Parvularcula flava sp. nov., an alphaproteobacterium isolated from surface seawater of the South China Sea

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An aerobic, coccoid to short rod, yellow-pigmented, non-sporulating and Gram-staining-negative bacterium, designated NH6-79^T, was isolated from surface seawater of the South China Sea. The isolate was motile with a polar flagellum. Growth was observed at 4–42 °C (optimum 37 °C), at pH 6.0–8.5 (optimum pH 7.0), and with 0.5–11 % (w/v) NaCl (optimum 4.5 %) and 1.5–17 % (w/v) sea salt (optimum 3.5–5 %). Strain NH6-79^T could decompose peptone to produce H_2S, but could not hydrolyse skimmed milk. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain NH6-79^T had the closest affinity to the genus *Parvularcula*, sharing the highest 16S rRNA gene sequence similarity with '*Parvularcula oceanus*' JLT2013 (94.1 %), *Parvularcula lutaonensis* CC-MMS-1^T (93.4 %), *Parvularcula dongshanensis* SH25^T (92.9 %) and *Parvularcula bermudensis* HTCC2503^T (92.7 %), and lower sequence similarities (<90 %) with all other genera. The dominant fatty acids were $C_{18:1}\omega7c$ and $C_{16:0}$. The polar lipid profile was mainly composed of three unidentified glycolipids. The predominant isoprenoid quinone was ubiquinone-10. The DNA G+C content was 60.7 mol%. Based on the polyphasic taxonomic characterization, strain NH6-79^T is considered to represent a novel species of the genus *Parvularcula*, for which the name *Parvularcula flava* sp. nov. is proposed. The type strain is NH6-79^T (=CGMCC 1.14984^T=JCM 30557^T=MCCC 1K00277^T).

During an investigation on bacterial diversity in the offshore surface seawater of China, a novel isolate designated NH6-79^T was isolated from the South China Sea and was considered to represent a novel species of the genus *Parvularcula* based on the preliminary phylogenetic analysis of 16S rRNA gene sequence. The genus *Parvularcula* was proposed by Cho & Giovannoni (2003) to form a new seventh order of the *Alphaproteobacteria*. At the time of writing, the genus *Parvularcula* comprises three species with validly published names, *P. bermudensis* (Cho & Giovannoni, 2003), *P. lutaonensis* (Arun *et al.*, 2009) and *P. dongshanensis* (Yu *et al.*, 2013), and one species with a name that is not yet validly published, '*P. oceanus*' (Li *et al.*, 2014). According to the descriptions of all these species, strains belonging to the genus *Parvularcula* are Gram-staining-negative, coccoid to short rod-shaped, motile with one polar flagellum, and additionally are chemoheterotrophic and have $C_{18:1}\omega7c$ as the major fatty acid. In this study, the polyphasic taxonomic identification of the novel *Parvularcula* strain, NH6-79^T, is described in detail.

Surface seawater was sampled from the NH6 sampling station (18° 06′ 56″ N 110° 45′ 38″ E) during a scientific cruise in the South China Sea in June 2009, transported without temperature control, and stored at 4 °C in the laboratory until used. Strain NH6-79^T was isolated through the same method described previously (Zhang *et al.*, 2015) and was preserved by freeze-drying. Unless otherwise mentioned, strain NH6-79^T and the *Parvularcula* reference strains were cultured in the modified marine 2216 medium (Liu *et al.*, 2013).

Amplification and sequencing of 16S rRNA gene, as well as sequence alignment and phylogenetic analysis based on 16S rRNA gene sequences were all performed as previously

Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PHB, poly-β-hydroxybutyrate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NH6-79^T is KM199855.

Two supplementary tables and three supplementary figures are available with the online Supplementary Material.

described (Zhang *et al.*, 2015). The DNA G+C content was determined by reversed-phase HPLC (Mesbah *et al.*, 1989) using the genomic DNA of *Escherichia coli* K-12 and salmon sperm DNA (Sigma) as calibration standards. Pairwise alignment based on the nearly full-length 16S rRNA gene sequence (1426 nt) of strain NH6-79^T indicated that this isolate shared the highest sequence similarity with '*Parvularcula oceanus*' JLT2013 (94.08 %) and showed 93.4–92.7 % sequence similarities to the type strains of other species of the genus *Parvularcula*. Similarity values between the 16S rRNA gene sequences of strain NH6-79^T and the type strains of other genera were lower than 90 %. Topologies exhibited in both neighbour-joining tree (Fig. 1) and maximum-likelihood tree (Fig. S1, available in the online Supplementary Material) illustrated that strain NH6-79^T clustered with the members of genus *Parvularcula* under the support of a high bootstrap value, which suggested that it should belong to this genus. The DNA G+C content of strain NH6-79^T was 60.7 mol%, which is similar to those of the type strains of recognized species of the genus *Parvularcula* (Table 1).

The temperature for optimal growth was tested at 25–45 °C in increments of 2 °C. The pH range for growth was determined at 0.5 pH intervals by supplementing with 30 mM buffering agents, including MES (pH 5.5–6.5), MOPS (pH 6.5–8.0), Tricine (pH 8.0–9.0) and Bis-Tris propane (pH 9.0–9.5). NaCl reliance was measured in the medium contained (l^{-1}): 1.0 g $MgCl_2{\cdot}6H_2O$, 5.0 g $MgSO_4{\cdot}7H_2O$, 0.7 g KCl, 0.15 g $CaCl_2{\cdot}2H_2O$, 0.5 g NH_4Cl, 0.1 g KBr, 0.27 g KH_2PO_4, 0.04 g $SrCl_2{\cdot}6H_2O$, 0.025 g H_3BO_3, 5.0 g trypticase peptone (BD) and 1.0 g yeast extract (BD) (pH 7.0) with 0–150 g NaCl increasing at a step of 5 g. The salinity range for growth was detected in the medium contained 0.5 % (w/v) trypticase peptone (BD) and 0.1 % (w/v) yeast extract (BD) (pH 7.0) with 0–20 % (w/v) sea salt (Sigma) increasing at a step of 0.5 % (w/v). Cultures incubated for 2 days were used to determine the optimal growth while those incubated for 14 days were used to determine the growth limits. As a result, growth of strain NH6-79^T was observed at 4–42 °C (optimum 37 °C), at pH 6.0–8.5 (optimum pH 7.0), with 0.5–11 % (w/v) NaCl (optimum 4.5 %) and 1.5–17 % (w/v) sea salt (optimum 3.5–5 %). No anaerobic growth was detected in the modified marine 2216 medium supplemented with 10 mg l^{-1} ferric citrate, NH_4NO_3, $NaNO_2$ and Na_2SO_3, 0.2 g l^{-1} sodium thioglycolate and cysteine, 3 g l^{-1} $Na_2S_2O_3$ and 1 g l^{-1} glucose by the Hungate tube method.

Using 4.5 % (w/v) NaCl, the medium described above in the NaCl reliance analysis was made into slopes. After incubation at 37 °C for 2 days, cells were harvested for morphology observation by transmission electron microscopy (JEM-1230; Jeol) after uranyl acetate staining and by optical microscopy (BX40; Olympus) after Gram staining. Intracellular poly-β-hydroxybutyrate (PHB) granules were checked by ultrathin sections and by Nile blue A staining (Ostle & Holt, 1982) using cells harvested from the modified marine 2216 agar plates. As shown in Fig. S2, strain NH6-79^T was coccoid to short rod-shaped with one polar flagellum and accumulated PHB as described in other members of the genus *Parvularcula* (Arun *et al.*, 2009; Li *et al.*, 2014).

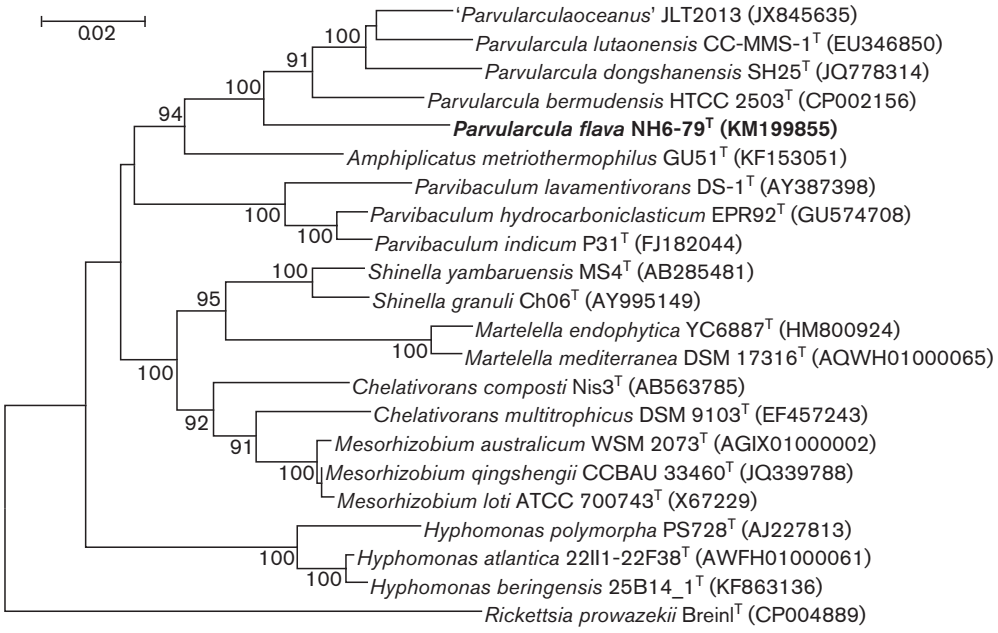

Fig. 1. Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of strain NH6-79^T and representatives of related taxa. Bootstrap values were expressed as a percentage of 1000 replicates and only those >80 % are given at the branch points. Bar, 0.02 substitutions per nucleotide position.

Table 1. Comparisons between strain NH6-79^T and strains of species of the genus *Parvularcula*

Strains: 1, NH6-79^T; 2, *P. bermudensis* KCTC 12087^T; 3, *P. dongshanensis* MCCC 1A06534^T; 4, *P. lutaonensis* KCTC 22245^T; 5, '*P. oceanus*' CGMCC 1.12400. All data are from this study except where otherwise indicated. +, Positive; w, weakly positive; −, negative or not detected. Summed feature 7 (ECL 18.845) is composed of unknown 18.846 and/or $C_{19:1}\omega6c$. DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; GL, unknown glycolipid; PGL, unknown phosphoglycolipid; L, unknown lipid. All strains were tested to be strictly aerobic in the present study. All strains have one polar flagellum.

Characteristic	1	2	3	4	5
Cell morphology	0.5–0.9×0.6–1.8 μm, coccoid to short rod	0.4–1.3×0.6–1.8 μm, coccoid to short rod*[a]	0.8–0.9×1.4–1.5 μm, short rod[b]	0.3–0.8×1–3 μm, short rod[c]	0.8–1.5×1.5–2.3 μm, short rod[d]
Color of colony	Yellow	Yellowish-brown[a]	Red[b]	Orange[c]	Orange[d]
Temperature range for growth (optimum) (°C)	4–42 (37)	10–37 (30)[a]	10–41 (28)[b]	25–50 (37)[c]	10–40 (30)[d]
NaCl range for growth (optimum) (%, w/v)	0.5–11 (4.5)	0.75–25 (3)[a]	0–12 (1–3)[b]	0.5–6 (3)[c]	0–9 (3–6)[d]
Hydrolysis of:					
DNA	−	−	+	w	+
Skimmed milk	−	+	+	+	+
β-Glucosidase activity (API ZYM)	−	−	+	w	+
Antibiotic sensitivity (μg per disc)					
Ciprofloxacin (5)	−	+	+	+	+
Kanamycin (30)	−	+	w	w	+
Neomycin (30)	−	+	+	+	+
Vibramycin (30)	−	+	w	w	+
Fatty acid profile					
Major components (>10 %)	$C_{18:1}\omega7c$, $C_{16:0}$	$C_{18:1}\omega7c$	$C_{18:1}\omega7c$, $C_{16:0}$	$C_{18:1}\omega7c$, $C_{16:0}$	$C_{18:1}\omega7c$, $C_{16:0}$
Different components (%)					
$C_{12:0}$	−	3.3	2.2	0.6	1.9
11-methyl $C_{18:1}\omega7c$	5.2	−	−	−	−
Summed feature 7	1.3	−	−	−	−
Polar lipid profile	7GL, PGL, 2L, PG	8GL, PGL, 3L	DPG, PG, 7GL, PGL, 4L	8GL, L	DPG, PG, 8GL, PGL, 3L
DNA G+C content (mol %, by HPLC)	60.7	60.8[a]	61.8[b]	59.0[c]	66.3[d]
Habitat	Surface seawater of South China Sea	Seawater (10 m depth) from the western Sargasso Sea[a]	Soft coral from Dongshan Island, China[b]	Coastal hot spring on a Pacific Ocean island[c]	Deep-sea water in southeastern Pacific[d]

*Data from: *a*, Cho & Giovannoni (2003); *b*, Yu *et al.* (2013); *c*, Arun *et al.* (2009); *d*, Li *et al.* (2014).

Unless otherwise stated, strains investigated in all the following tests were cultured at their optimal temperatures. Pigment was identified by the method described by Cho *et al.* (2003) except that cells cultivated on the modified marine 2216 agar for 3 days were used. Hydrolysis of aesculin, alginate, CM-cellulose, DNA, gelatin, skimmed milk, starch and Tweens 20, 40, 60 and 80, and activity of catalase were determined as previously described (Zhang *et al.*, 2015). Degradation of tyrosine was measured as for the substrates mentioned above except that 5 g l^{-1} tyrosine was added. H_2S production was checked on both 3 g l^{-1} $Na_2S_2O_3$ and 0.1 g l^{-1} cysteine. The MR-VP test was performed according to Lányi (1988).

Other physiological and biochemical characteristics were detected by API ZYM and API 20NE test systems (bioMérieux) and the GN2 MicroPlate (Biolog) according to the manufacturers' instructions. As a modification, cells suspended in 3 % (w/v) NaCl solution were used for inoculation, and 1 ml saline solution was supplemented into the AUX medium supplied by the manufacturer. Formula of the saline solution was in accordant with that of the inorganic salt of the medium described above in the NaCl reliance analysis, in which 24 % (w/v) NaCl was added. Sensitivity to antibiotics was checked by a two-layer plate method as previously performed (Zhang *et al.*, 2010) except that the modified marine 2216 agar was used. Detailed results of all physiological and biochemical characteristics are summarized in Table S1 and the species description.

After incubation in the modified marine 2216 at 37 °C and 140 r.p.m. for 2 days, cells were collected for extraction of isoprenoid quinones which were subsequently purified by TLC and identified using a HPLC-MS system (Agilent) (Komagata & Suzuki, 1988). Cells used for the analysis of fatty acids and polar lipids were harvested from the third quadrants of the modified marine 2216 agar plates. Fatty acids were saponified, methylated and extracted according to the standard protocol of the Sherlock Microbial Identification System (version 4.5, MIDI) and identified based on the TSBA40 method and the TSBA40 library (version 4.10, MIDI). Polar lipids were extracted as described by Kates (1986) and separated by two-dimensional TLC on silica gel 60 F_{254} plates (Merck) as described by Tindall (1990). Five kinds of spray reagents were used to visualize corresponding lipids, including molybdophosphoric acid for total lipids, α-naphthol/sulphuric acid and anisaldehyde for glycolipids, molybdenum blue for phospholipids and ninhydrin for aminolipids.

As listed in Table S2, $C_{18:1}\omega7c$ and $C_{16:0}$ were the major fatty acids (>10 %) detected in strain NH6-79^T. Q-10 was the predominant quinone (>98.7 %), which was a common characteristic in the genus *Parvularcula*. A trace amount of Q11 (approximately 1.3 %) could also be monitored. As showed in Fig. S3, the polar lipid profile of strain NH6-79^T was composed of three major unidentified glycolipids (GL1, 4, 5), four minor unidentified glycolipids (GL3, 6, 7, 8), one unidentified phosphoglycolipid (PGL), two unknown lipids (L1, 2) and phosphatidylglycerol (PG), which was most similar to the profile of *P. bermudensis* KCTC 12087^T. Moreover, sphingoglycolipids (SGL 1–3) reported by Li *et al.* (2014) in '*P. oceanus*' CGMCC 1.12400 and in type strains of the three recognized species of the genus *Parvularcula* were designated as unidentified glycolipids (GL 3–5) in this paper (Fig. S3), since no appraisal arguments could be provided. In addition, phosphatidylethanolamine (PE) reported by Yu *et al.* (2013) in *P. dongshanensis* MCCC 1A06534^T was not detected in our research (Fig. S3), because ninhydrin staining aimed at the polar lipids with free amino groups, such as PE, was completely negative in this strain (data not shown).

In addition to the phylogenetic proof described above, strain NH6-79^T also exhibits the typical characteristics of the genus *Parvularcula* (Table 1), such as cell morphology, pigmented colony, strict requirement for oxygen, and having $C_{18:1}\omega7c$ and $C_{16:0}$ as the major fatty acids. As summarized in Table 1, the polar lipid profiles of the *Parvularcula* members are divergent into two styles: those of *P. dongshanensis* MCCC 1A06534^T and '*P. oceanus*' CGMCC 1.12400 which contain diphosphatidylglcerol (DPG), and those of *P. bermudensis* KCTC 12087^T, *P. lutaonensis* KCTC 22245^T and strain NH6-79^T which lack DPG.

A number of phenotypic differences could be found between strain NH6-79^T and the current members of the genus *Parvularcula* (Table 1). For example, cells of strain NH6-79^T are yellow-pigmented, which is the most conspicuous feature. The optimal growth temperature of strain NH6-79^T is similar to that of *P. lutaonensis* KCTC 22245^T which was isolated from a coastal hot spring, and higher than those of the other strains of species of the genus *Parvularcula*. As for the chemotaxonomic discrepancies, several fatty acid components were detected only in strain NH6-79^T, including $C_{12:0}$, 11-methyl $C_{18:1}\omega7c$ and summed features 7 (Table 1). There are also some physiological and biochemical characteristics which are distinctive in strain NH6-79^T, such as no hydrolysis of skimmed milk and sensitivities to ciprofloxacin, kanamycin, neomycin and vibramycin. Furthermore, H_2S could be produced by strain NH6-79^T on the modified 2216 marine medium without supplement of any sulfur-bearing substrate, which is not found in the reference strains.

In conclusion, based on the results of this taxonomic research using a polyphasic approach, strain NH6-79^T is considered to represent a novel species of the genus *Parvularcula*, for which the name *Parvularcula flava* sp. nov. is proposed.

Description of *Parvularcula flava* sp. nov.

Parvularcula flava (fla′va. L. fem. adj. *flava* yellow, the colour of the pigment that the bacterium produces).

Cells are coccoid to short rods (0.5–0.9×0.6–1.8 μm), motile with one polar flagellum, non-sporulating, Gram-

stain-negative and strictly aerobic. After incubated on the modified marine 2216 agar at 37 °C for 7 days, colonies are convex with smooth and shiny surface, yellow-pigmented, translucent and uniformly circular with a diameter of approximately 1 mm. Growth occurs at 4–42 °C (optimum 37 °C), at pH 6.0–8.5 (optimum pH 7.0), and with 0.5–11 % (w/v) NaCl (optimum 4.5 %) and 1.5–17 % (w/v) sea salt (optimum 3.5–5 %). Produces carotenoid, but not bacteriochlorophyll α. Accumulates intracellular PHB granules. Hydrolyses of aesculin, gelatin, Tweens 20, 40, 60 and 80, and tyrosine, but not alginate, DNA, CM-cellulose, skimmed milk or starch. Catalase- and oxidase-positive. Produces H_2S from $S_2O_3^{2-}$, cysteine and peptone. Negative in methyl red test and positive in Voges-Proskauer test. Other physiological and biochemical characteristics are available in Table S1. The dominant fatty acids include $C_{18:1}\omega7c$ and $C_{16:0}$. The polar lipid profile is composed of seven unidentified glycolipids, one unidentified phosphoglycolipid, two unknown lipids and PG. The predominant isoprenoid quinone is ubiquinone-10.

The type strain is NH6-79^T (=CGMCC 1.14984^T=JCM 30557^T=MCCC 1K00277^T), which was isolated from surface seawater of the South China Sea. The DNA G+C content of the type strain is 60.7 mol%.

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References

Arun, A. B., Chen, W. M., Lai, W. A., Chou, J. H., Rekha, P. D., Shen, F. T., Singh, S. & Young, C. C. (2009). *Parvularcula lutaonensis* sp. nov., a moderately thermotolerant marine bacterium isolated from a coastal hot spring. *Int J Syst Evol Microbiol* **59**, 998–1001.

Cho, J. C. & Giovannoni, S. J. (2003). *Parvularcula bermudensis* gen. nov., sp. nov., a marine bacterium that forms a deep branch in the α-*Proteobacteria*. *Int J Syst Evol Microbiol* **53**, 1031–1036.

Kates, M. (1986). *Techniques of Lipidology*, 2nd edn. Amsterdam: Elsevier.

Komagata, K. & Suzuki, K. (1988). Lipids and cell-wall analysis in bacterial systematics. *Methods Microbiol* **19**, 161–207.

Li, S. H., Tang, K., Liu, K. S., Yu, C. P. & Jiao, N. Z. (2014). *Parvularcula oceani* sp. nov., isolated from deep-sea water of the Southeastern Pacific Ocean. *Antonie Van Leeuwenhoek* **105**, 245–251.

Liu, J. J., Zhang, X. Q., Pan, J., Sun, C., Zhang, Y., Li, C. Q., Zhu, X. F. & Wu, M. (2013). *Aequorivita viscosa* sp. nov., isolated from an intertidal zone, and emended descriptions of *Aequorivita antarctica* and *Aequorivita capsosiphonis*. *Int J Syst Evol Microbiol* **63**, 3192–3196.

Lányi, B. (1988). Classical and rapid identification methods for medically important bacteria. *Methods Microbiol* **19**, 1–67.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Ostle, A. G. & Holt, J. G. (1982). Nile blue A as a fluorescent stain for poly-beta-hydroxybutyrate. *Appl Environ Microbiol* **44**, 238–241.

Tindall, B. J. (1990). Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.

Yu, Z., Lai, Q., Li, G. & Shao, Z. (2013). *Parvularcula dongshanensis* sp. nov., isolated from soft coral. *Int J Syst Evol Microbiol* **63**, 2114–2117.

Zhang, X. Q., Ying, Y., Ye, Y., Xu, X. W., Zhu, X. F. & Wu, M. (2010). *Thermus arciformis* sp. nov., a thermophilic species from a geothermal area. *Int J Syst Evol Microbiol* **60**, 834–839.

Zhang, X. Q., Sun, C., Wang, C. S., Zhang, X., Zhou, X., Wu, Y. H., Xu, X. W. & Wu, M. (2015). *Sinimarinibacterium flocculans* gen. nov., sp. nov., a gammaproteobacterium from offshore surface seawater. *Int J Syst Evol Microbiol* **65**, 3541–3546.