Amphibacillus sediminis sp. nov., an endospore-forming bacterium isolated from lake sediment in Japan

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A facultatively anaerobic, moderately alkaliphilic, Gram-positive, spore-forming and rod-shaped bacterial strain, Shu-P-Ggiii25-2^T, was isolated from lake sediment in Shizuoka, Japan, and was characterized taxonomically using a polyphasic approach. Analysis of the 16S rRNA gene sequence indicated that the novel isolate clustered with the type strain of *Amphibacillus xylanus* and it exhibited sequence similarities of 94.9–95.6% to the type strains of species of the genus *Amphibacillus*. Isoprenoid quinones and oxidase and catalase activities were not detected for strain Shu-P-Ggiii25-2^T. The DNA G+C content was 42.3 mol%, the cell-wall peptidoglycan contained *meso*-diaminopimelic acid as the diagnostic diamino acid and the major cellular fatty acids were iso-C_{14:0}, iso-C_{15:0}, anteiso-C_{15:0}, C_{16:0} and iso-C_{16:0}. On the basis of phylogenetic position and phenotypic properties, strain Shu-P-Ggiii25-2^T represents a novel species of the genus *Amphibacillus*, for which the name *Amphibacillus sediminis* sp. nov. is proposed. The type strain is Shu-P-Ggiii25-2^T (=MBIC08269^T=IAM 15428^T=KCTC 13120^T). An emended description of the genus *Amphibacillus* is also given.

The genus *Amphibacillus* was first proposed by Niimura *et al.* (1990) and the genus currently comprises three recognized species, *Amphibacillus xylanus* (Niimura *et al.*, 1990), *Amphibacillus fermentum* and *Amphibacillus tropicus* (Zhilina *et al.*, 2001). Members of the genus *Amphibacillus* are moderately alkaliphilic and facultatively anaerobic (Zhilina *et al.*, 2001) and lack isoprenoid quinones and oxidase activity (Niimura *et al.*, 1990).

Here, we describe a novel strain, Shu-P-Ggiii25-2^T, which was isolated from sediment from Lake Hamana (Shizuoka, Japan) in the course of an environmental investigation. Based on partial 16S rRNA gene sequence analyses, the novel strain was found to be closely related to the genus *Amphibacillus*. The aim of the present study is to elucidate the taxonomic position of the isolate by using a polyphasic approach (physiological and chemotaxonomical analyses, fatty acid composition, quinone system, DNA G+C content and 16S rRNA gene sequence analysis). Based on

these substantial data, it is proposed that the isolate represents a novel species in the genus *Amphibacillus*.

Strain Shu-P-Ggiii25-2^T was isolated from sediment at a depth of 1 m in Lake Hamana in Shizuoka, Japan, in 2004. Suspended sediment was used for enrichment culture using GYPiii medium [5 g Bacto yeast extract (BD), 1 g Polypepton (soybean, Nihon Pharma), 1 g casein Polypepton (Nihon Pharma), 1 g fish meat extract, 2 g CaCO₃, 1.5 g Na₂CO₃, 10 g glucose, 70 g NaCl, 1 mg sodium pyruvate, 20 mg MgSO₄·7H₂O, 1 mg MnSO₄·4H₂O, 1 mg FeSO₄·7H₂O, 10 mg colistin, 10 mg sodium azide, 10 mg cycloheximide, 30 mg bromothymol blue (BTB), 5 mg Tween 80, 900 ml seawater for 1 l medium, pH 10] after incubation at 25 °C. When the colour of the pH indicator, BTB, changed, a small portion of culture was transferred to new medium. Strain Shu-P-Ggiii25-2^T was isolated from the second enrichment culture on GYPiii agar after incubation at 25 °C for three days. Isolate Shu-P-Ggiii25-2^T was routinely cultivated on trypticase soy agar (TSA; BBL) containing 50% Herbst's artificial seawater and incubated at 27 °C. Herbst's artificial seawater contains the following (1⁻¹ distilled water): 30 g NaCl, 0.7 g KCl, 5.3 g MgSO₄·7H₂O, 1.3 g CaCl₂·2H₂O and 10.8 g MgCl₂·6H₂O.

Abbreviation: BTB, bromothymol blue.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Shu-P-Ggiii25-2^T is AB243866.

Cell morphology and motility were examined using phase-contrast microscopy (BX60; Olympus). Growth under anaerobic conditions was determined after a week of incubation in an AnaeroPack (Mitsubishi Gas Chemical Co., Ltd). The temperature range for growth was assessed at various temperatures (5–60 °C). The pH range for growth was determined by adjusting the medium to various pH values (initial pH 6.0–10.0 at intervals of 0.5 pH units). Prior to sterilization, the pH was adjusted to various levels by using HCl and NaOH. Growth at various NaCl concentrations was investigated in trypticase soy agar containing 50 % Herbst's artificial seawater lacking NaCl. Catalase activity was tested by adding a drop of 3 % H₂O₂ to a single colony and was recorded as positive when the development of bubbles was observed. Oxidase activity was determined by cytochrome oxidase paper (Nissui Pharmaceutical Co., Ltd). API 20E and API 50CH microtest galleries (bioMérieux) were used according to the manufacturer's instructions to determine the physiological and biochemical characteristics of the novel strain. The API strips were recorded after 2 days of incubation at 30 °C. The results of the morphological, biochemical and physiological properties are given in the species description.

Based on a comparison of partial 16S rRNA gene sequences (Goto *et al.* 2000, 2002), strain Shu-P-Ggiii25-2^T was placed in the *Amphibacillus*–*Halolactobacillus* cluster. However, the sequences of the novel strain were found to be distinct from previously described species of both of these genera. The 16S rRNA gene sequence was determined using a 16S rRNA gene kit following the manufacturer's protocols (Applied Biosystems). The 16S rRNA gene sequence of strain Shu-P-Ggiii25-2^T was used for BLAST searches via the NCBI (National Center for Biotechnology Information). The sequences obtained were aligned by using the CLUSTAL_X software package (Thompson *et al.*, 1997) and evolutionary distances and *K*_{nuc} values (Kimura, 1980) were generated. Alignment gaps and ambiguous bases were not considered when the 1350 bases of the 16S

rRNA gene were compared. A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. The similarity values were calculated using MEGA3 (Kumar *et al.*, 2004). The almost complete 16S rRNA gene sequence of strain Shu-P-Ggiii25-2^T was determined and subjected to comparative analysis. The highest degree of 16S rRNA gene sequence similarity was found with *Paraliobacillus ryukyensis* (96.0 %), followed by that with *Amphibacillus tropicus* (95.6 %), *Halolactibacillus miurensis* (95.2 %), *Halolactibacillus halophilus* (95.1 %), *Amphibacillus fermentum* (94.9 %) and *Amphibacillus xylanus* (94.9 %). The phylogenetic tree shown in Fig. 1 indicates that strain Shu-P-Ggiii25-2^T is closely related to the genus *Amphibacillus*.

Genomic DNA was prepared according to the method of Marmur (1961) and the DNA G + C content was measured by HPLC as described by Mesbah *et al.* (1989). The cellular fatty acids were identified with the Sherlock Microbial Identification System (MIDI) and strain Shu-P-Ggiii25-2^T and the type species of the genus *Amphibacillus* were incubated on DSM 529 medium (DSM online catalogue; <http://www.dsmz.de/>) for 3 days at 30 °C. The analysis of cell-wall peptidoglycan was performed according to the methods of Schleifer & Kandler (1972). Respiratory quinone analyses were performed by using the method described by Collins & Jones (1981).

The DNA G + C content of strain Shu-P-Ggiii25-2^T was 42.3 mol%. The major fatty acid of strain Shu-P-Ggiii25-2^T was iso-C_{14:0} (18.2 %), iso-C_{15:0} (17.9 %), anteiso-C_{15:0} (17.8 %), C_{16:0} (14.1 %) and iso-C_{16:0} (9.2 %). This fatty acid profile is quite similar to those of the other recognized species of the genus *Amphibacillus* (Table 1). Strain Shu-P-Ggiii25-2^T possessed cell-wall peptidoglycans based on *meso*-diaminopimelic acid, which is the same type as the phylogenetically closely related neighbours *Amphibacillus xylanus* and *Amphibacillus tropicus* (Fig. 1). Isoprenoid

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain Shu-P-Ggiii25-2^T and other related taxa. Numbers at the nodes indicate the percentages of occurrence in 1000 bootstrapped trees; only values >50 % are shown. Bar, 1 substitution per 100 nucleotides.

Table 1. Fatty acid content of *Amphibacillus sediminis* sp. nov. and related organisms

Taxa: 1, *A. sediminis* sp. nov.; 2, *A. xylanus*; 3, *A. fermentum*; 4, *A. tropicus*; 5, *H. halophilus* (Ishikawa *et al.*, 2005); 6, *H. miurensis* (Ishikawa *et al.*, 2005). Fatty acids representing <1 % are not shown.

Fatty acid	1	2	3	4	5	6
C _{12:0}			2.4	4.3	2.5	2.1
C _{14:0}	16.3	15.0	25.9	9.6	4.1	4.0
C _{15:0}	1.2	1.5		5.8	1.4	1.6
C _{16:0}	14.1	15.0	22.9	31.4	43.1	37.2
C _{16:1}						1.3
C _{18:0}					4.6	5.5
C _{18:1Δ9}					2.7	2.2
C _{18:2}					1.1	1.2
iso-C _{13:0}	2.1	1.8	4.9		6.5	5.7
anteiso-C _{13:0}		2.0	9.6	3.5	19.1	18.8
iso-C _{14:0}	18.2	9.7	3.9	7.3		
iso-C _{15:0}	17.9	10.3	5.8	7.8	3.5	3.7
anteiso-C _{15:0}	17.8	28.2	18.7	22.1	6.2	7.6
iso-C _{16:0}	9.2	10.8	1.0	3.8		
iso-C _{17:0}					1.5	3.5
anteiso-C _{17:0}	1.5	4.1	1.0	1.1		2.5

quinones were not detected for the novel strain, as is also the case for the type species of the genera *Amphibacillus* (Niimura *et al.*, 1990; Zhilina *et al.*, 2001) and *Halolactibacillus* (Ishikawa *et al.*, 2005).

The chemotaxonomic characteristics of strain Shu-P-Ggiii25-2^T are consistent with those of the type species of the genus *Amphibacillus*, i.e. lacking quinones and cytochromes, and the novel strain exhibited similar cellular fatty acid contents (Tables 1 and 2). In addition, strain Shu-P-Ggiii25-2^T can be clearly distinguished from *Paraliobacillus ryukyuensis* by its lack of quinones, even though they share a high 16S rRNA gene sequence similarity. When compared with another neighbouring genus, *Halolactibacillus*, strain Shu-P-Ggiii25-2^T could be differentiated from the type species of this genus by its high content of the fatty acids C_{14:0}, iso-C_{14:0}, iso-C_{15:0} and

anteiso-C_{15:0} and relatively low content of fatty acids C_{16:0} and anteiso-C_{13:0} (Table 1). On the basis of phylogenetic analysis and physiological and chemotaxonomical characteristics, we conclude that strain Shu-P-Ggiii25-2^T represents a novel species of the genus *Amphibacillus*, for which the name *Amphibacillus sediminis* sp. nov. is proposed. An emended description of the genus *Amphibacillus* is given to reflect the moderately alkaliphilic and less biochemically reactive nature of *Amphibacillus sediminis* sp. nov.

Emended description of the genus *Amphibacillus* Niimura *et al.* 1990

Amphibacillus (Am.phi.ba.cil'lus. Gr. pref. *amphi* both sides or double; L. dim. n. *bacillus* a small rod; N.L. masc. n. *Amphibacillus* rod capable of both aerobic and anaerobic growth).

The description is based on that given by Niimura *et al.* (1990) with the following additions. Cells are Gram-positive, moderately alkaliphilic, facultatively anaerobic rods. Catalase activity is variable. The G + C content of the genomic DNA is 36–42 mol%. The type species of the genus is *Amphibacillus xylanus*.

Description of *Amphibacillus sediminis* sp. nov.

Amphibacillus sediminis (sed.i.mi'nis. L. gen. n. *sediminis* of a sediment).

Cells are Gram-positive long rods that are facultatively anaerobic and non-motile. Spherical endospores are formed in the terminal position. Colonies grown on trypticase soy agar containing 50 % Herbst's artificial seawater are circular, convex and white. The optimal temperature for growth is 27 °C; growth occurs at 17–55 °C, but not at 10 °C or 60 °C. Optimal pH for growth is 8.5; growth occurs at pH 7.0–9.0, but not at pH 6.5 or pH 9.5. NaCl is not required for growth but can be tolerated at concentrations of up to 6 % NaCl (w/v). Catalase and oxidase activities are negative. Acetoin is produced. H₂S and indole are not produced. Nitrate is not

Table 2. Differential characteristics of *Amphibacillus sediminis* sp. nov. and related organisms

1, *A. sediminis* sp. nov.; 2, *A. xylanus* (data from Niimura *et al.*, 1990); 3, *A. fermentum* (Zhilina *et al.*, 2001); 4, *A. tropicus* (Zhilina *et al.*, 2001); 5, *H. halophilus* (Ishikawa *et al.*, 2005); 6, *H. miurensis* (Ishikawa *et al.*, 2005); 7, *P. ryukyuensis* (Ishikawa *et al.*, 2002). +, Positive; –, negative.

Characteristic	1	2	3	4	5	6	7
Motility	–	–	+	+	+	+	+
Spore formation	+	+	–	+	–	–	+
Temperature range (°C)	17–55	25–45	18–56	18–56	5–40	5–45	10–47.5
pH range for growth	7.0–9.0	8.0–10.0	7.0–10.5	8.5–11.5	6.5–9.5	6.5–10.0	5.5–9.5
NaCl range (% w/v)	0–6	<6	1.0–19.7	1.0–20.9	0–24	0–25.5	0–22
Catalase activity	–	–	+	+	–	–	+
Major isoprenoid quinone	None	None	None	None	None	None	MK-7
DNA G + C content (mol%)	42.3	36.0	41.5	39.2	40.2	38.5	35.6

reduced. Urease, gelatinase, β -galactosidase and arginine dihydrolase activities are positive. Lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase activities and utilization of citrate are negative. Acid is produced from glycerol, glucose, fructose, mannose, aesculin, maltose and sucrose, but not from erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β -D-xyloside, galactose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, lactose, melibiose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate or 5-keto-gluconate. Cell walls contain peptidoglycans of the *meso*-diaminopimelic acid type. Isoprenoid quinones are not detected. The major cellular fatty acids are iso-C_{14:0}, iso-C_{15:0}, anteiso-C_{15:0}, C_{16:0} and iso-C_{16:0}. The genomic DNA G + C content of the type strain is 42.3 mol%.

The type strain, Shu-P-Ggiii25-2^T (=MBIC08269^T=IAM 15428^T=KCTC 13120^T), was isolated from sediment from Lake Hamana (Shizuoka, Japan).

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