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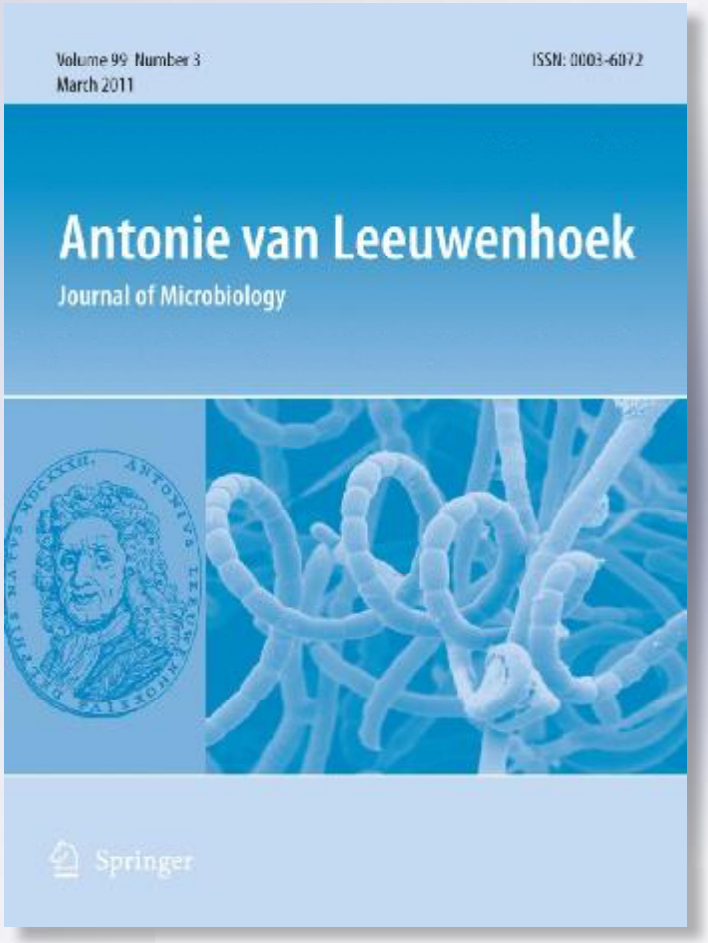
Antonie van Leeuwenhoek
Journal of Microbiology

ISSN 0003-6072
Volume 99
Number 3

Antonie van Leeuwenhoek
(2010) 99:473-480
DOI 10.1007/
s10482-010-9510-9



Springer

Antonie van Leeuwenhoek (2011) 99:473–480
DOI 10.1007/s10482-010-9510-9

ORIGINAL PAPER

Bacillus zhanjiangensis sp. nov., isolated from an oyster in South China Sea

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Received: 22 June 2010 / Accepted: 14 September 2010 / Published online: 24 September 2010

Abstract A novel Gram-stain-positive, motile, catalase- and oxidase-positive, endospore-forming, aerobic, rod-shaped bacterium, designated strain JSM 099021^T, was isolated from an oyster collected from Naozhou Island in the South China Sea. Growth occurred with 0–15% (w/v) NaCl (optimum 2–4%) and at pH 6.0–10.0 (optimum pH 7.5) and at 10–45°C (optimum 30–35°C). *meso*-Diaminopimelic acid was present in the cell-wall peptidoglycan. The predominant respiratory quinone was menaquinone 7 (MK-7) and the major polar lipids consisted of diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol. The major cellular fatty acids were anteiso-C15:0, anteiso-C17:0, iso-C15:0 and iso-C16:0. The genomic DNA G + C content was 39.5 mol%. A phylogenetic analysis based on 16S rRNA gene sequences indicated that strain JSM 099021^T belongs to the genus *Bacillus*, and was most closely related to the type strains of *Bacillus halmapalus* (sequence similarity 99.0%), *Bacillus horikoshii* (98.4%) and *Bacillus cohnii* (98.0%). The combination of phylogenetic analysis, DNA–DNA hybridization, phenotypic characteristics and chemotaxonomic data supported the proposal that strain JSM 099021^T represents a new species of the genus *Bacillus*, for which the name *Bacillus zhanjiangensis* sp. nov. is proposed. The type strain was JSM 099021^T (=DSM 23010^T = KCTC 13713^T).

Keywords *Bacillus zhanjiangensis* sp. nov. · South China Sea

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JSM 099021^T is HM460884.

Electronic supplementary material The online version of this article (doi:10.1007/s10482-010-9510-9) contains supplementary material, which is available to authorized users.

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Introduction

The genus *Bacillus* contains six phylogenetically distinct groups on the basis of 16S rRNA gene sequence analysis (Ash et al. 1991; Stackebrandt and Liesack 1993; Nielsen et al. 1994; Ventosa et al. 1998), and they are attracting interest because this group of bacteria has great biotechnological potential for the production of compatible solutes or hydrolytic enzymes (Horikoshi 1999; Margesin and Schinner 2001; Arahal and Ventosa 2002; 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, 2010; Huang et al. 2009; Xiao et al. 2009), a endospore-forming, Gram-stain-positive bacterium, designated strain JSM 099021^T, was isolated from an oyster collected from Naozhou Island (20° 52′ N–20° 56′ N 110° 33′ E–110° 38′ E), near a southern Chinese city, Zhanjiang. Based on the results of a polyphasic taxonomic study, this strain is considered to represent a novel species of the genus *Bacillus*.

Materials and methods

Strains and culture conditions

Strain JSM 099021^T was isolated from homogenates of an oyster using the dilution plating technique on marine agar 2216 (MA; Difco) at 30°C for 2 weeks. After primary isolation and purification, the isolate was maintained as serial transfers on MA slants, otherwise lyophilized cultures at 4°C and also deep-frozen at −80°C in 20% (v/v) glycerol. For comparison, three type strains, *Bacillus halmapalus* DSM 8723^T, *Bacillus horikoshii* DSM 8719^T and *Bacillus cohnii* DSM 6307^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 MA (pH 7.5) at 35°C.

Phenotypic characterization

Cell morphology was examined by using phase-contrast microscopy (DM3000, ×100 HCX PL Fluotar oil immersion objective, Ph3; Leica) with cells grown on MA plus 10 mg $MnSO_4$ for 3–7 days at 30°C and 7–10 days at room temperature (18–20°C). Flagella were examined according to the methods described by Smibert and Krieg (1994). The Gram staining and the KOH lysis test were carried out according to Smibert and Krieg (1994) and Gregersen (1978), respectively. Growth in the absence of NaCl was investigated on nutrient agar (NA) and in nutrient broth (NB) prepared according to the formula of Atlas (1993) except the addition of NaCl. Tolerance of NaCl was tested on NA as well as in NB at different NaCl concentrations [0.1 and 0.5% (w/v), and 1–30% (w/v) in increments of 1%]. Growth was tested at various temperatures (4, 5–55°C, in increments of 5°C) and at different pH (5.0–11.0, in increments of 0.5 pH units) on MA and NA and in NB. The buffer solutions described by Chen et al. (2007) were used for pH experiments. Methyl red and Voges–Proskauer tests and determination of H_2S production from L-cysteine, hydrolysis of aesculin, indole production, oxidation/fermentation of glucose, nitrate and nitrite reduction and activity of arginine dihydrolase, lysine and ornithine decarboxylase and phenylalanine deaminase were performed as described by Smibert and Krieg (1994). Hydrolysis of casein, cellulose, DNA, gelatin, hippurate, starch, Tween 20, 40, 60 and 80 and urea was determined as described by Cowan and 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 using the GasPak Anaerobic Systems (BBL) according to the manufacturer's instructions. Determination of acid production from carbohydrates and utilization of carbon and nitrogen sources was performed as described by Ventosa et al. (1982). Observation of motility and tests of catalase and oxidase activities were detected as described previously (Chen et al. 2007). Other enzymic activities were assayed using API ZYM strips (bioMérieux) according to the manufacturer's instructions with 3% (w/v) NaCl.

Determination of 16S rRNA gene sequence, phylogenetic analysis and DNA–DNA hybridization

The 16S rRNA gene sequence 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 using the software package MEGA 3.1 (Kumar et al. 2004) 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 and Nei 1987). Maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Kluge and Farris 1969) trees were generated using the tree making algorithms contained in the PHYLIP package (Felsenstein 2002). Bootstrap analysis was used to evaluate the tree topology by means of 1,000 resamplings (Felsenstein 1985). After the DNA was purified to an absorbance ratio of A260 nm versus A280 nm higher 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.

Chemotaxonomic characterization

Amino acids of whole-cell hydrolysates were analysed as described by Hasegawa et al. (1983). Isoprenoid quinones 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 appropriate detection reagents (Collins and Jones 1980). Fatty acids were determined according to Sasser (1990) using the Microbial Identification System (Microbial ID) with cells grown in NB in flasks on a rotary shaker (with shaking at 200 rpm) at 35°C for 2 days. 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).

Results and discussion

Phenotypic characteristics

Strain JSM 099021^T was strictly aerobic and the cells were motile, Gram-stain-positive rods, producing endospores which were ellipsoidal and located subterminally in slightly swollen sporangia. Cells tended to occur in long chains (4–8 cells) at early ages (1–3 days). Colonies were cream-white-pigmented, flat and semitranslucent with smooth, glistening surfaces and circular/slightly irregular margins, and 2–3 mm diameter after incubation for 2–3 days at 35°C on MA. The strain grew optimally in the presence of 2–4% (w/v) NaCl at pH 7.5 and at 30–35°C. Detailed phenotypic properties that differentiate strain JSM 099021^T from related *Bacillus* species are summarized in Table 1 and also mentioned in the species description below.

Phylogenetic analysis based on 16S rRNA gene sequence comparison and DNA–DNA relatedness

The almost-complete 16S rRNA gene sequence (1,506 bp) of the organism was determined (GenBank/EMBL/DDBJ accession number HM460884). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain JSM 099021^T should be assigned to the genus *Bacillus*, and was most closely related to the type strain of *B. halmapalus* (16S rRNA gene sequence similarity 99.0%; Nielsen et al. 1995), *B. horikoshii* (98.4%; Nielsen et al. 1995) and *B. cohnii* (98.0%; Spanka and Fritze 1993); lower than 96.1% sequence similarity was observed with other *Bacillus* species. In the neighbour-joining phylogenetic tree, strain JSM 099021^T formed a robust lineage with the type strains of *B. halmapalus*, *B. horikoshii* and *B. cohnii* (Fig. 1). The topology was similar to those of the phylogenetic trees constructed using maximum-likelihood and maximum-parsimony methods (Supplementary Fig. S1, available in Antonie van Leeuwenhoek Online). Levels of DNA–DNA relatedness between strain JSM 099021^T and strains *B. halmapalus* DSM 8723^T, *B. horikoshii* DSM 8719^T and *B. cohnii* DSM 6307^T were 22.4% (SD, 2.1%), 15.6% (SD, 1.8%) and 15.1% (SD, 2.0%), 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 099021^T represents a new species of the genus *Bacillus* according to accepted criteria (Wayne et al. 1987; Stackebrandt and Goebel 1994).

Table 1 Characteristics used to distinguish strain JSM 099021^T from the type strains of phylogenetically related *Bacillus* species

Characteristic	1	2	3	4
Diffusible pigment	−	−	−	+[b]
Long-chain morphology	+	−	−	−
Spore shape	Ellipsoidal	Ellipsoidal	Oval	Oval
Spore position	Subterminal	Subterminal	Subterminal	Terminal to subterminal
Sporangium	Slightly swollen	Slightly swollen	Slightly swollen	Swollen
Facultatively anaerobic	−	−	−	+
Nitrate reduction	−	−	−	+
Hydrolysis of				
Aesculin	+	−	−	−
Starch	+	+	+	−
Tween 20	−	−	+	+
Tween 40	+	−	+	+
Tween 60	+	−	+	+
Tween 80	+	+	−	+
Growth condition				
NaCl range (%, w/v)	0–15	0–5	0–10	0–10
NaCl optimum (%, w/v)	2–4	0	0	0–1
pH range	6.0–10.0	6.5–10.5	6.0–10.0	6.5–10.0
pH optimum	7.5	7.5–8.0	7.5	7.5–8.0
Temperature range (°C)	10–45	10–40	10–45	10–45
Temperature optimum (°C)	30–35	30–35	35	35
Acid production from:				
N-acetylglucosamine	+	+	+	−
Amygdalin	−	+	−	−
Cellobiose	+	+	+	−
D-fructose	+	+	+	−
Glycerol	+	−	−	−
Glycogen	+	+	+	−
Lactose	+	−	−	−
Maltose	+	+	+	−
D-mannitol	−	+	+	−
D-mannose	−	+	−	−
Melibiose	+	−	−	−
D-salicin	−	+	−	−
Sucrose	−	+	+	−
Trehalose	+	+	+	−
D-xylose	+	+	−	−
Source[a]	Oyster	Soil	Soil	Soil
DNA G + C content (mol%)[a]	39.5	38.6	41.3	34.6

Strains: 1 *B. zhanjiangensis* sp. nov. JSM 099021^T, 2 *B. halmapalus* DSM 8723^T, 3 *B. horikoshii* DSM 8719^T, 4 *B. cohnii* DSM 6307^T

"+", Positive; "−", negative

All strains are endospore-forming, motile, Gram-stain-positive rods. All strains form cream-white colonies, with growth in the presence of 0–5% (w/v) at pH 6.5–10.0 and at 10–40°C. All strains are positive for oxidation of glucose, activity of catalase and oxidase and hydrolysis of casein, gelatin and hippurate, but negative for fermentation of glucose, nitrite reduction, H_2S and indole production, methyl red and Voges–Proskauer test, hydrolysis of cellulose, DNA and urea and activity of arginine dihydrolase, phenylalanine deaminase and lysine and ornithine decarboxylase. All strains are negative for acid production from adonitol, L-arabinose, dulcitol, D-galactose, *myo*-inositol, melezitose, raffinose, L-rhamnose, D-ribose, D-sorbitol. All data were obtained from this study unless indicated otherwise

[a] Data for the type strains of *B. halmapalus*, *B. horikoshii* and *B. cohnii* were obtained from Nielsen et al. (1995) and Spanka and Fritze (1993), respectively

[b] Strain *B. cohnii* DSM 6307^T produced diffusible light brown pigments on MA and NA at 40–45°C

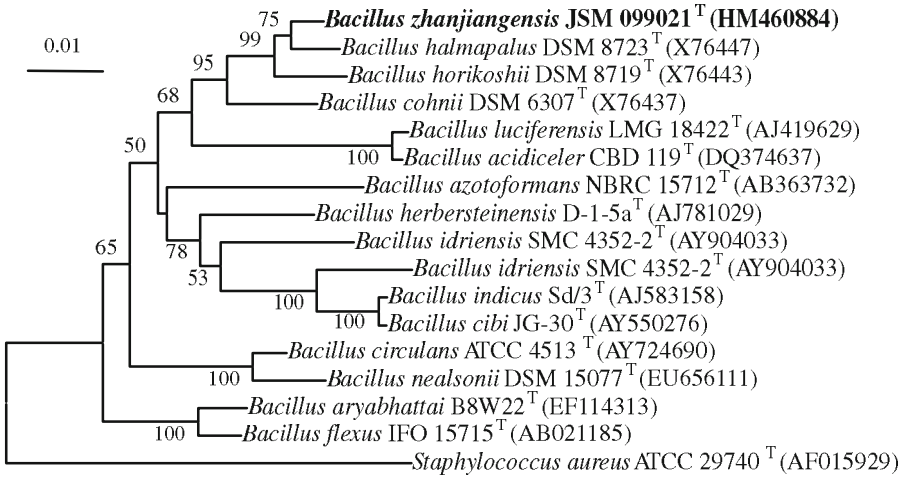

Fig. 1 Phylogenetic tree showing the phylogenetic positions of strain JSM 099021^T and related taxa based on 16S rRNA gene sequence analysis constructed using the neighbour-joining method. *Numbers* at nodes are bootstrap percentages (>50%) based on a neighbour-joining analysis of 1,000 resampled datasets. *Bar*, 1 substitution per 100 nucleotides

Chemotaxonomic characteristics and DNA base composition

Chemotaxonomic data for strain JSM 099021^T were consistent with the assignment of the strain to the genus *Bacillus*. The strain possessed a cell-wall type based on *meso*-diaminopimelic acid as the diagnostic diamino acid. Strain JSM 099021^T contained MK-7 (97.1%) as the predominant menaquinone, with MK-6 (1.0%) and MK-8 (1.9%) present in minor amounts. The polar lipids consisted of predominant amounts of diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol and minor amounts of phosphatidylinositol, phosphatidylinositol-mannoside and an unidentified phospholipid (Supplementary Fig. S2, available in Antonie van Leeuwenhoek Online). The fatty acid profiles of strain JSM 099021^T and the type strains of related species are given in Table 2. The major fatty acids (>5% of the total) of the novel isolate were anteiso-C15:0 (49.2%), anteiso-C17:0 (13.2%), iso-C15:0 (10.7%) and iso-C16:0 (7.3%), which are characteristic of numerous members within the genus *Bacillus* (Kämpfer 1994); iso-C14:0 (4.5%). The DNA G + C content of strain JSM 099021^T was 39.5 mol%.

Taxonomic conclusion

The results of the phylogenetic analysis and of morphological and chemotaxonomic investigations supported the affiliation of strain JSM 099021^T to the genus *Bacillus*. However, the ability to tolerate up to 15% (w/v) NaCl and to hydrolyze aesculin and to produce acid from glycerol, lactose and melibiose, as well as the discriminative long-chain morphology, together with several other phenotypic characteristics, differentiated the isolate clearly from its phylogenetic relatives (Table 1). Strain JSM 099021^T also differed with respect to its fatty acid profile, in which there were significant amounts of anteiso-C15:0 (49.2%) and anteiso-C17:0 (13.2%), whereas the amount of iso-C15:0 (10.7%) was noticeably less than those of the three related type strains (Table 2). In conclusion, the phylogenetic analysis based on 16S rRNA gene sequences, the DNA–DNA relatedness results and the phenotypic and chemotaxonomic data presented here support the proposal of strain JSM 099021^T representing a novel species of the genus *Bacillus*, for which we propose the name *Bacillus zhanjiangensis* sp. nov.

Description of *B. zhanjiangensis*

B. zhanjiangensis (zhan.ji.ang.en'sis. N.L. masc. adj. *zhanjiangensis* pertaining to Zhanjiang, a Chinese city near which the sample was collected).

Cells are catalase- and oxidase-positive, aerobic, Gram-stain-positive rods, approximately 0.6–1.0 μm wide and 3.0–6.0 μm long, producing endospores

Table 2 Fatty acid profiles of strain JSM 099021^T and related *Bacillus* species

Fatty acid[a]	1	2	3	4
Straight-chain				
C15:0	–	–	–	0.9
C16:0	4.3	1.2	0.8	1.0
C16:0 N alcohol	1.3	0.7	0.3	–
C17:0	0.3	–	–	0.7
C18:0	0.8	0.5	0.2	–
Unsaturated				
C16:1ω7*c* alcohol	0.6	3.9	10.3	2.1
C16:1ω11*c*	0.5	1.3	1.6	5.1
C18:1ω9*c*	0.6	0.9	0.6	–
Branched				
iso-C13:0	–	0.9	0.1	–
iso-C14:0	4.5	2.0	9.8	2.8
iso-C15:0	10.7	46.6	40.5	29.3
anteiso-C15:0	49.2	9.3	5.2	18.5
iso-C16:0	7.3	2.6	10.1	15.0
iso-C17:1ω10*c*	0.5	16.0	11.3	1.5
iso-C17:0	4.0	8.7	1.7	18.3
anteiso-C17:0	13.2	1.9	1.5	2.2
Summed feature 4	0.7	2.1	5.6	1.3

Strains: 1 *B. zhanjiangensis* sp. nov. JSM 099021^T, 2 *B. halmapalus* DSM 8723^T, 3 *B. horikoshii* DSM 8719^T, 4 *B. cohnii* DSM 6307^T. Values are percentages of total fatty acid content, those representing <0.5% in all strains being omitted. "–", Not detected. All data were from this study

[a] Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 4 contained iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B

which are ellipsoidal and locate subterminally in slightly swollen sporangia. Cells tend to occur in long chains (4–8 cells). Motile by means of peritrichous flagella. Colonies are cream-white-pigmented, flat and semitranslucent, have smooth, glistening surfaces and circular/slightly irregular margins, and are 2–3 mm diameter on MA. No diffusible pigments are produced. Growth occurs with 0–15% (w/v) NaCl (optimum 2–4%) and at pH 6.0–10.0 (optimum pH 7.5) and at 10–45°C (optimum 30–35°C). Nitrate and nitrite is not reduced. Negative for tests of glucose fermentation, methyl red, Voges–Proskauer, H_2S and indole production. Aesculin, casein, gelatin, hippurate, starch, Tween 40, 60 and 80 are hydrolyzed, but cellulose, DNA, Tween 20 and urea are not. Acids are produced from cellobiose, D-fructose, D-glucose, glycogen, lactose, maltose, melibiose, trehalose, D-xylose, glycerol, and *N*-acetylglucosamine, but not from L-arabinose, D-galactose, D-mannose, melezitose, raffinose, L-rhamnose, D-ribose, sucrose, amygdalin, D-salicin, adonitol, dulcitol, *myo*-inositol, D-mannitol or D-sorbitol. The following compounds are utilized as sole sources of carbon and energy or sole sources of carbon, nitrogen and energy: cellobiose, dextrin, D-fructose, D-glucose, maltose, D-mannose, sucrose, starch, trehalose, glycerol, glycogen, citrate, gluconate, *N*-acetylglucosamine and L-asparagine; the following are not utilized: L-arabinose, D-galactose, lactose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, D-xylose, D-salicin, adonitol, D-arabitol, *myo*-inositol, D-mannitol, D-sorbitol, acetate, butyrate, malate, propionate, succinate, L-alanine, L-arginine, L-glutamic acid, glycine, L-histidine, hydroxy-L-proline, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-serine and L-valine. Constitutive enzymes expressed are *N*-acetyl-β-glucosaminidase, alkaline phosphatase, α-chymotrypsin, esterase lipase (C8), leucine arylamidase, lipase (C14), naphthol-AS-BI-phosphohydrolase and trypsin; acid phosphatase, arginine dihydrolase, cystine arylamidase, esterase (C4), α-fucosidase, α- and β-galactosidase, α- and β-glucosidase, β-glucuronidase, lysine decarboxylase, α-mannosidase, ornithine decarboxylase, phenylalanine deaminase and valine arylamidase are not observed. *meso*-Diaminopimelic acid is present in the cell-wall peptidoglycan as the diagnostic diamino acid. Possesses MK-7 as the predominant menaquinone, and diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol as the major polar lipids. Major fatty acids are anteiso-C15:0, anteiso-C17:0, iso-C15:0 and iso-C16:0. The DNA G + C content of the type strain is 39.5 mol% (HPLC method).

The type strain, JSM 099021^T (=DSM 23010^T = KCTC 13713^T), was isolated from homogenates of an oyster collected from Naozhou Island in the South China Sea, near a southern Chinese city, Zhanjiang.

Acknowledgments This work was supported by grants from the National Basic Research Program of China (2010CB833800), National Natural Science Foundation of China (NSFC) (30970007), Key Program of Shanghai Agrobiological Gene Center (SAGC200903) and Key Laboratory of Ecotourism's Application Technology of Hunan Province (SL2010Z01). We are grateful to Mr Qi-Hui Chen and Zhu-Xiang Liu for their excellent technical assistance.

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