Natribacillus halophilus gen. nov., sp. nov., a moderately halophilic and alkalitolerant bacterium isolated from soil

Akinobu Echigo,^{1,2} Hiroaki Minegishi,² Yasuhiro Shimane,³ Masahiro Kamekura⁴ and Ron Usami^{1,2,3}

Correspondence
Akinobu Echigo
echigo@toyo.jp

¹Faculty of Science and Engineering, Toyo University, 2100 Kujirai, Kawagoe, Saitama 350-8585, Japan

²Bio-Nano Electronics Research Centre, Toyo University, 2100 Kujirai, Kawagoe, Saitama 350-8585, Japan

³Graduate School of Interdisciplinary New Science, Toyo University, 2100 Kujirai, Kawagoe, Saitama 350-8585, Japan

⁴Halophiles Research Institute, 677-1 Shimizu, Noda, Chiba 278-0043, Japan

A moderately halophilic and alkalitolerant bacterium, designated strain HN30^T, was isolated from garden soil in Japan. Cells of strain HN30^T were motile, endospore-forming, aerobic, rod-shaped and Gram-positive, and contained A1 γ meso-diaminopimelic acid-type murein. Growth occurred in 7–23 % (w/v) NaCl (optimum, 10–15 %, w/v), at pH 6.5–10.0 (optimum, pH 8.0–8.5) and at 20–40 °C (optimum, 30 °C). The isoprenoid quinone was menaquinone-7. The polar lipids were phosphatidylglycerol and diphosphatidylglycerol. The major cellular fatty acids were anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{16:0} and C_{16:0}. The DNA G + C content of strain HN30^T was 47 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain HN30^T was most closely related to *Geomicrobium halophilum* BH1^T (93 % sequence similarity). 16S rRNA gene sequence similarities with other recognized species were less than 89 %. Phylogenetic and phenotypic characteristics indicated that strain HN30^T represents a novel species in a new genus, for which the name *Natribacillus halophilus* gen. nov., sp. nov. is proposed; the type strain is HN30^T (=JCM 15649^T=DSM 21771^T).

Gram-positive, rod-shaped, endospore-forming, aerobic bacteria have generally been assigned to the family *Bacillaceae*, a systematically diverse taxon. Species of the genus *Bacillus* may be divided into several distinct groups based on phylogenetic analysis using 16S rRNA gene sequences (Ash *et al.*, 1991) and many moderately halophilic species belong to rRNA group 6 (Nielsen *et al.*, 1994). Subsequently, many of these species have been accommodated into newly proposed genera. The family *Bacillaceae* now comprises more than 38 genera and a number of moderately halophilic species have been isolated from various saline environments (Ventosa *et al.*, 1998). For example, *Aquisalibacillus elongatus* was isolated from a saline lake (Márquez *et al.*, 2008), *Bacillus ainingensis* was from sediment from a salt lake (Xue *et al.*, 2008), *Bacillus*

qingdaonensis was from a crude sea salt sample (Wang *et al.*, 2007), *Bacillus salarius* was from a saline lake (Lim *et al.*, 2006), *Filobacillus milosensis* was from surface sediment located on the beach (Schlesner *et al.*, 2001), *Gracilibacillus halotolerans* was from the Great Salt Lake (Wainø *et al.*, 1999), *Lentibacillus salicampi* was from a salt field (Yoon *et al.*, 2002), *Salsuginibacillus kocurii* was from soda-lake sediment (Carrasco *et al.*, 2007) and *Tenuibacillus multivorans* was from saline soil (Ren & Zhou, 2005).

In previous studies, we have isolated many moderately halophilic micro-organisms from soil samples in Japan (Echigo *et al.*, 2005) and identified three novel haloalkaliphilic species, *Alkalibacillus silvisoli* (Usami *et al.*, 2007), *Halalkalibacillus halophilus* (Echigo *et al.*, 2007) and *Geomicrobium halophilum* (Echigo *et al.*, 2010). In this paper, we describe a novel moderately halophilic and alkalitolerant bacterium, designated strain HN30^T, isolated from ordinary soil in Japan. On the basis of phylogenetic and phenotypic characteristics, we propose that the strain represents a novel species in a new genus of the family *Bacillaceae*.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain HN30^T, *Bacillus qingdaonensis* JCM 14087^T and *Bacillus salarius* DSM 16461^T are AB449109, AB571874 and AB571873, respectively.

Three supplementary figures and a supplementary table are available with the online version of this paper.

About 0.5 g of a soil sample taken from a surface soil of an ordinary garden in Okabe City, Saitama, Japan, was placed on an agar plate of JCM medium no. 611, which contained (in g l⁻¹): 5.0 Casamino acids (Difco), 5.0 yeast extract (Difco), 1.0 sodium glutamate monohydrate, 3.0 trisodium citrate dihydrate, 2.0 KCl, 0.2 MgSO₄·7H₂O, 0.036 FeCl₂·4H₂O, 200.0 NaCl and 20.0 Bacto agar (Difco). After autoclaving, the pH was adjusted to 9.5 by aseptically adding 1/10 volume of sterile 10 % (w/v) Na₂CO₃ solution to the medium. The soil sample was spread with a sterile spatula and incubated at 37 °C for 3 weeks. Only two colonies appeared; they were picked up, transferred to fresh agar plates, and pure cultures of the two strains (HN31 and HN30^T) were obtained by plating serial dilutions and repeated transfers on agar plates. Partial sequencing (about 600 bp of 5'-terminal) of PCR-amplified 16S rRNA genes showed that the sequence of strain HN31 was 97.2 % similar to that of *F. milosensis* SH 714^T (AJ238042) at the time of isolation (Echigo *et al.*, 2005) [in this study, it was 99.0 % similar to that of *Piscibacillus halophilus* HS224^T (FM864227); Amoozegar *et al.*, 2009], whereas similarities of the partial 16S rRNA gene sequence of strain HN30^T were less than 93 % with any other known sequences. Since we ourselves have not been able to isolate any other strains with 16S rRNA gene sequences that are similar to HN30^T and strains with high similarities have not been registered in databases, strain HN30^T only was examined in this study. The closely related strains, *Geomicrobium halophilum* BH1^T (Echigo *et al.*, 2010), *Bacillus salarius* DSM 16461^T (Lim *et al.*, 2006), *Bacillus aidingensis* DSM 18341^T (Xue *et al.*, 2008) and *Bacillus qingdaonensis* JCM 14087^T (Wang *et al.*, 2007) were used as references.

Growth characteristics were determined following the minimal standards for describing new taxa of aerobic, endospore-forming bacteria (Logan *et al.*, 2009) with JCM liquid medium no. 611 as a basal medium. The NaCl concentration range for growth was determined at 0–30 % (w/v) [at 1 % (w/v) intervals], at pH 8.5 and 37 °C. Growth pH was determined at pH 5.0–11.0 (at intervals of 0.5), adjusted with 10 % (w/v) H₂SO₄ or 10 % (w/v) KOH, at 10 % (w/v) NaCl and 37 °C. Growth temperature was determined at 10–70 °C (at 5 °C intervals), at 10 % (w/v) NaCl and pH 8.5. Physiological and chemotaxonomic analyses were carried out in JCM medium no. 611 with 10 % (w/v) NaCl incubated at 37 °C. Cells of strain HN30^T were maintained on an agar slope of JCM medium no. 611 [10 % (w/v) NaCl, pH 9.5] at 5 °C.

Cell morphology was studied using a phase-contrast microscope (Axiovert 135; Zeiss). Colonies of strain HN30^T formed on agar plates were approximately 1.0–1.5 mm in diameter, cream-like and opaque. Cells of strain HN30^T were rod-shaped (approximately 1.0–1.5 × 3.0–5.0 µm), motile and Gram-positive. Oval terminal endospores were produced within swollen sporangia [at stationary phase in 10 % (w/v) NaCl, pH 9.5, 37 °C] (see Supplementary Fig. S1, available in IJSEM Online). Strain HN30^T grew in 7–23 % (w/v) NaCl, with optimum growth

in 10–15 % (w/v) NaCl. Growth occurred at pH 6.5–10.0, with optimum growth at pH 8.0–8.5. The temperature range for growth was 20–40 °C, with optimum growth at 30 °C. Anaerobic growth was not observed in an anaerobic jar after incubation for 7 days at 37 °C. Sole carbon source utilization was tested in a modified JCM liquid medium no. 611 without Casamino acids and yeast extract.

Tests for catalase, oxidase and decarboxylase activities, hydrolysis of starch, pullulan, casein, gelatin, Tween 80, tributyrin, DNA, hippurate, aesculin, urea, tyrosine, xanthine and hypoxanthine, indole and H₂S production, and reduction of nitrate were performed according to the procedures of Smibert & Krieg (1981, 1994), Oren *et al.* (1997) and Schlesner *et al.* (2001) in media containing 10 % (w/v) NaCl, at pH 9.5. The results are included in the species description.

HPLC analysis of isoprenoid quinones, GC/MS analysis of fatty acid methyl esters and TLC analysis of polar lipids were performed according to the modified procedures of Minnikin *et al.* (1984), Tamaoka (1986) and Komagata & Suzuki (1987), using cells cultivated at 30 °C for 3 days in JCM liquid medium no. 611 [10 % (w/v) NaCl, pH 9.5]. The isoprenoid quinone of strain HN30^T was menaquinone-7 (MK-7). No other significant peaks were observed. The polar lipids were phosphatidylglycerol and diphosphatidylglycerol (Supplementary Fig. S2, available in IJSEM Online). The cellular fatty acid profile was characterized by saturated branched fatty acids, anteiso-C_{15:0} (45.3 %), anteiso-C_{17:0} (24.3 %), iso-C_{16:0} (10.1 %) and C_{16:0} (8.3 %) (Supplementary Table S1, available in IJSEM Online).

Preparation of peptidoglycan and determination of its structure were performed according to the modified procedures of Schleifer & Kandler (1972), Schleifer (1985) and Schlesner *et al.* (2001) as described previously (Echigo *et al.*, 2007; Usami *et al.*, 2007). Strain HN30^T possessed A1γ, meso-diaminopimelic acid-type murein, which was in common with the great majority of endospore-forming Gram-positive strains of the family Bacillaceae.

Total DNA was extracted by the method of Saito & Miura (1963). The DNA G+C content of strain HN30^T, determined by the HPLC method of Tamaoka & Komagata (1984), was 47 mol%. 16S rRNA genes were amplified by PCR with the following forward and reverse primers: 5'-AGAGTTTGATCCTGGCTCAG-3' (positions 8–27 according to *Escherichia coli* numbering) and 5'-GGCTACCTTGTTACGACTT-3' (positions 1510–1492). Amplified DNA was cloned using the TA Cloning kit (Invitrogen) and sequenced using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kits (Applied Biosystems) with the following primers: 5'-GGAAACAGCTATGAC-CATG-3' (vector side), 5'-GACTACCAGGGTATCTAATC-3' (positions 805–786), 5'-AGGGTTGCGCTCGTTG-3' (positions 1115–1100) and 5'-GTAAAACGACGGCCAGT-3' (vector side) on the ABI PRISM 310 Genetic Analyzer

(Applied Biosystems). Ten clones gave exactly the same sequence. The 16S rRNA gene sequence of strain HN30^T (1557 bp) was most closely related to that of *Geomicrobium halophilum* BH1^T [93 % sequence similarity by FASTA (Pearson & Lipman, 1988; Lipman & Pearson, 1985) and 91 % by EzTaxon server 2.1 (Chun *et al.*, 2007)], followed by *B. salarius* BH169^T (89 and 89 %, respectively), *B. ainingensis* 17-5^T (88 and 87 %, respectively) and *B. qingdaonensis* JCM 14087^T (87 and 87 %, respectively). These values indicate that strain HN30^T could belong to a new genus (Ludwig *et al.*, 1998). Sequences of related strains retrieved from the DDBJ (Miyazaki *et al.*, 2003) were aligned using CLUSTAL_X 2.0.10 (Larkin *et al.*, 2007). The neighbour-joining tree was reconstructed according to Saitou & Nei (1987) and evaluated by bootstrap sampling methods (Felsenstein, 1985) based on 1000 replicates. Maximum-likelihood analyses were performed with RAxML 7.0.4 using a GTR + I model (Stamatakis *et al.*, 2005) and support values were obtained by bootstrapping (100 replicates) using CONSENSE in PHYLIP (Felsenstein, 2002). The neighbour-joining tree (Fig. 1) and maximum-likelihood tree (see Supplementary Fig. S3 available in IJSEM Online) showed that strain HN30^T was related to *Geomicrobium halophilum* BH1^T but formed another distinctly separate branch.

Since Ash *et al.* (1991) demonstrated that species of the genus *Bacillus* could be subdivided into at least five groups, numerous new genera have been described. We

believe that there is a consensus that the genus *Bacillus sensu stricto* should be restricted to species that share high 16S rRNA gene sequence similarities with the type species, *Bacillus subtilis*, and its chemotaxonomic traits (Albert *et al.*, 2007; Kämpfer *et al.*, 2006; L'Haridon *et al.*, 2006). The 16S rRNA gene sequence of strain HN30^T showed 87 % similarity with that of *B. subtilis* DSM 10^T (accession no. AJ276351). Phenotypic properties of strain HN30^T also indicated that the isolate could be distinguished from strains of the most closely related species, *Geomicrobium halophilum* BH1^T (Echigo *et al.*, 2010), *B. salarius* DSM 16461^T (Lim *et al.*, 2006), *B. ainingensis* DSM 18341^T (Xue *et al.*, 2008) and *B. qingdaonensis* JCM 14087^T (Wang *et al.*, 2007) (Table 1; Supplementary Table S1 available in IJSEM Online). Cells of strain HN30^T were rod-shaped and occurred singly, whereas cells of *Geomicrobium halophilum* were bean-like in shape and formed irregular clusters. The predominant isoprenoid quinone of strain HN30^T was MK-7, whereas MK-7 and MK-6 were predominant in *Geomicrobium halophilum* and *B. qingdaonensis*. The major cellular fatty acid of strain HN30^T was anteiso-C_{15:0}, whereas *Geomicrobium halophilum* has been shown to possess iso-C_{15:0} (Supplementary Table S1, available in IJSEM Online). The above phylogenetic and phenotypic characteristics indicated that strain HN30^T represents a novel species in a new genus, for which the name *Natribacillus halophilus* gen. nov., sp. nov. is proposed.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain HN30^T and other related strains. Bootstrap values are based on 1000 replicates; only those greater than 600 are shown. *Brevibacillus brevis* NBRC 15304^T (AB271756) was used as the outgroup. Bar, 0.01 changes per nucleotide position.

Table 1. Differential characteristics of strain HN30^T and related species

Taxa: 1, strain HN30^T; 2, *Geomicrobium halophilum* BH1^T; 3, *Bacillus salarius* BH169^T; 4, *Bacillus aidingensis* 17-5^T; 5, *Bacillus qingdaonensis* JCM 14087^T. Data for the four reference species were taken from Echigo *et al.* (2010), Lim *et al.* (2006), Wang *et al.* (2007) and Xue *et al.* (2008), except where marked.

Characteristic	1*	2	3	4	5
Colonial pigmentation	Cream	Brown	Cream	Cream	Cream white
Cell morphology	Rods	Bean-like	Short rods	Short rods	Rods
Motility	+	—	—	+	—
Spore formation	+	—	+	+	—
NaCl concentration for growth					
Range (%)	7–23	5–25	3–20	8–33	2.5–20.5
Optimum (%)	10–15	10–15	10–12	12	12
Growth temperature:					
Range (°C)	20–40	20–40	15–40	22–44	25–45
Optimum (°C)	30	30	30	37	37
Growth pH:					
Range	6.5–10.0	6.0–10.0	6.8–9.5	6.0–9.5	6.5–10.5
Optimum	8.0–8.5	8.5–9.0	8.0	7.2	9.0
Reduction of nitrate	—	—	—	+	+
Acid production from:					
D-Fructose	—	+	+	+	+
D-Glucose	—	+	+	+	+
Lactose	+	—	+	—	+
D-Mannitol	—	—	+	+	+
D-Mannose	—	—	+	+	+
Oxidase test	+	+	+	—	—
Antibiotic sensitivity (µg per disc):					
Gentamicin (50)	+	+	+	—	+
Kanamycin (50)	—	—	—	+	+
Neomycin (25)	—	—	—	—	+
Novobiocin (25)	—	+	+	+	+
Streptomycin (100)	—	+	+	—	+
Tetracycline (50)	+	+	—	+	+
DNA G + C content (mol%)	47	45	43	48.1	48
Isoprenoid quinone	MK-7	MK-7, MK-6	MK-7	MK-7	MK-7, MK-6

*Data from this study.

Description of *Natribacillus* gen. nov.

Natribacillus [Na.tri.ba.cil'lus. N.L. n. *natron* (arbitrarily derived from the Arabic n. *natrun* or *natron*) soda, sodium carbonate; L. masc. n. *bacillus* rod; N.L. masc. n. *Natribacillus* sodium (-requiring) rod, referring to the high sodium ion requirement and the cell shape].

Cells are motile, Gram-positive, endospore-forming and rod-shaped, approximately 1.0–1.5 × 3.0–5.0 µm. Cell walls contain A1γ meso-diaminopimelic acid-type murein. Alkalitolerant, mesophilic, aerobic and halophilic. Catalase- and oxidase-positive. Reduction of nitrate and gas formation are not observed. The isoprenoid quinone is MK-7. Major cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{16:0} and C_{16:0}. The type species is *Natribacillus halophilus*.

Description of *Natribacillus halophilus* sp. nov.

Natribacillus halophilus (ha.lo'phi.lus. Gr. n. *hals* salt; Gr. adj. *philos* loving; N.L. masc. adj. *halophilus* salt loving).

Exhibits the following characteristics in addition to those given in the genus description. Colonies formed on agar are cream-like and opaque. Oval terminal endospores are produced within swollen sporangia in the stationary phase. Growth occurs in 7–23 % (w/v) NaCl, with optimum growth in 10–15 % (w/v) NaCl. Growth occurs at pH 6.5–10.0, with optimum growth at pH 8.0–8.5. Temperature range for growth is 20–40 °C, with optimum growth at 30 °C. Ornithine decarboxylase-, arginine decarboxylase- and lysine decarboxylase-negative. Tyrosine and gelatin are hydrolysed; starch, pullulan, casein, Tween 80, tributyrin, DNA, hippurate, aesculin, urea, xanthine and hypoxanthine

are not hydrolysed. Indole and H₂S are not produced. Polar lipids are phosphatidylglycerol and diphosphatidylglycerol. D-Mannitol, raffinose, trehalose, D-sorbitol and sucrose can be utilized as sole carbon sources; L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, glycerol, lactose, maltose, D-mannose, ribose and D-xylose cannot be utilized. Acid is produced from lactose and sucrose but not from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, glycerol, maltose, D-mannitol, D-mannose, raffinose, ribose, D-sorbitol, trehalose or D-xylose. Sensitive to (µg per disc) ampicillin (50), bacitracin (25), chloramphenicol (25), erythromycin (25), gentamicin (50), penicillin G (25), rifampicin (50), tetracycline (50) and vancomycin (25); resistant to (µg per disc) anisomycin (50), kanamycin (50), neomycin (25), novobiocin (25), pravastatin (50) and streptomycin (100).

The type strain is HN30^T (=JCM 15649^T=DSM 21771^T), isolated from ordinary soil of a garden in Okabe City, Saitama Prefecture, Japan. The DNA G+C content of the type strain is 47 mol%.

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