Parvularcula lutaonensis sp. nov., a moderately thermotolerant marine bacterium isolated from a coastal hot spring

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Strain CC-MMS-1^T, a motile, Gram-negative, orange-pigmented bacterium, was isolated from water samples collected from a rare coastal hot spring at Luta, an island off the eastern coast of Taiwan. 16S rRNA gene sequence studies showed that the novel strain was closely related to *Parvularcula bermudensis* HTCC2503^T (95.1 % sequence similarity). The fatty acid profile was slightly different from *P. bermudensis* HTCC2503^T. The results of physiological and biochemical tests allowed clear phenotypic differentiation of the novel isolate from *P. bermudensis* HTCC2503^T. It is evident from the genotypic and phenotypic data that the strain should be classified as a novel species in the genus *Parvularcula*. The name proposed for this taxon is *Parvularcula lutaonensis* sp. nov. The type strain is CC-MMS-1^T (=BCRC 17814^T=KCTC 22245^T).

Strain CC-MMS-1^T was isolated during the characterization of micro-organisms from water samples collected from a rare coastal hot spring (water temperature 55 °C; pH 7.3; salinity 32 ‰) located in a small volcanic island off the east coast of Taiwan. The isolate was isolated and maintained on marine agar 2216 (MA; Difco) after incubation at 37 °C for 48 h. Subcultivation was performed on MA at 37 °C for 2–3 days. CC-MMS-1^T was able to grow at 25–50 °C but not at 55 or 20 °C on MA.

Gram-staining was performed as described by Gerhardt *et al.* (1994). Poly- β -hydroxybutyrate granule accumulation in cells was observed using light microscopy after staining with Sudan black. Cell morphology was observed under a Zeiss light microscope at $\times 1000$, using cells that had been grown for 3 days at 37 °C on MA. Details of the cell morphology are given in the species description. The pH range for growth was determined by measuring the optical densities (OD₅₉₅) of cultures grown in marine broth 2216 (Difco; MB) which was adjusted prior to sterilization to various pH values (pH 3–11, in increments of 0.5 pH units) using appropriate biological buffers (Chung *et al.*, 1995). Growth at various temperatures (10–55 °C) was

measured in MB. Anaerobic growth was checked by using the Oxoid AnaeroGen system (Miller *et al.*, 1995) in MB, recording growth by measuring the optical density (OD₅₉₅) of the culture with respect to time.

Phenotypic characteristics were investigated using Biolog-GN MicroPlates (Biolog), API ZYM, API 20E and API 20NE tests (bioMérieux). The results of the physiological characterization are given in the species description and in Table 1. Strain CC-MMS-1^T was capable of producing acid from various carbohydrates. However, carbon substrate oxidation tests with organic acids as substrates showed few positive results. In API 20E tests, strain CC-MMS-1^T was positive for β -glucosidase, citrate utilization, acetoin production and gelatinase, while in API 20NE tests, strain CC-MMS-1^T was positive for aesculin hydrolysis, gelatinase, β -galactosidase, cytochrome oxidase and assimilation of glucose, mannose and maltose. In API ZYM enzyme profiling, strain CC-MMS-1^T was positive for alkaline phosphatase, butyrate esterase (C4), caprylate esterase (C8), leucine arylamidase, valine arylamidase, trypsin, α -chymotrypsin, naphthol-AS-B-1-phosphohydrolase, β -glucuronidase and α -glucosidase.

To determine the DNA G + C content, DNA was prepared and degraded enzymatically into nucleosides as described by Mesbah *et al.* (1989), and the nucleoside mixture was separated with HPLC. The G + C content of strain

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of CC-MMS-1^T is EU346850.

A supplementary figure showing the polar lipid profile of strain CC-MMS-1^T is available with the online version of this paper.

Table 1. Genotypic, phenotypic and nutritional characteristics that distinguish strain CC-MMS-1^T from *P. bermudensis* HTCC2503^T

Data from this study and Cho & Giovannoni (2003). +, Positive; −, negative.

Characteristic	CC-MMS-1 ^T	<i>P. bermudensis</i> HTCC2503 ^T
Colony characteristics		
Pigmentation	Orange	Yellowish brown
Morphology	Smooth/shiny	Dry/wrinkled
Rate of growth	Fast	Slow
Temperature range (°C)	25–50	10–37
Nitrate reduction	−	+
Poly-β-hydroxybutyrate granules	+	−
Hydrolysis of:		
Aesculin	+	−
Urease	−	+
Oxidation of (Biolog GN2):		
Adonitol	−	+
L-Alaninamide	−	+
L-Alanyl glycine	−	+
L-Arabinose	−	+
D-Arabitol	−	+
L-Asparagine	−	+
L-Aspartic acid	−	+
α-Cyclodextrin	+	−
D-Fructose	+	−
L-Fucose	−	+
D-Galacturonic acid	−	+
L-Glutamic acid	−	+
D-Gluconic acid	+	−
D-Glucosaminic acid	+	−
Glycerol	+	−
DL-α-Glycerol phosphate	−	+
Glycyl-L-aspartic acid	−	+
Glycyl-L-glutamic acid	−	+
β-Hydroxybutyric acid	+	−
myo-Inositol	−	+
Inosine	−	+
α-Ketobutyric acid	−	+
DL-Lactic acid	+	−
Lactulose	+	−
L-Leucine	−	+
Propionic acid	−	+
D-Psicose	+	−
L-Rhamnose	−	+
L-Serine	−	+
Sucrose	−	+
Trehalose	+	−
Urocanic acid	−	+
Xylitol	−	+
DNA G + C content (mol%)	59.0	60.8

CC-MMS-1^T was 59 ± 1%. A continuous stretch of 1408 bp of the 16S rRNA gene of strain CC-MMS-1^T was sequenced as described previously (Young *et al.*, 2005).

Analysis of the sequence data was performed by using the software package MEGA version 2.1 (Kumar *et al.*, 2001) after multiple alignments of data by CLUSTAL_X (Thompson *et al.*, 1997) with 16S rRNA gene sequences from type species of genera within the class *Alphaproteobacteria* and the type strain of *Rickettsia prowazekii* from the class *Gammaproteobacteria*. A distance-matrix method (distance options according to the Kimura two-parameter model), including clustering by neighbour-joining (Fig. 1) and a discrete character-based maximum-parsimony method (not shown), were used to generate phylogenetic trees. In each case, bootstrap values were calculated based on 1000 replications. Sequence similarity calculations indicated that the 16S rRNA gene sequence for strain CC-MMS-1^T showed the greatest degree of similarity to that of *Parvularcula bermudensis* HTCC2503^T (95.1 %) of the deeply branched order 'Parvularculales'. The sequence similarities between strain CC-MMS-1^T and the other type species were lower (<90 %). DNA–DNA relatedness experiments were not performed between strain CC-MMS-1^T and its closest phylogenetic neighbour *P. bermudensis* HTCC2503^T because the level of 16S rRNA gene sequence similarity between the strains was less than 96 %, which is below the cut-off value (97 %) suggested by Stackebrandt & Goebel (1994) for genomic distinction of species.

Fatty acid methyl esters were prepared, separated and identified according to the instructions of the Microbial Identification System (MIDI). The fatty acid profile of strain CC-MMS-1^T was similar to that of *P. bermudensis* HTCC2503^T (Cho & Giovannoni, 2003), but showed some differences (Table 2). Isoprenoid quinones of strain CC-MMS-1^T were extracted, separated and identified as described by Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985). Ubiquinone 10 (Q-10) was the predominant respiratory quinone. Polar lipids were extracted and analysed by two-dimensional TLC according to Embley & Wait (1994). The predominant polar lipids of strain CC-MMS-1^T were four unknown phospholipids and four unknown glycolipids (see Supplementary Fig. S1, available in IJSEM Online).

Strain CC-MMS-1^T is clearly different from *P. bermudensis* HTCC2503^T, in both phenotype and genotype, and represents a second member of the genus *Parvularcula* and order 'Parvularculales', for which the name *Parvularcula lutaonensis* sp. nov. is proposed.

Description of *Parvularcula lutaonensis* sp. nov.

Parvularcula lutaonensis (lu.ta.o.nen'sis. N.L. fem. adj. *lutaonensis* pertaining to Luta, a small volcanic island off the eastern coast of Taiwan).

Cells are Gram-negative, aerobic, short rods, 1–3 µm in length and 0.3–0.8 µm in diameter. Cells are motile by a single polar flagellum and are non-spore-forming. Good growth occurs after 48 h incubation on MA at 37 °C. Colonies on MA at 38 °C are orange, smooth, shiny and

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences constructed after multiple alignments of data by CLUSTAL_X (Thompson *et al.*, 1997). Distance calculations and clustering with the neighbour-joining method were performed by using MEGA version 2.1. Bootstrap values based on 1000 replications (>50 %) are shown at the branching points. Bar, 0.02 substitutions per nucleotide.

convex with spreading edges, 1.0–2.0 mm in diameter and non-fluorescent. The optimal temperature for growth is 37 °C. Growth occurs at pH 7 and 9, but not at pH 6 or 10; optimal growth occurs at pH 7. Growth occurs in the presence of 0.5–6 % (w/v) NaCl; optimal growth occurs in the presence of 3 % (w/v) NaCl. No anaerobic growth is observed on MA supplemented with nitrate. Accumulates poly- β -hydroxybutyrate granules as inclusion bodies. Positive in tests for oxidase and negative for catalase. Does not reduce nitrate to nitrite. The fatty acid profile of strain CC-MMS-1^T is presented in Table 2. The following carbon sources are oxidized: α -cyclodextrin, dextrin, glycogen, cellobiose, D-fructose, gentiobiose, α -D-glucose, lactulose, maltose, D-mannitol, D-mannose, β -methyl-D-glucoside, D-psicose, D-sorbitol, trehalose, turanose, D-gluconic acid, D-glucosaminic acid, β -hydroxybutyric acid, α -ketoglutaric acid, DL-lactic acid and glycerol. The following substrates are not oxidized as carbon sources: Tweens 40 and 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, i-erythritol, L-fucose, D-galactose, myo-inositol, α -D-lactose, melibiose, raffinose, L-rhamnose, sucrose, xylitol, pyruvic acid methyl

Table 2. Major fatty acids of strain CC-MMS-1^T and *P. bermudensis* HTCC2503^T

Data from this study and Cho & Giovannoni (2003). Values are percentages of total fatty acid content. —, Not reported.

Fatty acid	CC-MMS-1 ^T	<i>P. bermudensis</i> HTCC2503 ^T
C _{12:0}	1.2	5.2
C _{12:0} 3-OH	2.1	—
C _{14:0}	—	2.9
C _{16:0}	15.7	8.6
iso-C _{17:0}	1.9	—
C _{18:1} ω 9c	—	6.0
C _{18:1} ω 7c	73.4	73.3
C _{18:0}	5.7	4.0

ester, succinic acid monomethylester, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucuronic acid, α -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketobutyric acid, α -ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxyl-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, DL-carnitine, γ -aminobutyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, DL- α -glycerol phosphate, α -D-glucose 1-phosphate and D-glucose 6-phosphate. Positive reactions (API 20E and 20NE) are seen for β -glucosidase, citrate utilization, acetoin production, gelatinase, aesculin, β -galactosidase and assimilation of glucose, mannose, maltose and cytochrome oxidase. Negative reactions are seen for reduction of nitrates to nitrogen, indole production, glucose fermentation, arginine dihydrolase, urease, assimilation of arabinose, mannitol, N-acetylglucosamine, potassium gluconate, capric acid, adipic acid, malate, trisodium citrate, phenylacetic acid, lysine decarboxylase, ornithine decarboxylase, H₂S production, tryptophan deaminase, oxidation of glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose. In API ZYM enzyme reactions, strain CC-MMS-1^T is positive for alkaline phosphatase, butyrate esterase (C4), caprylate esterase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, naphthol-AS-B1-phosphohydrolase, α -glucosidase and β -galactosidase, but negative reactions are seen for myristate lipase (C14), acid phosphatase, α -galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Genotypic, phenotypic and nutritional features that distinguish the type strain from *P. bermudensis* HTCC2503^T are given in Table 1.

The type strain, CC-MMS-1^T (=BCRC 17814^T=KCTC 22245^T), was isolated from a rare coastal hot spring on a Pacific Ocean island.

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