# *Bacillus nanhaiensis* sp. nov., isolated from an oyster

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A novel Gram-stain-positive, slightly halophilic, facultatively alkaliphilic, catalase-positive, oxidase-negative, endospore-forming, motile, rod-shaped, aerobic bacterium, designated strain JSM 082006$^T$, was isolated from an oyster collected from Naozhou Island in the South China Sea. The isolate grew in 0–18 % (w/v) NaCl (optimum, 0.5–4.0 %), at pH 6.0–10.5 (optimum, pH 8.0) and at 15–45 °C (optimum, 30 °C). *meso*-Diaminopimelic acid was present in the cell-wall peptidoglycan. The major cellular fatty acids were anteiso-$C_{15:0}$, iso-$C_{15:0}$ and $C_{16:0}$. Strain JSM 082006$^T$ contained MK-7 as the predominant respiratory quinone and diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine as the major polar lipids. The genomic DNA G+C content was 40.2 mol%. Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain JSM 082006$^T$ should be assigned to the genus *Bacillus* and that it was most closely related to the type strains of *Bacillus barbaricus* (sequence similarity 99.1 %) and *Bacillus arsenicus* (97.5 %), followed by those of *Bacillus rigui* (96.6 %) and *Bacillus solisalsi* (96.1 %). Phylogenetic analysis, DNA–DNA relatedness values, phenotypic characteristics and chemotaxonomic data support the view that strain JSM 082006$^T$ represents a novel species of the genus *Bacillus*, for which the name *Bacillus nanhaiensis* sp. nov. is proposed; the type strain is JSM 082006$^T$ (=DSM 23009$^T$ =KCTC 13712$^T$).

Halophilic, halotolerant, alkaliphilic and/or alkalitolerant species of the genus *Bacillus* are widely distributed throughout various types of saline environments (Ash *et al.*, 1991; Nielsen *et al.*, 1994; Ventosa *et al.*, 1998; Arahal & Ventosa, 2002; Romano *et al.*, 2005; Lim *et al.*, 2006a, b; Carrasco *et al.*, 2007; Yumoto, 2007; Aino *et al.*, 2008; Chen *et al.*, 2009e; Liu *et al.*, 2009) and these bacteria have attracted increasing interest, attributable to their ability to grow under extreme conditions as well as to the potential use of their enzymes in biotechnological applications (Horikoshi, 1999; Margesin & Schinner, 2001; Nogi *et al.*, 2005; Krulwich *et al.*, 2007). During an investigation of the diversity of the microbial population of invertebrates inhabiting the South China Sea (Chen *et al.*, 2009a, b, c, d, e; Huang *et al.*, 2009; Xiao *et al.*, 2009), a slightly halophilic, facultatively alkaliphilic, endospore-forming, Gram-stain-positive bacterium, designated strain JSM 082006$^T$, was isolated from an oyster collected from Naozhou Island, China. Data from the present taxonomic study indicate that this strain represents a novel species of the genus *Bacillus*.

Strain JSM 082006$^T$ was isolated from homogenates of an oyster by plating 1 : 10 serial dilutions of the sample on marine agar 2216 (MA; Difco) cultivated at 30 °C for 2 weeks. After primary isolation, the strain was purified by repeated streaking and subculturing on nutrient agar (NA; Atlas, 1993) plates (4–5 times) and examining the cultures by light microscopy. The isolate was preserved both on NA slants at 4 °C and as 20 % (v/v) glycerol stocks at −80 °C.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JSM 082006$^T$ is GU477780.

For comparison, two type strains, *Bacillus barbaricus* DSM 14730$^T$ and *Bacillus arsenicus* DSM 15822$^T$, were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Unless indicated otherwise, morphological, physiological, molecular and chemotaxonomic studies were performed with cells grown on NA (pH 8.0) at 30 °C.

Cell morphology was examined by using light microscopy (model DM3000; Leica). Gram staining and the KOH lysis test were carried out according to Smibert & Krieg (1994) and Gregersen (1978), respectively. Flagella and endospores were examined according to the methods of Leifson and Schaeffer-Fulton, respectively (Smibert & Krieg, 1994). Growth was tested at various temperatures (4, 5–55 °C, in increments of 5 °C) and pH (5.0–11.0, in increments of 0.5 pH units) on NA as well as in nutrient broth (NB; Atlas, 1993). Growth in the absence of NaCl was investigated on NA prepared according to the formula of Atlas (1993) except that NaCl was excluded. Tolerance of NaCl was tested on NA at different NaCl concentrations [0.1 and 0.5 % (w/v), and 1–30 % (w/v), in increments of 1 %]. Methyl red and Voges–Proskauer tests, determination of $H_2S$ production from L-cysteine, hydrolysis of aesculin, indole production, and nitrate and nitrite reduction were tested as recommended by Smibert & Krieg (1994). Hydrolysis of casein, cellulose, DNA, gelatin, starch, Tweens 20, 40, 60 and 80, and urea was determined as described by Cowan & Steel (1965). Growth under anaerobic conditions was determined on MA and NA supplemented with 0.5 % (w/v) glucose and with or without 0.1 % (w/v) nitrate by using the GasPak Anaerobic System (BBL) according to the manufacturer's instructions. Determination of acid production from carbohydrates and utilization of carbon and nitrogen sources were performed as recommended by Ventosa *et al.* (1982). Observation of motility and tests for catalase and oxidase activities were carried out as described previously (Chen *et al.*, 2007). Other enzymic activities were assayed by using API ZYM strips (bioMérieux) according to the manufacturer's instructions.

Strain JSM 082006$^T$ was slightly halophilic and facultatively alkaliphilic, with optimum growth occurring in 0.5–4.0 % (w/v) NaCl and at pH 8.0. Colonies were pale yellow-pigmented, flat, translucent with glistening surfaces and circular/slightly irregular margins, and 2–3 mm in diameter after incubation for 2–3 days at 30 °C on NA. Cells were Gram-stain-positive, endospore-forming, motile, aerobic, straight rods. Detailed phenotypic properties that differentiate strain JSM 082006$^T$ from related species of the genus *Bacillus* are summarized in Table 1 and also mentioned in the species description below.

Genomic DNA was isolated according to Hopwood *et al.* (1985) and the G+C content was determined using the HPLC method (Mesbah *et al.*, 1989). The 16S rRNA gene was amplified by PCR and sequenced as described by Cui *et al.* (2001). Pairwise sequence similarities were calculated using a global alignment algorithm, implemented at the EzTaxon server (Chun *et al.*, 2007). Phylogenetic analysis was performed by using the software package MEGA version 4.1 (Tamura *et al.*, 2007) after multiple alignment of sequence data by CLUSTAL X (Thompson *et al.*, 1997). Distances were calculated using distance options according to Kimura's two-parameter model (Kimura, 1980) and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) trees were generated by using the treeing algorithms contained in the PHYLIP package (Felsenstein, 2002). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by means of 1000 resamplings (Felsenstein, 1985). After the DNA was purified to an absorbance ratio ($A_{260}/A_{280}$) greater than 1.8, DNA–DNA hybridization experiments were performed using the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992) using a UV-1206 spectrophotometer (Shimadzu) equipped with a TB-85 thermo-bath. Every hybridization experiment was performed with five replications and the highest and lowest values in each experiment were excluded. The DNA–DNA relatedness values were expressed as the means of the remaining three values.

The DNA G+C content of strain JSM 082006$^T$ was 40.2 mol%. The almost-complete 16S rRNA gene sequence (1429 bp) was determined. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain JSM 082006$^T$ should be assigned to the genus *Bacillus* and that it was most closely related to the type strains of *B. barbaricus* (16S rRNA gene sequence similarity of 99.1 %; Täubel *et al.*, 2003) and *B. arsenicus* (97.5 %; Shivaji *et al.*, 2005), followed by those of *Bacillus rigui* (96.6 %; Baik *et al.*, 2010) and *Bacillus solisalsi* (96.1 %; Liu *et al.*, 2009); sequence similarities observed with other species of the genus *Bacillus* were less than 96 %. The neighbour-joining phylogenetic tree further confirmed that strain JSM 082006$^T$ was phylogenetically related closely to species of the genus *Bacillus* and the isolate formed a robust lineage with the type strains of *B. barbaricus* and *B. arsenicus* (Fig. 1). The topology was similar to those of phylogenetic trees reconstructed by using maximum-likelihood and maximum-parsimony methods (not shown). Levels of DNA–DNA relatedness between strain JSM 082006$^T$ and the type strains of *B. barbaricus* and *B. arsenicus* were 34.1 % (SD of 3.2 %) and 18.5 % (SD of 2.5 %), respectively, values that are well below the threshold value (70 %) recommended by Wayne *et al.* (1987) for the definition of members of a species. Therefore, it would appear that, on the basis of the phylogenetic and DNA–DNA hybridization data, strain JSM 082006$^T$ represents a novel species of the genus *Bacillus* according to accepted criteria (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

Amino acids of whole-cell hydrolysates were analysed as described by Hasegawa *et al.* (1983). Isoprenoid quinones

**Table 1.** Characteristics used to distinguish strain JSM 082006$^T$ from the type strains of phylogenetically related species of the genus *Bacillus*

Strains: 1, *B. nanhaiensis* sp. nov. JSM 082006$^T$; 2, *B. barbaricus* DSM 14730$^T$; 3, *B. arsenicus* DSM 15822$^T$. +, Positive; −, negative. All strains are Gram-stain-positive rods that produce subterminal oval endospores. All strains are positive for catalase activity and hydrolysis of starch, but negative for nitrate and nitrite reduction, $H_2S$ and indole production, methyl red and Voges–Proskauer tests, and hydrolysis of cellulose, DNA, Tweens 20, 40 and 60, and urea. All strains produce acid from D-glucose, glycogen and maltose, but not from adonitol, L-arabinose, cellobiose, dulcitol, D-galactose, glycerol, *myo*-inositol, lactose, D-mannitol, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, D-salicin, D-sorbitol or D-xylose. All strains utilize D-glucose, maltose, dextrin and L-histidine as sole carbon or nitrogen and energy sources, but the following are not utilized: L-arabinose, cellobiose, D-galactose, lactose, melezitose, melibiose, raffinose, L-rhamnose, sucrose, D-xylose, adonitol, D-arabitol, glycerol, D-sorbitol, D-salicin, acetate, butyrate, citrate, propionate, succinate, L-arginine, L-asparagine, L-glutamic acid, glycine, hydroxy-L-proline, L-isoleucine, L-methionine, L-serine and L-valine. Except where marked, all data are from this study.

| Characteristic | 1 | 2 | 3 |
|---|---|---|---|
| Colony colour | Pale yellow | Brownish | Cream |
| Swollen sporangium | − | + | − |
| Motility | + | − | + |
| Oxidase | − | − | + |
| Facultatively anaerobic | − | + | − |
| Growth in NaCl (w/v): | | | |
| 2 % | + | − | + |
| 18 % | + | − | − |
| Growth at: | | | |
| 15 °C | + | − | − |
| pH 10.5 | + | + | − |
| Hydrolysis of: | | | |
| Aesculin | + | + | − |
| Casein | − | + | − |
| Gelatin | + | − | + |
| Tween 80 | − | − | + |
| Acid production from: | | | |
| *N*-Acetylglucosamine | − | + | − |
| D-Fructose | − | + | − |
| Starch | + | + | − |
| Sucrose | + | − | + |
| Trehalose | − | + | − |
| Utilization of: | | | |
| D-Fructose | − | + | + |
| Trehalose | − | + | + |
| *myo*-Inositol | − | − | + |
| Gluconate | − | + | − |
| D-Mannose | − | + | − |
| D-Mannitol | + | − | + |
| D-Ribose | − | + | − |
| L-Alanine | + | + | − |
| L-Leucine | − | + | − |
| L-Phenylalanine | − | + | − |
| L-Proline | − | + | − |
| DNA G+C content (mol%) | 40.2 | 42.0* | 35.0† |

*Data from Baik *et al.* (2010).
†Data from Shivaji *et al.* (2005).

were analysed by HPLC as described by Groth *et al.* (1996). Polar lipids were extracted according to the method of Minnikin *et al.* (1979) and were identified by two-dimensional TLC and spraying with the appropriate detection reagents (Collins & Jones, 1980). Fatty acids in the isolate and *B. barbaricus* DSM 14730$^T$ were determined according to Sasser (1990) using the Microbial Identification System (Microbial ID) with cells grown in NB (pH 8.0; Atlas, 1993) in flasks on a rotary shaker (with shaking at 200 r.p.m.) at 30 °C for 2 days.

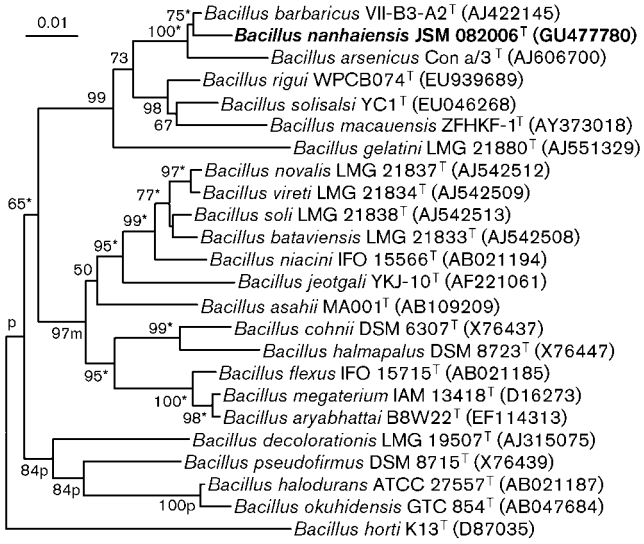


**Fig. 1.** Phylogenetic tree showing the phylogenetic positions of strain JSM 082006$^T$ and related taxa based on 16S rRNA gene sequence analysis reconstructed by using the neighbour-joining method. 'm' or 'p' labels indicate branches that were also found with the maximum-likelihood (Felsenstein, 1981) or maximum-parsimony (Kluge & Farris, 1969) algorithms, respectively; asterisks indicate branches that were recovered with all three methods. Numbers at nodes are bootstrap percentages (>50 %) based on a neighbour-joining analysis of 1000 resampled datasets. Bar, 1 substitution per 100 nt.

Chemotaxonomic data for strain JSM 082006$^T$ were consistent with assignment of the strain to the genus *Bacillus*. The strain possessed a cell-wall type based on *meso*-diaminopimelic acid. Strain JSM 082006$^T$ contained MK-7 (95.1 %) as the predominant menaquinone, with MK-8 (4.1 %) and MK-6 (0.8 %) present in minor amounts. Diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine were predominant in the polar lipid profile; an unknown aminophospholipid, an unknown phospholipid and an unknown polar lipid were also detected. The fatty acid profile of strain JSM 082006$^T$ was similar to that of *B. barbaricus* DSM 14730$^T$, although there were differences in the proportions of some components (Table 2). Major fatty acids (>10 % of the total) of strain JSM 082006$^T$ were anteiso-$C_{15:0}$ (40.7 %), iso-$C_{15:0}$ (17.8 %) and $C_{16:0}$ (14.0 %).

The results of the phylogenetic analysis, and morphological and chemotaxonomic investigations support the affiliation of strain JSM 082006$^T$ to the genus *Bacillus*. However, the pale yellow pigmentation of strain JSM 082006$^T$, as well as the ability to tolerate up to 18 % (w/v) NaCl and grow at 15 °C, clearly differentiated the isolate from its phylogenetic relatives *B. barbaricus* and *B. arsenicus* (Table 1). The presence of noticeable amounts of unbranched saturated fatty acids (making up 19.2 % of the total) and the absence of $C_{16:1}\omega7c$ in the fatty acid profile of strain JSM 082006$^T$ (Table 2), together with several other phenotypic characteristics (Table 1), also differentiated the novel strain clearly from *B. barbaricus*, its closest phylogenetic relative. In conclusion, phylogenetic analysis based on 16S rRNA gene sequences, DNA–DNA relatedness results, and the phenotypic and chemotaxonomic data presented here support the proposal that strain JSM 082006$^T$ represents a novel species of the genus *Bacillus*, for which the name *Bacillus nanhaiensis* sp. nov. is proposed.

## Description of *Bacillus nanhaiensis* sp. nov.

*Bacillus nanhaiensis* (nan.hai.en′sis. N.L. masc. adj. *nanhaiensis* pertaining to Nanhai, the Chinese name for the South China Sea, the source of the sample from which the type strain was isolated).

Cells are Gram-stain-positive, catalase-positive, oxidase-negative, aerobic, straight rods, approximately 0.4–0.6 μm wide and 4.0–6.0 μm long, occurring singly, as pairs or as short chains, producing oval endospores that lie in subterminal unswollen sporangia. Motile by means of a single polar flagellum. Colonies are pale yellow-pigmented, flat and translucent, have glistening surfaces and circular/slightly irregular margins, and are 2–3 mm in diameter on NA. No diffusible pigments are produced. Slightly halophilic and facultatively alkaliphilic; growth occurs in 0–18 % (w/v) NaCl (optimum 0.5–4.0 %), at pH 6.0–10.5 (optimum pH 8.0) and at 15–45 °C (optimum 30 °C). Nitrate and nitrite are not reduced. Negative for methyl red, Voges–Proskauer, $H_2S$ and indole production tests. Aesculin, gelatin and starch are hydrolysed, but casein, cellulose, DNA, Tweens 20, 40, 60 and 80, and urea are not. Acids are produced from D-glucose, glycogen, maltose, starch and sucrose, but not from *N*-acetylglucosamine, adonitol, L-arabinose, cellobiose, dulcitol, D-fructose, D-galactose, glycerol, *myo*-inositol, lactose, D-mannitol, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, D-salicin, D-sorbitol, trehalose or D-xylose. D-Glucose, maltose, dextrin, D-mannitol, L-alanine and L-histidine are utilized as sole sources of carbon and energy or sole sources of carbon, nitrogen and energy; L-arabinose, cellobiose, D-fructose, D-galactose, lactose, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, sucrose, trehalose, D-xylose, adonitol, D-arabitol, glycerol, *myo*-inositol, D-sorbitol, D-salicin, acetate, butyrate, citrate, gluconate, propionate, succinate, L-arginine, L-asparagine, L-glutamic acid, glycine, hydroxy-L-proline, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-serine and L-valine are not utilized. Constitutive enzymes expressed are acid phosphatase, alkaline phosphatase, esterase (C4) and esterase lipase (C8); α-chymotrypsin, cystine arylamidase, α-fucosidase, α- and β-galactosidase, α- and β-glucosidase, *N*-acetyl-β-glucosaminidase, β-glucuronidase, leucine arylamidase, lipase (C14), α-mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase activities are not observed. *meso*-Diaminopimelic acid is present in the cell-wall peptidoglycan as the diagnostic diamino acid. Possesses MK-7 as the

**Table 2.** Fatty acid composition of strain JSM 082006$^T$ and *Bacillus barbaricus* DSM 14730$^T$

Strains: 1, *B. nanhaiensis* sp. nov. JSM 082006$^T$; 2, *B. barbaricus* DSM 14730$^T$. Data are percentages of the total fatty acid content; fatty acids representing <0.5 % in both strains have been omitted. −, Not detected. All data are from this study.

| Fatty acid | 1 | 2 |
|---|---|---|
| Saturated | | |
| $C_{14:0}$ | 2.8 | 0.8 |
| $C_{16:0}$ | 14.0 | 3.5 |
| $C_{16:0}$ N alcohol | 1.8 | 0.6 |
| $C_{17:0}$ | 0.6 | − |
| Unsaturated | | |
| $C_{16:1}\omega7c$ alcohol | 0.6 | 1.0 |
| $C_{16:1}\omega7c$ | − | 6.5 |
| $C_{16:1}\omega11c$ | 1.5 | 7.1 |
| $C_{18:1}\omega9c$ | 0.8 | 0.2 |
| Branched | | |
| iso-$C_{14:0}$ | 7.2 | 5.2 |
| iso-$C_{15:0}$ | 17.8 | 18.1 |
| anteiso-$C_{15:0}$ | 40.7 | 46.2 |
| iso-$C_{16:0}$ | 4.6 | 2.3 |
| iso-$C_{17:0}$ | 1.1 | 1.6 |
| anteiso-$C_{17:0}$ | 2.5 | 4.5 |
| Summed feature 3* | 1.3 | − |
| Summed feature 4* | 0.5 | 1.6 |

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 comprises $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$. Summed feature 4 comprises iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B.

predominant menaquinone and diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine as the predominant polar lipids. Major fatty acids (>10 % of the total) are anteiso-$C_{15:0}$, iso-$C_{15:0}$ and $C_{16:0}$.

The type strain is JSM 082006$^T$ (=DSM 23009$^T$ =KCTC 13712$^T$), isolated from homogenates of an oyster collected from Naozhou Island in the South China Sea, China. The DNA G+C content of the type strain is 40.2 mol% (HPLC method).

## Acknowledgements

This work was supported by grants from the National Basic Research Program of China (2010CB833800), National Natural Science Foundation of China (NSFC) (30970007) and International Cooperation Research Program of Yunnan Province (2009AC017). We are grateful to Mr Ke Huang for his excellent technical assistance.

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