Pontibacillus litoralis sp. nov., a facultatively anaerobic bacterium isolated from a sea anemone, and emended description of the genus *Pontibacillus*

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A facultatively anaerobic, moderately halophilic, Gram-positive, endospore-forming, motile, catalase- and oxidase-positive, rod-shaped bacterium, strain JSM 072002^T, was isolated from a sea anemone (*Anthopleura xanthogrammica*) collected from the South China Sea. Strain JSM 072002^T was able to grow with 0.5–15 % (w/v) NaCl and at pH 6.0–10.0 and 15–50 °C; optimum growth was observed with 2–5 % (w/v) NaCl and at pH 7.5 and 35 °C. *meso*-Diaminopimelic acid was present in the cell-wall peptidoglycan. The major cellular fatty acids were iso-C_{15:0} and anteiso-C_{15:0}. The predominant respiratory quinone was menaquinone 7 and the genomic DNA G + C content was 41.3 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain JSM 072002^T should be assigned to the genus *Pontibacillus* and revealed relatively low 16S rRNA gene sequence similarities (<97 %) with the type strains of the three recognized *Pontibacillus* species (*Pontibacillus chungwhensis* BH030062^T, 96.8 %; *Pontibacillus marinus* KCTC 3917^T, 96.7 %; *Pontibacillus halophilus* JSM 076056^T, 96.0 %). The combination of phylogenetic analysis, DNA–DNA relatedness values, phenotypic characteristics and chemotaxonomic data supports the view that strain JSM 072002^T represents a novel species of the genus *Pontibacillus*, for which the name *Pontibacillus litoralis* sp. nov. is proposed. The type strain is JSM 072002^T (=DSM 21186^T=KCTC 13237^T). An emended description of the genus *Pontibacillus* is also presented.

The genus *Pontibacillus* was first proposed by Lim *et al.* (2005a) and was defined as containing Gram-positive, moderately halophilic, strictly aerobic, motile, endospore-forming rods with *meso*-diaminopimelic acid cell-wall type

and menaquinone 7 (MK-7) as the predominant respiratory quinone (Lim *et al.*, 2005a, b). At the time of writing, the genus comprised three species with validly published names: *Pontibacillus chungwhensis* (Lim *et al.*, 2005a), *Pontibacillus marinus* (Lim *et al.*, 2005b) and *Pontibacillus halophilus* (Chen *et al.*, 2009b). 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; Xiao *et al.*, 2009), a facultatively anaerobic, moderately halophilic, endospore-forming, Gram-positive bacterium, designated JSM 072002^T, was isolated from a sea anemone collected from a tidal flat of Naozhou Island, China. Based

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain JSM 072002^T and *Pontibacillus marinus* KCTC 3917^T are EU583724 and FJ808613, respectively.

A supplementary figure showing maximum-parsimony and maximum-likelihood phylogenetic trees based on 16S rRNA gene sequences is available with the online version of this paper.

on the results of a polyphasic taxonomic study, this strain is considered to represent a novel species of the genus *Pontibacillus*.

Strain JSM 072002^T was isolated from homogenates of a surface-sterilized sea anemone (*Anthopleura xanthogrammica*) by plating 1:10 serial dilutions of the sample on marine agar 2216 (MA, pH 7.5; Difco) cultivated at 37 °C for 7 days. After primary isolation, the strain was purified by repeated streaking and subculturing on MA plates (4–5 times) and examining the cultures by light microscopy. The isolate was preserved both on MA slants at 4 °C and as 20% (v/v) glycerol stocks at –80 °C. Three reference strains, *P. chungwhensis* KCTC 3890^T, *P. marinus* KCTC 3917^T and *P. halophilus* JSM 076056^T, were obtained from the KCTC (Korean Collection for Type Cultures) and the YIM (Yunnan Institute of Microbiology, China), respectively. Unless otherwise indicated, morphological, molecular and chemotaxonomic studies were performed with cells grown on MA at 35 °C.

Cell morphology was examined by using light microscopy. The Gram reaction was carried out by using the staining method of Smibert & Krieg (1994) combined with the KOH lysis test (Buck, 1982). Flagella and endospores were examined according to the methods of Leifson and Schaeffer-Fulton, respectively (Smibert & Krieg, 1994). Growth was tested at various temperatures (5–55 °C, in increments of 5 °C) and different pH values (5.0–11.0, in increments of 0.5 pH units) on MA as well as in marine broth 2216 (pH 7.5; Difco). Tolerance of and requirement for salts was determined as described by Lim *et al.* (2005a, b). Growth was also tested with NaCl as the sole salt. Growth under anaerobic conditions was determined on MA supplemented with or without 0.1% (w/v) nitrate by using the GasPak Anaerobic Systems (BBL) according to the manufacturer's instructions. Hydrolysis of casein, DNA, gelatin, hypoxanthine, starch, Tweens 20, 40, 60 and 80, urea and xanthine was determined as described by Cowan & Steel (1965). Methyl red and Voges–Proskauer tests and determination of aesculin hydrolysis, indole and H₂S production, nitrate and nitrite reduction, phenylalanine deaminase and lysine and ornithine decarboxylases were performed as recommended by Smibert & Krieg (1994). Oxidation/fermentation of glucose and respiration on fumarate, nitrate and nitrite were studied using the procedures described by Mata *et al.* (2002) with MH medium (Quesada *et al.*, 1983) containing 4% (w/v) sea salts (Rodriguez-Valera *et al.*, 1981) as the basal medium. Determination of acid production from carbohydrates and utilization of carbon and nitrogen sources was performed as recommended by Ventosa *et al.* (1982). Observation of motility and tests for antibiotic susceptibility, anaerobic growth and catalase and oxidase activities were performed as described by Chen *et al.* (2007). Other enzyme activities were assayed by using API ZYM strips (bioMérieux) according to the manufacturer's instructions except that the suspension medium was supplemented with 4% (w/v) NaCl.

Colonies were creamy white-pigmented, flat and non-translucent with glistening surfaces and circular/slightly

irregular margins. Cells were facultatively anaerobic, moderately halophilic, motile, endospore-forming, catalase- and oxidase-positive, Gram-positive rods, with growth occurring with 0.5–15% (w/v) NaCl (optimum, 2–5%), at pH 6.0–10.0 (optimum, pH 7.5) and at 15–50 °C (optimum, 35 °C). Methyl red, nitrate reduction and fermentation of glucose tests gave positive results. The results of other phenotypic tests are summarized in the species description and in Table 1.

DNA was isolated according to the method of Hopwood *et al.* (1985) and the G+C content was determined by using the HPLC method (Mesbah *et al.*, 1989). The 16S rRNA gene sequence was amplified by PCR and sequenced as described by Cui *et al.* (2001). The resulting sequence was compared with those obtained from public databases (GenBank/EMBL/DDBJ) to find the most similar sequences. Multiple alignment was carried out with CLUSTAL X (Thompson *et al.*, 1997). Gaps at the 5' and 3' ends of the alignment were omitted from further analysis. Distances were calculated using distance options according to Kimura's two-parameter model (Kimura, 1980). A neighbour-joining (Saitou & Nei, 1987) tree was constructed by using the software package MEGA version 3.1 (Kumar *et al.*, 2004). 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). The confidence values for the branches of the phylogenetic trees were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. DNA–DNA hybridization was carried out by using photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989).

The DNA G+C content of strain JSM 072002^T was 41.3 mol%. Almost-complete 16S rRNA gene sequences of strain JSM 072002^T (1475 bp) and *P. marinus* KCTC 3917^T (1430 bp) were determined. Phylogenetic analyses based on 16S rRNA gene sequences revealed that the isolate was a member of the family *Bacillaceae* and was most closely related to the type strains of the three recognized species of the genus *Pontibacillus*. The four strains constituted an independent lineage in the phylogenetic trees and strain JSM 072002^T occupied a distinct clade (Fig. 1 and Supplementary Fig. S1, available in IJSEM Online). Strain JSM 072002^T showed relatively low levels (<97%) of 16S rRNA gene sequence similarity with *P. chungwhensis* BH030062^T (96.8%), *P. marinus* KCTC 3917^T (96.7%) and *P. halophilus* JSM 076056^T (96.0%), which should permit classification of this strain as representing a novel species of the genus *Pontibacillus* (Stackebrandt & Goebel, 1994; Rosselló-Mora & Amann, 2001).

Amino acids of whole-cell hydrolysates were analysed as described by Hasegawa *et al.* (1983). Menaquinones were isolated by using the methods of Minnikin *et al.* (1984) and then separated by HPLC (Kroppenstedt, 1982). Fatty acid compositions were determined as described by Sasser (1990) by using the Microbial Identification System

Table 1. Differential characteristics between strain JSM 072002^T and recognized *Pontibacillus* species

Strains: 1, JSM 072002^T (*Pontibacillus litoralis* sp. nov.; data from this study); 2, *P. chungwhensis* BH030062^T (Lim *et al.*, 2005a); 3, *P. marinus* BH030004^T (Lim *et al.*, 2005b); 4, *P. halophilus* JSM 076056^T (Chen *et al.*, 2009b). +, Positive; –, negative.

Characteristic	1	2	3	4
Colony pigmentation	Creamy white	Yellow	Cream	Yellow
Spore shape	Ellipsoidal	Spherical	Spherical	Ellipsoidal
Spore position	Subterminal	Terminal	Terminal	Subterminal
Conditions for growth				
NaCl range (% w/v)	0.5–15	1–15	1–9	1–25*
NaCl optimum (% w/v)	2–5	2–5	2–5	4–9*
Temperature range (°C)	15–50	15–40	15–45	15–40
Temperature optimum (°C)	35	30	35–40	25–30
pH range	6.0–10.0	6.5–9.5	6.0–9.0	6.0–10.0
pH optimum	7.5	7.5–8.5	7.0–7.5	7.0–8.0
Anaerobic growth	+	–	–	–
Oxidase	+	–	+	+
Nitrate reduction to nitrite	+	–	+	–
Hydrolysis of:				
Aesculin	+	–	+	+
Casein	–	+	–	–
Gelatin	+	–	–	+
Starch	–	+	–	+
Tween 80	–	+	+	–
Acid production from:				
D-Fructose	+	–	+	–
D-Glucose	+	+	–	–
Glycerol	–	+	–	–
Maltose	+	+	+	–
D-Mannose	–	–	–	+
Melibiose	–	–	+	–
Raffinose	–	–	+	–
D-Ribose	–	+	+	+
Trehalose	+	+	+	–
Major fatty acids†				
iso-C _{14:0}	5.6	7.3	4.0	16.6
iso-C _{15:0}	55.1	28.5	65.1	31.5
anteiso-C _{15:0}	11.1	31.1	10.2	10.5
iso-C _{16:0}	7.5	10.2	2.2	16.6
C _{16:1} ω7c alcohol	8.2	7.3	8.8	10.1
DNA G + C content (mol%)	41.3	41.0	42.0	45.5

*Data from this study.

†Major fatty acids are in bold; data are percentages of the total fatty acid content.

(Microbial ID). The chemotaxonomic data for strain JSM 072002^T were consistent with its assignment to the genus *Pontibacillus* (Lim *et al.*, 2005a, b). The peptidoglycan of the organism contained *meso*-diaminopimelic acid as the diagnostic diamino acid. MK-7 (98.6 %) was the predominant respiratory quinone, with MK-8 (1.4 %) present in minor amounts. Major fatty acids (>10 % of the total) of strain JSM 072002^T were iso-C_{15:0} (55.1 %) and anteiso-C_{15:0} (11.1 %) (Table 1), with C_{16:1}ω7c alcohol (8.2 %), iso-C_{16:0} (7.5 %), iso-C_{14:0} (5.6 %), iso-C_{17:0} (3.2 %), C_{16:0} (2.0 %), iso-C_{17:1}ω10c (2.8 %), C_{18:1}ω9c (1.8 %) and summed feature 4 (iso-C_{17:1} I and/or anteiso-C_{17:1} B;

1.8 %) present in minor amounts and C_{16:1}ω11c and anteiso-C_{17:0} being detected at a level of <1 %. This fatty acid profile was similar to those of the type strains of the three recognized *Pontibacillus* species, although there were differences in the proportions of some fatty acids (Lim *et al.*, 2005b; Chen *et al.*, 2009b).

The results of the phylogenetic analysis and the morphological and chemotaxonomic studies supported the affiliation of strain JSM 072002^T to the genus *Pontibacillus* (Lim *et al.*, 2005a, b). However, the isolate differed markedly from the three recognized *Pontibacillus* species by the

Fig. 1. Phylogenetic tree showing the position of strain JSM 072002^T among related taxa based on 16S rRNA gene sequences constructed by using the neighbour-joining method. Percentages at nodes are levels of bootstrap support based on 1000 resampled datasets. Bar, 1 substitution per 100 nucleotide positions.

strain's capability of facultative anaerobiosis, as well as by the creamy white pigmentation, the comparatively wide growth-temperature range (15–50 °C) and by the strain's inability to produce acid from D-ribose (Table 1). To establish the precise taxonomic position of strain JSM 072002^T, DNA–DNA hybridizations were performed between the isolate and *P. chungwhensis* KCTC 3890^T and *P. marinus* KCTC 3917^T and the levels of DNA–DNA relatedness were 43.5 and 36.7 %, respectively, which were below the threshold value of 70 % recommended by Wayne *et al.* (1987) for assigning strains to the same species. Together with the relatively low levels (<97 %) of 16S rRNA gene sequence similarity between strain JSM 072002^T and *Pontibacillus* species, these results allow us to assign the isolate to the genus *Pontibacillus* as a representative of a novel species, for which we propose the name *Pontibacillus litoralis* sp. nov.

Description of *Pontibacillus litoralis* sp. nov.

Pontibacillus litoralis (li.to.ra'lis. L. masc. adj. *litoralis* of or belonging to the seashore).

Cells are facultatively anaerobic, moderately halophilic, catalase- and oxidase-positive, Gram-positive rods, approximately 0.4–0.8 µm wide and 3.5–5.0 µm long. Motile by means of peritrichous flagella and bear

ellipsoidal endospores that lie in subterminal swollen sporangia. Colonies are creamy white-pigmented, flat and non-translucent with glistening surfaces and circular/slightly irregular margins, 1–2 mm in diameter after incubation at 35 °C on MA for 2–5 days. No diffusible pigments are produced. Growth occurs at pH 6.0–10.0 (optimum, pH 7.5) and 15–50 °C (optimum, 35 °C). Growth occurs in the presence of 0.5–15 % (w/v) NaCl in complex medium [optimum, 2–5 % (w/v) NaCl]. Does not grow with NaCl as a sole salt. Positive for methyl red test, nitrate reduction and fermentation of glucose, but negative for H₂S and indole production, nitrite reduction, Voges–Proskauer test, phenylalanine deaminase and lysine and ornithine decarboxylases. Respiration on nitrate, nitrite and fumarate under anaerobic conditions is not observed. Aesculin, gelatin and Tween 40 are hydrolysed, but casein, DNA, hypoxanthine, starch, Tweens 20, 60 and 80, urea and xanthine are not. Acid is produced from D-fructose, D-glucose, maltose, sucrose, trehalose and turanose, but not from adonitol, L-arabinose, 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. Utilizes D-fructose, D-glucose, glycerol, maltose and D-mannitol as sole carbon sources and L-alanine and L-asparagine as sole nitrogen sources, but not L-arabinose, cellobiose, dextrin, D-fructose, D-galactose,

lactose, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, D-salicin, sucrose, trehalose, D-xylose, adonitol, D-arabitol, *myo*-inositol, D-sorbitol, acetate, butyrate, citrate, gluconate, propionate, L-arginine, L-glutamic acid, glycine, L-histidine, hydroxy-L-proline, L-isoleucine, L-leucine, L-methionine, L-proline, L-serine and L-valine. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase. Negative for acid phosphatase, α -chymotrypsin, cystine arylamidase, α -fucosidase, α - and β -galactosidases, *N*-acetyl- β -glucosaminidase, α - and β -glucosidases, β -glucuronidase, lipase (C14), α -mannosidase, trypsin and valine arylamidases. Cells are susceptible to (μ g per disc): ampicillin (30), carbenicillin (30), chloramphenicol (30), gentamicin (10), kanamycin (30), lincomycin (2), rifampicin (5) and streptomycin (10), but not to nalidixic acid (20), novobiocin (30), polymyxin B (30) or tetracycline (30). *meso*-Diaminopimelic acid is present in the cell-wall peptidoglycan as the diagnostic diamino acid. The predominant menaquinone is MK-7. The major cellular fatty acids are iso-C_{15:0} and anteiso-C_{15:0}. The DNA G + C content of the type strain is 41.3 mol% (HPLC method).

The type strain, JSM 072002^T (=DSM 21186^T=KCTC 13237^T), was isolated from homogenates of a surface-sterilized sea anemone (*Anthopleura xanthogrammica*) collected from a tidal flat of a China island, Naozhou, located at the edge of the South China Sea, China.

Emended description of the genus *Pontibacillus* Lim *et al.* 2005

The description is as given by Lim *et al.* (2005a, b) with the following changes. Aerobiosis or facultative anaerobiosis. Positive or negative for oxidase. Spherical or ellipsoidal endospores are formed in terminal or subterminal swollen sporangia. The genomic DNA G + C content is 41.0–45.5 mol%.

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