Gracilibacillus thailandensis sp. nov., from fermented fish (*pla-ra*)

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A novel strain, designated TP2-8^T, was isolated from fermented fish (*pla-ra*) in Thailand. It stained Gram-positive and the cells were aerobic, endospore-forming rods. The strain grew at pH 6–8 (optimum pH 7), 15–55 °C (optimum 37 °C) and 1–22 % (w/v) NaCl (optimum 5–10 %). It contained *meso*-diaminopimelic in the cell-wall peptidoglycan. MK-7 and cellular fatty acids anteiso-C_{15:0}, iso-C_{15:0} and anteiso-C_{17:0} were major components. Polar lipids diphosphatidylglycerol and phosphatidylglycerol and unidentified lipids were detected. The DNA G + C content was 37.6 mol%. Comparison of the 16S rRNA gene sequence of strain TP2-8^T with those of other members of the family *Bacillaceae* indicated that it was a member of the genus *Gracilibacillus* (94.9–99.2 % sequence similarity) and was closely related to *Gracilibacillus saliphilus* YIM 91119^T (99.2 % similarity), *G. lacisalsi* BH312^T (98.6 %), *G. orientalis* XH-63^T (97.7 %), '*G. quinghaiensis*' YIM C229 (97.7 %) and *G. boracitolerans* T-16X^T (97.2 %). Strain TP2-8^T showed low DNA–DNA relatedness (≤49 %) to *G. saliphilus* YIM 91119^T, *G. lacisalsi* DSM 19029^T, *G. orientalis* CCM 7326^T, '*G. quinghaiensis*' DSM 17858 and *G. boracitolerans* JCM 21714^T. On the basis of the physiological and biochemical characteristics and molecular data presented, strain TP2-8^T is proposed to represent a novel species, *Gracilibacillus thailandensis* sp. nov. (type strain TP2-8^T = JCM 15569^T = PCU 304^T = TISTR 1881^T).

The genus *Gracilibacillus* was described by Wainø *et al.* (1999) to accommodate moderately halophilic, endospore-forming bacteria. At the time of writing, the genus comprises seven species with validly published names: *Gracilibacillus halotolerans*, the type species, from surface mud of the Great Salt Lake (Wainø *et al.*, 1999), *G. dipsosauri*, from a desert iguana (Lawson *et al.*, 1996), *G. orientalis* (Carrasco *et al.*, 2006), *G. lacisalsi* (Jeon *et al.*, 2008) and *G. saliphilus* (Tang *et al.*, 2009), from saline lakes, *G. boracitolerans*, from soil (Ahmed *et al.*, 2007) and *G. halophilus*, from a saline soil (Chen *et al.*, 2008b). '*Gracilibacillus quinghaiensis*' has been described

from a salt-lake sediment (Chen *et al.*, 2008a), but this name has not yet been validly published. These bacteria thrive in a maximum of 11–30 % NaCl and at 50 °C. Moderately halophilic bacteria are also found in many kinds of fermented food, including *Jeotgalibacillus alimentarius*, from jeotgal (Yoon *et al.*, 2001), *Lentibacillus juripiscarius* and *Lentibacillus halophilus*, from fish sauce (Namwong *et al.*, 2005; Tanasupawat *et al.*, 2006), *Lentibacillus kapialis*, from fermented shrimp paste (Pakdeeto *et al.*, 2007), and *Piscibacillus salipiscarius*, from fermented fish (*pla-ra*) (Tanasupawat *et al.*, 2007). In the course of a study on bacterial diversity in fermented foods in Thailand, we isolated a novel *Gracilibacillus*-like strain, designated TP2-8^T, from *pla-ra*, a traditional fermented fish, containing 7.8–17.9 % NaCl (w/v) (Phithakpol *et al.*, 1995). In this paper, we describe its phenotypic and chemotaxonomic characteristics, including DNA–DNA relatedness and 16S rRNA gene sequence analyses.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TP2-8^T is FJ182214.

16S rRNA gene sequence-based maximum-parsimony and maximum-likelihood trees, fatty acid profiles of strain TP2-8^T and related strains and detailed DNA–DNA hybridization results are available as supplementary material with the online version of this paper.

Strain TP2-8^T was isolated from *pla-ra* collected from a market in Nonthaburi province, Thailand. The strain was isolated from the sample by the spread-plate technique on JCM no. 377 agar plates (1⁻¹: 100 g NaCl, 5 g Casamino acids, 5 g yeast extract, 1 g glutamic acid, 2 g KCl, 3 g trisodium citrate, 20 g MgSO₄·7H₂O, 36 mg FeCl₂·4H₂O, 0.36 mg MnCl₂·4H₂O, 20 g agar; pH 7.2) and incubated at 30 °C for 3–7 days. Cell shape, size and arrangement were examined on JCM no. 377 agar at 30 °C after 3 days. The modification of Hucker & Conn (1923) was used for Gram staining. Cell form and spore formation were observed under scanning electron microscopy. Flagella were examined as described by Forbes (1981). Growth at various NaCl concentrations was investigated in JCM no. 377 broth, with MgSO₄·7H₂O omitted and with the addition of various concentrations of NaCl (0, 0.5 %, w/v, and 1–25 % at intervals of 1 %). Growth at pH 5–10 (at intervals of 1 pH unit) and 15, 25, 30, 37, 40, 45, 50 and 55 °C was investigated in JCM no. 377 broth (Namwong *et al.*, 2005). Catalase and oxidase activity, indole production, the methyl red/Voges–Proskauer reaction, H₂S production and nitrate reduction were determined as described by Barrow & Feltham (1993). JCM no. 377 medium with 0.25 % agar, 1 % glucose and bromocresol purple as indicator was used for the oxidative/fermentative (OF) test. Hydrolysis of aesculin, casein, gelatin, starch, Tween 80, urea, xanthine and hypoxanthine was determined as described by Namwong *et al.* (2005). Arginine decarboxylase was determined by using the medium reported by Thornley (1960). Acid production from carbohydrates was determined in the medium described by Leifson (1963). All tests were carried out in medium supplemented with 10 % NaCl (except for the investigation of effects of NaCl on growth). The ability to utilize various compounds was examined in a medium described by Carrasco *et al.* (2006) supplemented with 10 % NaCl. Carbohydrates were used at a final concentration of 0.5 % (w/v). When amino acids were used as substrates, the basal medium contained neither KNO₃ nor (NH₄)₂HPO₄. Growth under anaerobic conditions on JCM no. 377 agar plates with or without KNO₃ (1 %, w/v when present) was performed in a Gaspak anaerobic jar (BBL). Antibiotic susceptibility was determined according to the conventional Kirby–Bauer method (Bauer *et al.*, 1966). Plates were incubated at 30 °C for 48 h and inhibition zones were interpreted according to the manufacturer's manual (Oxoid).

Cell biomass was produced in JCM medium no. 377 at 30 °C for 3 days for chemotaxonomic characterization. Diaminopimelic acid in the cell wall and the menaquinone profile were determined as described by Komagata & Suzuki (1987). Polar lipids were extracted as described by Minnikin *et al.* (1984) and identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Komagata & Suzuki, 1987). For quantitative analysis of cellular fatty acid compositions, cell mass was harvested and fatty acid methyl esters were prepared and identified according to the instructions of the Microbial

Identification System (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Chromosomal DNA was isolated and purified from cells grown in JCM medium no. 377 according to the method of Saito & Miura (1963). The DNA G+C content was determined by reversed-phase HPLC (Tamaoka & Komagata, 1984). DNA–DNA hybridization was conducted in microwell plates as reported by Ezaki *et al.* (1989). The 16S rRNA gene of the isolate was amplified, purified and sequenced as described by Namwong *et al.* (2005). The sequence determined was aligned with selected sequences obtained from the GenBank/EMBL/DDBJ databases employing CLUSTAL_X version 1.83 (Thompson *et al.*, 1997). The alignment was edited manually, and positions with gaps and ambiguous bases were eliminated prior to construction of phylogenetic trees. Phylogenetic trees based on the neighbour-joining method (Saitou & Nei, 1987) as well as the maximum-parsimony and maximum-likelihood methods were constructed by using the NJplot program (Perrière & Gouy, 1996) in the MEGA 4 software (Tamura *et al.*, 2007). Confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

Cells of strain TP2-8^T were Gram-stain-positive rods, approximately 0.3–0.4 µm wide and 3–4 µm long after 3 days of incubation at 37 °C on JCM no. 377 agar (Fig. 1). Oval endospores were produced at a terminal position in swollen sporangia. Cells were motile by means of peritrichous flagella. Colonies were circular, translucent and white to cream in colour (diameter 0.2–0.5 mm). The strain utilized glucose oxidatively. Phenotypic characteristics are listed in the species description and in Table 1. This strain contained MK-7 as the major menaquinone. Major cellular fatty acids were anteiso-C_{15:0} (37.6 %), iso-C_{15:0} (16.1 %) and anteiso-C_{17:0} (12.9 %). The strain showed a similar fatty acid profile to that of its closest relative, *G. saliphilus* YIM 91119^T (Supplementary Table S1, available in IJSEM Online). Polar lipid fractions contained diphosphatidylglycerol, phosphatidylglycerol, an unidentified glycolipid and an unidentified polar lipid. The DNA

Fig. 1. Scanning electron micrograph of cells of strain TP2-8^T grown on JCM no. 377 medium at 37 °C. Bar, 1 µm.

Table 1. Differential characteristics of TP2-8^T and type strains of *Gracilibacillus* species

Strains: 1, TP2-8^T (data from this study); 2, *G. saliphilus* YIM 91119^T (Tang *et al.*, 2009); 3, *G. laciisalsi* DSM 19029^T (Jeon *et al.*, 2008); 4, *G. orientalis* CCM 7326^T (Carrasco *et al.*, 2006); 5, '*G. quinghaiensis*' DSM 17858 (Chen *et al.*, 2008a); 6, *G. boracitolerans* JCM 21714^T (Ahmed *et al.*, 2007); 7, *G. halotolerans* JCM 7302^T (Wainø *et al.*, 1999); 8, *G. dipsosauri* JCM 7303^T (Wainø *et al.*, 1999); 9, *G. halophilus* DSM 17856^T (Chen *et al.*, 2008b). Data were obtained from the sources listed unless indicated. +, Positive; w, weakly positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9
Spore shape*	O	S	S	S	E	S	E	S	E
NaCl concentration for growth (% w/v)									
Range	1–22	1–22†	0.5–18†	3–20†	0.5–8	0–11	0–20	0–15	7–30
Optimum	5–10	10–15†	5–7†	10†	1–3	0.5–3	0	3	15
Temperature for growth (°C)									
Range	15–55	4–45	15–50	15–45†	4–45	11–37	6–50	28–50	28–60
Optimum	37	37	40	37†	37	25–28	47	45	45–50
pH for growth									
Range	6–8	6–8	5.5–10	5–9†	6–8.5	6–10	5–10	ND	6–9
Optimum	7	7	7.5–8	7†	7–7.5	7.5–8.5	7.5	7.5	7
Nitrate reduction†	+	+	+	–	+	–	+	+	+
Hydrolysis of:†									
Gelatin	+	–	–	+	–	–	+	+	+
Starch	–	+	–	–	–	+	+	–	+
Urea	–	+	–	–	+	–	+	–	ND
Acid from:†									
Glycerol	+	–	+	–	W	+	ND	–	W
Lactose	–	+	–	–	–	+	ND	+	–
Melezitose	+	–	–	–	–	+	ND	–	–
Carbon source utilization:†									
L-Aspartic acid	–	+	+	–	ND	ND	ND	ND	ND
Glycine	–	+	–	–	ND	ND	ND	ND	ND
L-Lysine	–	+	–	–	ND	ND	ND	ND	ND
Raffinose	+	–	+	+	ND	ND	ND	ND	ND
Sodium acetate	–	+	–	+	ND	ND	ND	ND	ND
DNA G + C content (mol%)	37.6	40.1	39	37.1	40.9	35.8	38	39.4	42.3

*E, Ellipsoidal; O, oval; S, spherical.

†Data from this study.

G + C content was 37.6 mol%. Comparison of the 16S rRNA gene sequence of strain TP2-8^T (1500 nt) with those of other members of the family *Bacillaceae* indicated that it was placed in the genus *Gracilibacillus* and was closely related to *G. saliphilus* YIM 91119^T (99.2 % similarity), *G. laciisalsi* BH312^T (98.6 %), *G. orientalis* XH-63^T (97.7 %), '*G. quinghaiensis*' YIM C229 (97.7 %), *G. boracitolerans* T-16X^T (97.2 %), *G. dipsosauri* DD1^T (96.4 %), *G. halotolerans* NN^T (95.8 %) and *G. halophilus* YIM-C55.5^T (94.9 %) (Fig. 2). Phylogenetic trees based on other algorithms are shown as Supplementary Figs S1 and S2. In agreement with the phylogenetic assignment, the phenotypic properties of strain TP2-8^T, such as its cell morphology, the NaCl concentration range for growth, the presence of diaminopimelic acid in the cell wall, major menaquinone composition, fatty acid profile and DNA G + C content, are consistent with the assignment of strain TP2-8^T to the genus *Gracilibacillus*. DNA–DNA hybridization studies showed that strain TP2-8^T had low levels of DNA–DNA

relatedness (≤ 49 %) to *G. saliphilus* YIM 91119^T, *G. laciisalsi* DSM 19029^T, *G. orientalis* CCM 7326^T, '*G. quinghaiensis*' DSM 17858, *G. boracitolerans* JCM 21714^T, *G. halotolerans* JCM 7303^T, *G. dipsosauri* JCM 7302^T and *G. halophilus* DSM 17856^T (Supplementary Table S2), well below the level of 70 % recommended for species delineation by Wayne *et al.* (1987). In addition, strain TP2-8^T and its closest relative, *G. saliphilus* YIM 91119^T, showed differences in sensitivity to ampicillin (10 µg), gentamicin (10 µg), novobiocin (30 µg) and chloramphenicol (30 µg) (this study; Tang *et al.*, 2009). Strain TP2-8^T was distinguished from *Gracilibacillus* species by spore shape, ranges of NaCl concentration, temperature and pH for growth, biochemical characteristics, acid production and carbon source utilization and G + C content, as shown in Table 1. On the basis of its phenotypic and genotypic properties, strain TP2-8^T represents a novel species of the genus *Gracilibacillus*, for which we propose the name *Gracilibacillus thailandensis* sp. nov.

Fig. 2. Phylogenetic tree showing relationships between strain TP2-8^T, *Gracilibacillus* species and related taxa based on 16S rRNA gene sequences. The branching pattern was generated by the neighbour-joining method. Bootstrap percentages $\geq 53\%$ based on 1000 replications are shown at nodes. Bar, 0.01 substitutions per nucleotide position. Maximum-likelihood and maximum-parsimony trees are available as Supplementary Figs S1 and S2.

Description of *Gracilibacillus thailandensis* sp. nov.

Gracilibacillus thailandensis (thai.lan.den'sis. N.L. masc. adj. *thailandensis* pertaining to Thailand, where the type strain was isolated).

Cells are Gram-stain-positive rods, approximately 0.3–0.4 μm wide and 3–4 μm long. Oval endospores are produced in a terminal position in swollen sporangia. Motile by means of peritrichous flagella. Colonies are circular, translucent, white and 0.2–0.5 mm in diameter. Strictly aerobic. Utilizes glucose oxidatively. Grows at pH 6–8 (optimum pH 7), 15–55 $^{\circ}\text{C}$ (optimum 37 $^{\circ}\text{C}$) and 1–22% (w/v) NaCl (optimum 5–10%), but not in 0.5% NaCl or below. No growth under anaerobic conditions even if KNO_3 is added. Catalase, oxidase, methyl red test, nitrate reduction and hydrolysis of aesculin and gelatin are positive. Negative for indole production, Voges–Proskauer reaction, hydrolysis of casein, starch, Tween 80, urea, L-tyrosine, xanthine and hypoxanthine, arginine dihydrolase activity and production of H_2S . Susceptible to ampicillin (10 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (30 μg), novobiocin (30 μg) and rifampicin (30 μg), but resistant to bacitracin (10 U), chloramphenicol (30 μg) and nalidixic acid (30 μg). Acid is produced from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, glycerol, maltose, D-mannitol, D-mannose, melibiose, melezitose, raffinose, rhamnose, ribose, salicin, sucrose, trehalose and D-xylose but not from inulin, myo-inositol, lactose, sorbitol, ribitol or arabinol. Utilizes L-arginine, cellobiose, galactose, lactose, D-mannose, melezitose, raffinose, sodium citrate and trehalose but not glycine, L-aspartic acid, L-cysteine, L-glutamic, hippuric acid, L-lysine, L-ornithine, L-phenylalanine, sodium acetate, sodium pyruvate, sodium benzoate, L-threonine or D-xylose as sole sources of carbon and nitrogen (the latter where appropriate). Contains diaminopimelic acid in the cell wall and MK-7 as the major menaquinone. Major cellular fatty acids are anteiso- $\text{C}_{15:0}$, iso- $\text{C}_{15:0}$ and anteiso- $\text{C}_{17:0}$. The major

polar lipids are diphosphatidylglycerol, phosphatidylglycerol, an unidentified glycolipid and an unidentified polar lipid. The DNA G+C content of the type strain is 37.6 mol%.

The type strain, TP2-8^T (=JCM 15569^T =PCU 304^T =TISTR 1881^T), was isolated from *pla-ra*, a traditional fermented fish, in Thailand.

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