanosine to thymidine (Mesbah & Whitman, 1989).

The 16S rRNA gene was amplified and analysed as described by Xu *et al.* (2007). PCR products were cloned into pMD 19-T vector (TaKaRa) and then sequenced. An almost-complete 16S rRNA gene sequence of strain A1g^T (1484 nt) was obtained and was compared with closely related sequences of reference organisms from the EzTaxon service (Chun *et al.*, 2007). Sequence data were aligned with CLUSTAL W 1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods with the MEGA 4 program package (Tamura *et al.*, 2007) and by using the maximum-likelihood method (Felsenstein, 1981) with the TreePuzzle 5.2 program. Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura, 1980) for the neighbour-joining method. Bootstrap analysis was used to evaluate the tree topology by means of 1000 resamplings.

Cells of strain A1g^T were Gram-stain-positive, sporulating rods that were motile by means of polar flagella (Fig. 1). The NaCl concentration, pH and temperature ranges for growth in PY broth were 0–17 % (w/v), pH 6.0–9.0 and 10–45 °C. The cell-wall diamino acid was *meso*-diaminopimelic acid. The isoprenoid quinone of strain A1g^T was MK-7 and the DNA G+C content was 36.3 mol%. These chemotaxonomic characteristics were in accordance with those given for the genus *Oceanobacillus* (Yumoto *et al.*, 2005; Lee *et al.*, 2006). Detailed results are given in the species description below and in Table 1.

16S rRNA gene sequence comparisons showed that strain A1g^T should be placed within the genus *Oceanobacillus*, being related most closely to the type strains of *O. oncorhynchi* subsp. *incaldanensis* (97.9 % similarity), *O. oncorhynchi* subsp. *oncorhynchi* (97.5 %) and *O. iheyensis* (96.3 %); levels of 16S rRNA gene sequence similarity with respect to the type strains of other recognized *Oceanobacillus* species were 94.2–95.3 %. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain A1g^T had closest affinity with the type strains of *O. oncorhynchi* and *O. iheyensis* with high levels of bootstrap support (Fig. 2). The topologies of the

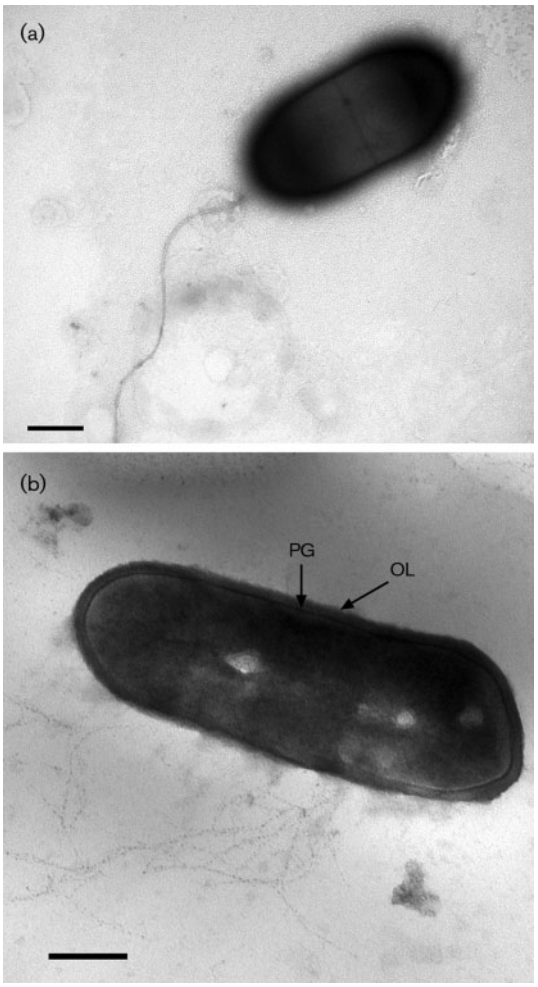

Fig. 1. Transmission electron micrographs of cells of strain A1g^T. (a) Exponentially growing cell, showing polar flagella. Bar, 0.5 μm. (b) Ultrathin section showing outer membrane of the cell. PG, Peptidoglycan; OL, outer layer. Bar, 0.2 μm.

phylogenetic trees built by using the maximum-parsimony and maximum-likelihood methods also supported the notion that strain A1g^T formed a stable clade with the type strains of *O. oncorhynchi* and *O. iheyensis* (Supplementary Fig. S1 in IJSEM Online).

DNA–DNA hybridization experiments were performed by the thermal denaturation and renaturation method of De Ley *et al.* (1970) as modified by Huß *et al.* (1983), by using a Beckman DU 800 spectrophotometer. Levels of DNA–DNA relatedness between strain A1g^T and *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T, *O. oncorhynchi* subsp. *oncorhynchi* JCM 12661^T and *O. iheyensis* DSM 14371^T were 29, 45 and 38 %, respectively, significantly below the value of 70 % which is considered to be the threshold for the delineation of species (Wayne *et al.*, 1987).

The major fatty acids of strain A1g^T were anteiso-$C_{15:0}$ (37.7 %), anteiso-$C_{17:0}$ (18.9 %), iso-$C_{16:0}$ (15.8 %) and iso-$C_{15:0}$ (6.8 %). This profile was different from those of *O. iheyensis* DSM 14371^T and *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T (Supplementary Table S1). The iso-$C_{14:0}$ content of strain A1g^T (3.8 %) was lower than that of *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T (17.5 %) and *O. iheyensis* DSM 14371^T (17.0 %). Additionally, strain A1g^T could be differentiated from recognized *Oceanobacillus* species on the basis of several phenotypic characteristics, such as cultural conditions, nitrate reduction, hydrolysis of substrates, acid production from sugars or alcohols, susceptibility to antibiotics and enzyme activities (Table 1).

On the basis of the genotypic and phenotypic data presented in this study, strain A1g^T should be assigned to a novel species within the genus *Oceanobacillus*, for which the name *Oceanobacillus neutriphilus* sp. nov. is proposed.

Description of *Oceanobacillus neutriphilus* sp. nov.

Oceanobacillus neutriphilus (neu.tri.phi′lus. L. adj. *neuter -tra -trum* neither, used to refer to neutral pH; Gr. adj. *philos* loving; N.L. masc. adj. *neutriphilus* preferring neutral pH).

Cells are Gram-positive-staining, aerobic rods, 0.7–1.2 μm wide and 1.5–2.5 μm long with rounded ends, that are motile by means of polar flagella and produce ellipsoidal spores in a central position. Colonies on MA are 1–2 mm in diameter, of low convexity, smooth, circular with regular borders and cream-coloured after 48 h. Growth occurs in PY medium at NaCl concentrations of 0–17.0 % (w/v) with optimum growth at 3.0–5.0 %. The pH and temperature ranges for growth are 6.0–9.0 and 10–45 °C (optimum growth at pH 7.0 and 37 °C). No growth is detected below pH 5.5 or above pH 9.5. Positive for oxidase and catalase. Hydrolyses aesculin, Tween 40 and Tween 60, but not casein, DNA, gelatin, starch, Tween 80 or tyrosine. Nitrate is not reduced to nitrite. H_2S is not produced from thiosulfate. Positive for *o*-nitrophenyl-*β*-D-galactopyranosidase. Negative for arginine dihydrolase, indole production, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and urease. Utilizes D-fructose, D-glucose, maltose, mannitol, D-mannose and sucrose as sole carbon and energy sources, but not acetate, L-alanine, L-arabinose, citrate, L-cysteine, ethanol, formate, fumarate, glutamate, L-glutamine, glycine, L-histidine, isoleucine, lactate, lactose, malate, malonate, L-methionine, *myo*-inositol, L-ornithine, propionate, pyruvate, raffinose, L-serine, L-sorbitol, L-sorbose, starch, succinate or L-valine. Acid is produced from D-glucose, maltose, D-mannose and sucrose, but not from L-arabinose, ethanol, *myo*-inositol, lactose, raffinose, L-sorbitol or L-sorbose. Susceptible to (μg per disc unless indicated otherwise) amoxicillin (10), ampicillin (10), carbenicillin (100), cefotaxime (30), chloramphenicol (30), erythromycin (15), kanamycin (30), neomycin (30), nitrofurantoin (300), novobiocin (30) and rifampicin (5), but not to cefoxitin (30), nystatin

Table 1. Differential characteristics between strain A1g^T and the type strains of related members of *Oceanobacillus*

Strains: 1, A1g^T; 2, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T; 3, *O. oncorhynchi* subsp. *oncorhynchi* JCM 12661^T; 4, *O. iheyensis* DSM 14371^T. Data were obtained in the present study unless indicated. +, Positive; −, negative; W, weakly positive.

Characteristic	1	2	3	4
Spore position*	C	NS	T	T
NaCl range for growth (%, w/v)	0–17.0	0–20.0	0–22.5	0–18.0
NaCl optimum (%, w/v)	3.0–5.0	10.0	7.5–10.0	3.0–5.0
Growth temperature range (°C)	10–45	10–45	10–45	15–42
pH optimum	7.0	9.0	9.5	8.0–8.5
Nitrate reduction	−	+	+	−
o-Nitrophenyl-*β*-D-galactopyranosidase	+	−	−	−
Hydrolysis of:				
Casein	−	−	−	+
Gelatin	−	−	−	+
Tween 60	+	−	−	+
Urea	−	−	+	−
Acid production from:				
Arbutin	+	+	+	−
Cellobiose	W	+	+	−
Gentiobiose	−	+	+	−
Glycerol	W	−	−	+
D-Mannitol	+	+	−	W
Raffinose	−	−	+	−
Salicin	+	+	+	−
Sucrose	+	+	+	−
D-Tagatose	+	+	+	−
Trehalose	+	+	+	−
Sensitivity to:				
Cefotaxime (30 μg)	+	+	−	+
Cefoxitin (30 μg)	−	+	+	+
Nitrofurantoin (300 μg)	+	+	−	+
Penicillin G (10 μg)	−	+	+	+
Streptomycin (10 μg)	−	−	+	−
API ZYM results				
α-Chymotrypsin	+	+	+	−
α-Glucosidase	+	+	+	−
β-Glucosidase	+	+	+	−
DNA G+C content (mol%) (HPLC)	36.3	40.1[a]†	38.5[b]	35.8[c]

*C, Central; NS, not sporulated; T, terminal.
†Data from: *a*, Romano *et al.* (2006); *b*, Yumoto *et al.* (2005); *c*, Lu *et al.* (2001).

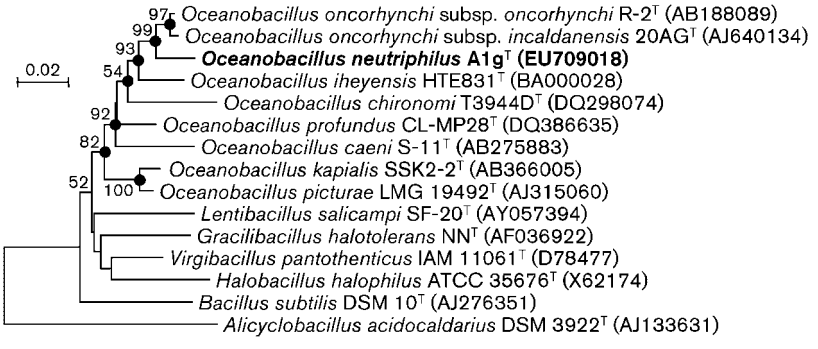

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships between strain A1g^T and related taxa. Bootstrap values at nodes are based on 1000 replicates; only values >50% are shown. Filled circles indicate nodes recovered with bootstrap values >50% in the maximum-parsimony and maximum-likelihood trees. Bar, 0.02 substitutions per nucleotide position.

(100), penicillin (10), polymyxin B (300 IU), streptomycin (10) or tetracycline (30). The following constitutive enzyme activities are detected in API ZYM tests: acid phosphatase, alkaline phosphatase, α-chymotrypsin, esterase (C4), esterase lipase (C8), α-glucosidase, β-glucosidase and naphthol-AS-BI-phosphohydrolase. *N*-Acetyl-β-glucosaminidase, cystine arylamidase, α-fucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, lipase (C14), leucine arylamidase, α-mannosidase, trypsin and valine arylamidase activities are not observed. Oxidative acid production in API 50 CH tests is positive for *N*-acetylglucosamine, arbutin, aesculin, D-fructose, D-glucose, maltose, D-mannitol, salicin, sucrose, D-tagatose and trehalose and weakly positive for cellobiose and glycerol. Principal fatty acids (>5 %) are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{16:0}$ and iso-$C_{15:0}$. The isoprenoid quinone is MK-7. The cell-wall diamino acid is *meso*-diaminopimelic acid. The DNA G+C content of the type strain is 36.3 mol%.

The type strain, A1g^T (=CGMCC 1.7693^T =JCM 15776^T), was isolated from activated sludge of a bioreactor treating salt-containing wastewater.

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