Bacillus nanhaiisediminis sp. nov., an alkalitolerant member of *Bacillus* rRNA group 6

Jianli Zhang,¹ Jiewei Wang,¹ Fei Song,¹ Caiyuan Fang,¹ Yuhua Xin² and Yabo Zhang¹

Correspondence

Jianli Zhang

zhangjianli@bit.edu.cn

¹School of Life Science, Beijing Institute of Technology, Beijing 100081, PR China

²China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, PR China

A Gram-stain-positive, rod-shaped bacterium, designated strain NH3^T, was isolated from a sediment sample from the South China Sea and was subjected to a polyphasic taxonomic study. The isolate grew optimally at 37 °C and pH 9. Strain NH3^T had cell-wall peptidoglycan based on *meso*-diaminopimelic acid and MK-7 as the predominant menaquinone. The cellular fatty acid profile included significant amounts of iso-C_{15:0} and iso-C_{14:0}. The major polar lipids were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The DNA G+C content of strain NH3^T was 40.3 mol%. Phylogenetic analysis of the 16S rRNA gene sequence revealed that strain NH3^T was a member of rRNA group 6 of the genus *Bacillus*, which includes alkalitolerant, alkaliphilic and halotolerant species. The closest phylogenetic relatives were *Bacillus akibai* 1139^T (96.82% 16S rRNA gene sequence similarity), *B. pseudofirmus* DSM 8715^T (96.76%), *B. okhensis* Kh10-101^T (96.76%) and *B. alkalidiazotrophicus* MS 6^T (96.47%). Strain NH3^T could be distinguished from these phylogenetically close neighbours based on a number of phenotypic properties. On the basis of phenotypic and chemotaxonomic characteristics and phylogenetic data, we conclude that strain NH3^T (=CGMCC 1.10116^T =JCM 16507^T) merits classification as the type strain of a novel species, for which the name *Bacillus nanhaiisediminis* sp. nov. is proposed.

During the past decade, studies on the ecology, physiology and taxonomy of alkaliphilic and alkalitolerant microbes have revealed an impressive diversity. Since Vedder (1934) first isolated the aerobic, endospore-forming, obligately alkaliphilic bacterium *Bacillus alcalophilus*, many obligately or facultatively alkaliphilic *Bacillus* strains have been isolated in order to investigate their physiological adaptation to high pH and to utilize their enzymes industrially (Horikoshi, 1971, 1999; Yamamoto *et al.*, 1972; Hayashi *et al.*, 1988). Nielsen *et al.* (1994) established a new rRNA group, designated rRNA group 6, that contains some alkaliphilic and alkalitolerant bacilli. *Bacillus* species that belong to rRNA group 6 have been found to form a phylogenetic clade that is distinct from other related genera, as well as *Bacillus* rRNA group 1, which includes *Bacillus subtilis*, the type species of the genus (Nielsen *et al.*, 1994; Schlesner *et al.*, 2001). At the time of writing, more than 20 alkaliphilic *Bacillus* species have been identified (Vedder, 1934; Spanka & Fritze, 1993; Nielsen *et al.*, 1995;

Agnew *et al.*, 1995; Fritze, 1996; Switzer Blum *et al.*, 1998; Yumoto *et al.*, 1998, 2003; Li *et al.*, 2002; Olivera *et al.*, 2005; Nowlan *et al.*, 2006; Borchert *et al.*, 2007). A sampling campaign during an ecological survey of sea-mud bacteria in the South China Sea led to the isolation of a pale-yellow-pigmented bacterium, designated strain NH3^T. The aim of the present study was to determine the exact taxonomic status of strain NH3^T by using a polyphasic approach. Phenotypic and chemotaxonomic characteristics and phylogenetic analysis based on 16S rRNA gene sequences showed that the new isolate merited classification as the type strain of a novel *Bacillus* species.

Strain NH3^T was isolated on marine agar 2216 (MA; Difco), which had been seeded with a sea-mud suspension and incubated at 37 °C for 3 days. The sediment sample was collected at a depth of 900 m. Strain NH3^T was maintained on Luria–Bertani (LB) agar (per litre distilled water: 10 g tryptone, 5 g yeast extract and 10 g NaCl; pH 9.0) at 4 °C and as glycerol suspensions (20%, v/v) at –20 °C. Cell biomass of strain NH3^T for most of the chemotaxonomic and molecular-systematic studies was prepared as described by Zhang *et al.* (2002), with the modification that the strain was grown in shake flasks of LB broth.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NH3^T is GQ292773.

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

Micromorphological and colonial properties were examined on LB agar, nutrient agar and MA for 36–48 h at 37 °C. Cellular morphology was examined by light microscopy (BH-2; Olympus). Gram staining was performed as described by Gerhardt *et al.* (1981). The presence of flagella and spores was assessed according to the methods of Das *et al.* (1996) and Kanso & Patel (2003). Cells of strain NH3^T were motile and Gram-stain-positive. Cells were rod-shaped (0.5–0.7 × 1.3–2.5 µm) with round ends. Spores were observed from cultures grown under different conditions (Supplementary Fig. S1, available in IJSEM Online). Cultures were resistant to heat (70–100 °C for 10 min), supporting the notion that strain NH3^T was a spore-former. Colonies were smooth, circular to slightly irregular, slightly raised, pale yellow and 0.5–2.0 mm in diameter after 36–48 h of cultivation at 37 °C on LB agar.

Strain NH3^T was examined for a range of phenotypic properties. Utilization of sole carbon sources from carbohydrates was assessed according to the procedures described by Gordon *et al.* (1974, 1977) and Zhang *et al.* (2009). Catalase activity was determined on freshly growing colonies by using 3% (v/v) hydrogen peroxide solution. Hydrolysis reactions and determination of oxidative or fermentative catabolism were carried out according to Smibert & Krieg (1994) and La Scola *et al.* (2003). Growth under various pH, temperature and NaCl regimes was determined on LB agar plates incubated for up to 5 days. Anaerobic growth was tested in TYEG medium (Wynter *et al.*, 1996). Resistance to lysozyme was determined by the method of Gordon *et al.* (1973). Resistance to antibiotics was examined by placing impregnated filter-paper discs (Goodfellow & Orchard, 1974) on LB agar; the results were recorded following incubation at 37 °C for up to 3 days. Strain NH3^T grew optimally at 37 °C (range 10–43 °C) and did not grow in the presence of more than 11% (w/v) NaCl. Growth was observed at pH 6.5–10 but not at pH 6, and the optimum pH for growth (pH 9) confirmed strain NH3^T to be an alkalitolerant bacterium. The organism did not grow anaerobically in TYEG medium. Strain NH3^T was catalase-positive but oxidase-negative. A range of carbohydrates were used as sole carbon sources. The phenotypic properties of strain NH3^T are summarized in the species description and compared with those of related species of the genus *Bacillus* in detail in Table 1.

Peptidoglycan was prepared and analysed according to the method described by Komagata & Suzuki (1987). Isoprenoid quinones were extracted and purified according to Collins *et al.* (1987); purified menaquinones were identified by reversed-phase HPLC (Wu *et al.*, 1989). For quantitative analysis of cellular fatty acids, cell mass was harvested and fatty acids were extracted, purified, methylated and identified by using the standard Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Polar lipids were extracted from freeze-dried cell material and separated by two-dimensional silica-gel TLC as described by Tindall (1990). Chromatographs of the polar

lipids were stained with ninhydrin, molybdenum blue and α -naphthol. The G + C content of the DNA was determined by means of the thermal denaturation method (Marmur & Doty, 1962) with DNA from *Escherichia coli* AS 1.365 as a reference. Strain NH3^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. The predominant isoprenoid quinone was unsaturated menaquinone with seven isoprene units (MK-7). Strain NH3^T contained iso-C_{15:0}, iso-C_{14:0} and iso-C_{16:0} as the major fatty acids (see Supplementary Table S1 for detailed results); these branched compounds are typical of the fatty acids found in the cell membranes of *Bacillus* species (Kämpfer, 1994; Albert *et al.*, 2005). The major polar lipids of strain NH3^T were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. Minor amounts of one unknown aminophospholipid and one glycolipid were found (Supplementary Fig. S2). These chemotaxonomic features of strain NH3^T were typical of those found in members of the genus *Bacillus* (Priest *et al.*, 1988; Heyrman *et al.*, 2004; Albert *et al.*, 2005, 2007; Wieser *et al.*, 2005; Lim *et al.*, 2006a, b; Xue *et al.*, 2008). The DNA G + C content of strain NH3^T was 40.3 mol%, a value that falls within the range defined for the genus *Bacillus* (Nielsen *et al.*, 1995; Priest *et al.*, 1988).

Preparation of genomic DNA, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR product from strain NH3^T were carried out according to Rainey *et al.* (1996). The PCR product was sequenced directly by using the method of Lu *et al.* (2001). Sequence gel electrophoresis was performed; the nucleotide sequences were obtained automatically by using an Applied Biosystems DNA sequencer (model 377) and software provided by the manufacturer. Identification of phylogenetic neighbours and calculation of levels of pairwise 16S rRNA gene sequence similarity were achieved by using the EzTaxon server 2.1 (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Multiple alignment with sequences from closely related species was performed by using CLUSTAL X 1.8 (Thompson *et al.*, 1997). Phylogenetic trees were reconstructed with the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Kluge & Farris, 1969) algorithms in MEGA version 4 (Tamura *et al.*, 2007). A maximum-likelihood (Felsenstein, 1981) tree was generated by using the treeing algorithms contained in the PHYLIP package (Felsenstein, 2004). Evolutionary distance matrices were generated according to Kimura (1980). Topologies of the resultant unrooted trees were evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining dataset, based on 1000 resamplings. An almost-complete 16S rRNA gene sequence was obtained for strain NH3^T (1419 nt). 16S rRNA gene sequence analysis showed that strain NH3^T was related phylogenetically to members of the family *Bacillaceae* and belonged within the phyletic group classically defined as the genus *Bacillus*, particularly *Bacillus* species that have been designated as belonging to rRNA group 6. In the neighbour-joining phylogenetic tree, it was apparent that strain NH3^T formed a distinct phyletic line

Table 1. Phenotypic characteristics that differentiate strain NH3^T from the type strains of phylogenetically related *Bacillus* species

Strains: 1, NH3^T; 2, *B. akibai* JCM 9157^T; 3, *B. alkalidiazotrophicus* NCCB 100213^T; 4, *B. okhensis* JCM 13040^T; 5, *B. pseudofirmus* DSM 8715^T. All strains are rod-shaped and positive for utilization of D-glucose and D-mannose. Data were obtained in this study unless indicated otherwise. +, Positive; –, negative; w, weakly positive; v, variable.

Characteristic	1	2	3	4	5
Cell width (µm)	0.5–0.7	0.6–0.8	0.6–1.2	0.6–0.8	0.6–0.8
Cell length (µm)	1.3–2.5	3.0–4.0	3.0–8.0	2.0–2.9	3.0–6.0
Colony pigmentation	Pale yellow	Yellow	Cream	Cream	Yellow
Colony margin	Slightly irregular	Entire	Irregular	Irregular	Irregular
Spore formation	+	+	+	–	+
Ranges for growth					
Temperature (°C)	10–43	20–45	15–43	25–40	10–45
NaCl (% w/v)	0–11	0–7	0.5–7	0–10	0–16
pH	6.5–10	8–10	7.8–10.6	7–10	8–10
pH optimum	9	9–10	9.5	9	9
Nitrate reduction	+	+	–	–	–
Hydrolysis of:					
Casein	–	–	–	+	+
Gelatin	–	–	–	+	+
Starch	–	+	+	+	+
Growth on sole carbon sources					
D-Fructose	–	+	+	+	+
myo-Inositol	–	–	–	–	w
Lactose	–	–	w	+	–
D-Mannitol	+	+	+	–	v
Melezitose	+	+	+	–	–
Melibiose	+	–	+	–	–
Raffinose	+	–	+	–	–
α-L-Rhamnose	+	–	–	+	–
D-Ribose	–	–	–	+	+
D-Sorbitol	+	+	–	–	–
Sucrose	–	+	+	–	+
Trehalose	+	+	+	–	+
D-Xylose	–	+	–	+	+
DNA G + C content (mol%)	40.3	34.4 ^{a*}	37.1 ^b	41 ± 1 ^c	39.0–40.8 ^d

*Data taken from: a, Nogi *et al.* (2005); b, Sorokin *et al.* (2008); c, Nowlan *et al.* (2006); d, Nielsen *et al.* (1995) (range of values for 23 strains, including the type strain).

within the *Bacillus* rRNA group 6 clade (Fig. 1). Strain NH3^T was related most closely to *Bacillus akibai* 1139^T (96.82 % 16S rRNA gene sequence similarity), *B. pseudofirmus* DSM 8715^T (96.76 %), *B. okhensis* Kh10-101^T (96.76 %) and *B. alkalidiazotrophicus* MS 6^T (96.47 %). DNA–DNA hybridization experiments between strain NH3^T and its nearest phylogenetic neighbours were not performed because strains with 16S rRNA gene sequences that differ by more than 3 % are unlikely to exhibit more than 70 % relatedness at the whole-genome level (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002; Stackebrandt & Ebers, 2006).

A range of phenotypic properties distinguished strain NH3^T from its closest phylogenetic neighbours (Table 1). In particular, *B. akibai* JCM 9157^T grew at 20–45 °C, tolerated 7 % NaCl, hydrolysed starch and utilized

D-fructose, sucrose and D-xylose as sole carbon sources, but not melibiose, raffinose or α-L-rhamnose; *B. pseudofirmus* DSM 8715^T grew at 10–45 °C, tolerated 16 % NaCl, hydrolysed casein, gelatin and starch, utilized D-fructose, myo-inositol (weakly), D-ribose, sucrose and D-xylose as sole carbon sources, but not α-L-rhamnose or D-sorbitol, and did not reduce nitrate; *B. okhensis* JCM 13040^T grew at 25–40 °C, tolerated 10 % NaCl, hydrolysed casein, gelatin and starch, utilized D-fructose, lactose, D-ribose and D-xylose as sole carbon sources and did not reduce nitrate; *B. alkalidiazotrophicus* NCCB 100213^T grew at 15–43 °C, tolerated 7 % NaCl, hydrolysed starch, utilized D-fructose, lactose (weakly) and sucrose as sole carbon sources, but not α-L-rhamnose or D-sorbitol, and did not reduce nitrate; strain NH3^T grew at 10–43 °C, tolerated 11 % (w/v) NaCl, did not hydrolyse casein, gelatin or starch, utilized α-L-rhamnose and D-sorbitol as sole carbon sources, but not

Fig. 1. Phylogenetic tree showing the relationships between strain NH3^T and the type strains of closely related *Bacillus* species, constructed by using the neighbour-joining method and based on nearly complete 16S rRNA gene sequences. Asterisks indicate branches of the tree that were also recovered by using both the maximum-parsimony and the maximum-likelihood tree-making algorithms; 'mp' and 'ml' denote branches of the tree that were also recovered by using the maximum-parsimony or maximum-likelihood method, respectively. Numbers at nodes indicate percentage levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values >50% are given. Bar, 0.005 substitutions per site.

D-fructose, *myo*-inositol, D-ribose, sucrose or D-xylose, and reduced nitrate.

On the basis of the data presented, we conclude that strain NH3^T merits recognition as the type strain of a novel species of the genus *Bacillus*, for which the name *Bacillus nanhaiisediminis* sp. nov. is proposed.

Description of *Bacillus nanhaiisediminis* sp. nov.

Bacillus nanhaiisediminis (nan.hai.i.se.di'mi.nis. N.L. n. *nanhaium* Nan Hai, the Chinese name for the South China Sea; L. n. *sedimen* -inis a sediment; N.L. gen. n. *nanhaiisediminis* of a sediment from the South China Sea).

Cells are Gram-stain-positive, aerobic rods, 0.5–0.7 µm wide and 1.3–2.5 µm long. Cells are motile by means of peritrichous flagella. Ellipsoidal endospores are formed subterminally or centrally. Colonies are pale yellow, convex, smooth, circular to slightly irregular and 0.5–2.0 mm in diameter after 36–48 h at 37 °C on LB agar. Grows well on nutrient agar. Diffusible pigments are not formed. Positive for catalase and phenylalanine dehydrogenase. Negative in assays for oxidase, egg-yolk reaction, arginine dihydrolase, lipase and ONPG hydrolysis. Grows at 10–43 °C (optimum 37 °C) and at pH 6.5–10.0 (optimum pH 9.0). Hydrolyses urea but not gelatin, casein, starch, aesculin, arbutin, tyrosine, adenine, guanine,

xanthine or hypoxanthine. Nitrate is reduced. Indole reaction is positive, but methyl red and Voges–Proskauer reactions are negative. Hydrogen sulfide is not produced. Utilizes L-arabinose, cellobiose, D-galactose, glucosamine hydrochloride, D-glucose, 3-methyl glucose, glycerol, maltose, D-mannitol, D-mannose, melezitose, melibiose, raffinose, α-L-rhamnose, D-sorbitol, trehalose, D-xylitol, acetate, fumaric acid, pyruvic acid, sorbic acid and succinate as sole carbon and energy sources, but not aesculin, arbutin, *meso*-erythritol, D-fructose, *N*-acetyl-D-glucosamine, glycogen, *myo*-inositol, lactose, D-ribose, L-sorbose, sucrose, D-xylose, benzoin, citrate, creatine, lactic acid, oxalate or tartrate. Growth occurs in the presence of 0–11% (w/v) NaCl but not 12%. Resistant to (µg per disc unless otherwise stated) aztreonam (30) and streptomycin sulphate (10), but susceptible to amikacin (30), amoxicillin plus clavulanic acid (10), ampicillin (10), cefotaxime (30), chloramphenicol (30), ciprofloxacin (5), clindamycin hydrochloride (2), erythromycin (15), gentamicin sulphate (10), kanamycin sulphate (30), mezlocillin (75), ofloxacin (5), penicillin G (10 IU), rifampicin (5), tetracycline hydrochloride (30), tobramycin sulphate (10) and lysozyme (0.005%, w/v). The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. MK-7 is the major menaquinone. The main fatty acids are iso-C_{15:0}, iso-C_{14:0}, iso-C_{16:0}, anteiso-C_{15:0}, C_{16:1}ω7*c* alcohol, C_{16:0}, C_{16:1}ω11*c* and iso-C_{17:0}. The polar lipids are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol, and minor amounts of one unknown aminophospholipid and one glycolipid. The DNA G+C content of the type strain is 40.3 mol%.

The type strain, NH3^T (=CGMCC 1.10116^T =JCM 16507^T), was isolated from a sediment sample collected from the South China Sea.

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