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Hymenobacter pallidus sp. nov., isolated from a freshwater fish culture pond

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Abstract

A bacterial strain designated LYH-12^T was isolated from a freshwater fish culture pond in Taiwan, ROC and characterized by taking a polyphasic taxonomy approach. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain LYH-12^T belonged to the genus *Hymenobacter* and was most closely related to *Hymenobacter xinjiangensis* X2-1g^T and *Hymenobacter rigui* WPCB131^T with a sequence similarity of 96.6 % and less than 96.5 % with other members of the genus. Cells of strain LYH-12^T were Gram-stain-negative, aerobic, non-motile rods that were covered by large capsules and formed light pink-coloured colonies. Growth occurred at 10–37 °C (optimum, 20–30 °C), at pH 6.5–7.5 (optimum, pH 7) and with 0–1 % NaCl (optimum, 0.5 %). Strain LYH-12^T contained iso-$C_{15:0}$, $C_{16:1}\omega5c$, $C_{16:0}$, iso-$C_{17:0}$ 3-OH, summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and anteiso-$C_{17:1}\omega9c$ as the predominant fatty acids. The only isoprenoid quinone detected was MK-7. The polar lipid profile consisted of phosphatidylethanolamine, one uncharacterized aminophospholipid, four uncharacterized aminolipids, two uncharacterized phospholipids and three uncharacterized lipids. The major polyamine was homospermidine. The DNA G+C content of the genomic DNA was 64.3 mol%. On the basis of the phylogenetic inference and phenotypic data, strain LYH-12^T should be classified as a novel species, for which the name *Hymenobacter pallidus* sp. nov. is proposed. The type strain is LYH-12^T (=BCRC 80919^T=LMG 29171^T=KCTC 42898^T).

The genus *Hymenobacter*, first proposed by Hirsch *et al.* [1] and emended subsequently by Buczolits *et al.* [2] and Han *et al.* [3], belongs to the family *Cytophagaceae*, order *Cytophagales*, class *Cytophagia*, phylum *Bacteroidetes* [4]. At the time of writing, this genus comprises 36 recognized species (www.bacterio.net/hymenobacter.html). Members of the genus *Hymenobacter* forming pink- to red-pigmented colonies are Gram-stain-negative and have rod-shaped cells. The present study was carried out to clarify the taxonomic position of a strain of the genus *Hymenobacter* by taking a polyphasic taxonomic approach.

During our investigations on the biodiversity of bacteria in the water of a fish culture pond in the Sanyi Township (GPS location: 22° 54′ 40″ N, 121° 14′ 13″ E) in the vicinity of MiaoLi County, Taiwan, a light pink-pigmented bacterium, designated LYH-12^T, was isolated and selected for detailed taxonomy analyses. Strain LYH-12^T was maintained on R2A agar (BD Difco) at 25 °C, and preserved at −80 °C as a suspension in R2A broth (BD Difco) with 20 % (v/v) glycerol.

Genomic DNA was isolated using a bacterial genomic DNA purification kit (DP02-150, GeneMark Technology), and the 16S rRNA gene sequence was analysed as described by Chen *et al.* [5]. Primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′) were used for amplification of bacterial 16S rRNA genes by PCR [6, 7]. The PCR product was purified and cloned into the pBluescript II vector (Stratagene), and direct sequencing was performed by using 520F, 800R [7], T3 promoter and T7 promoter primers with an ABI Prism 3730 DNA sequencer (Applied Biosystems). The sequenced length of the 16S rRNA gene was 1471 bp for strain LYH-12^T and this gene sequence was compared to those available from the EzTaxon-e [8], the Ribosomal Database Project [9] and the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Analysis of the sequence data was performed by using the software package BioEdit [10] and MEGA 7 [11], after multiple alignments of the data by CLUSTAL X version 2.0 [12]. Distances (corrected according to Kimura's two-parameter model; [13]) were calculated and clustering was performed

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Abbreviations: AL, aminolipids; APL, aminophospholipid; L, lipid; PE, phosphatidylethanolamine; PL, phospholipids.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Hymenobacter pallidus* strain LYH-12^T is LN879491.
Four supplementary figures is available with the online Supplementary Material.

by using the neighbour-joining method [14]. The maximum-likelihood [15], maximum-parsimony [16] and minimum-evolution [17] trees were generated by using the tree-making algorithms contained in the PHYLIP software package [18]. In each case bootstrap values were calculated based on 1000 replications.

The 16S rRNA gene sequence analysis indicated that strain LYH-12^T formed a separate phylogenetic branch within the genus *Hymenobacter* in the neighbour-joining tree (Fig. 1). The overall topologies of the maximum-likelihood and maximum-parsimony trees were similar. These findings were confirmed by analysis based on the minimum-evolution algorithm (see Fig. S1, available in the online Supplementary Material). Sequence similarity calculations (over 1400 bp) indicated that strain LYH-12^T was most closely related to *Hymenobacter xinjiangensis* X2-1g^T (96.6 % 16S rRNA gene sequence similarity) and *Hymenobacter rigui* WPCB131^T (96.6 %). Sequence similarities <96.5 % were observed with the type strains of all other species listed in Fig. 1.

The type strains *H. xinjiangensis* X2-1g^T (=JCM 23206^T) and *H. rigui* WPCB131^T (=KCTC 12533^T) were obtained from culture collections, and both type strains were used as reference strains and evaluated together under identical experimental conditions to those for strain LYH-12^T.

Cell morphology of strain LYH-12^T was observed by phase-contrast microscopy (DM 2000; Leica) using cells grown in R2A agar at lag, exponential and stationary phases. Flagellar motility was tested using the hanging drop method, and the Spot Test flagella stain (BD Difco) was used for flagellum staining. Gliding motility was studied using phase-contrast microscopy as described by Bernardet *et al.* [19]. The Gram Stain Set S kit (BD Difco) and Ryu's non-staining KOH method [20] were used to perform the Gram reaction. The presence of a capsule was assessed by using Hiss's staining method [21]. Poly-β-hydroxybutyrate granule accumulation was examined under light microscopy after staining of the cells with Sudan black. Colony morphology was observed on R2A agar by using a stereoscopic microscope (SMZ 800; Nikon). The presence of flexirubin and carotenoid types of pigments was investigated as described by Reichenbach [22] and Schmidt *et al.* [23].

The physiological characteristics of strain LYH-12^T and two closely related strains were examined by growing bacteria at various pH values, temperatures and NaCl concentrations. The pH range for bacterial growth was estimated by measuring the optical densities (wavelength 600 nm) of R2A broth cultures. The pH of the medium was adjusted prior to sterilization to pH 4.0–9.0 (at intervals of 0.5 pH unit) using the following biological buffers, acetate buffer, citrate buffer, phosphate buffer and Tris-Hydrochloride buffer [24]. The temperature range for growth was determined on R2A agar at 4–50 °C. To investigate the tolerance to NaCl, R2A broth was prepared according to the formula of the BD Difco medium with NaCl concentration adjusted to 0, 0.5 % and 1.0–6.0 %, w/v (at intervals of 1.0 %). Growth under anaerobic conditions was determined after incubating strain LYH-12^T in the Oxoid AnaeroGen system. Bacterial growth was studied on R2A, nutrient, Luria–Bertani and trypticase soy agars (all from Difco) under aerobic condition at 25 °C.

Strain LYH-12^T and the closely related strains were examined for activities of catalase, oxidase, DNase, urease and lipase (corn oil), and hydrolysis of starch, casein, gelatin, lecithin and Tweens 20, 40, 60 and 80 using standard approaches [25]. Chitin hydrolysis was assessed on chitinase-detection agar [26] and visualized by the formation of clear zones around the colonies. Hydrolysis of carboxymethylcellulose (CM-cellulose) was tested as described by Bowman [27] using R2A agar as the basal medium. Utilization of carbon sources was investigated in a basal medium containing (l^{-1}): 0.4 g KH_2PO_4, 0.53 g Na_2HPO_4, 0.3 g NH_4Cl, 0.3 g NaCl, 0.1 g $MgCl_2$. $6H_2O$, 0.11 g $CaCl_2$ and 1 ml trace element solution, pH 7.0 [28]. Substrates were added at a concentration of 0.1 % (w/v) and the tubes incubated under aerobic conditions at 25 °C. Occurrence of bacterial growth was checked for 12 days at 1 day intervals. The experiment was done in duplicate. Additional biochemical tests were investigated using API ZYM and API 20NE kits (both from bioMérieux). All commercial phenotypic tests were performed according to the manufacturers' instructions.

Strain LYH-12^T was a Gram-stain-negative, aerobic, rod-shaped bacterium. Cells were covered by large capsules (see Fig. S2). Poly-β-hydroxybutyrate accumulation was observed. Motility was not observed. Cells grew well on R2A agar, nutrient agar, Luria–Bertani agar and trypticase soy agar. Detailed results from the phenotypic and biochemical analyses of strain LYH-12^T are provided in Table 1 and in the species description.

The fatty acid profiles of strain LYH-12^T and the two reference strains were determined using cells grown on R2A agar at 25 °C for 3 days, when bacterial cultures reached the stationary stage of growth. Fatty acid methyl esters were prepared and separated according to the MIDI standard protocol (Sherlock Microbial Identification System, version 6.0), analysed by gas chromatography (Hewlett-Packard 5890 Series II) and identified by using the RTSBA6.00 database of the Microbial Identification system [29].

The predominant cellular fatty acids (>5 % of the total fatty acids) of strain LYH-12^T were iso-$C_{15:0}$ (32.5 %), $C_{16:1}\omega5c$ (12.8 %), $C_{16:0}$ (7.0 %), summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; 6.5 %), iso-$C_{17:0}$ 3-OH (5.8 %) and anteiso-$C_{17:1}$ $\omega9c$ (5.3 %). The complete fatty acid composition is shown in Table 2. The fatty acid profile of strain LYH-12^T differed markedly from those of these two closely related strains by the presence of anteiso-$C_{17:1}\omega9c$. In addition, strain LYH-12^T and *H. xinjiangensis* X2-1g^T contained lower amounts of summed feature 4 (iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B) when compared to *H. rigui* WPCB131^T.

The polar lipids of strain LYH-12^T, *H. xinjiangensis* X2-1g^T and *H. rigui* WPCB131^T were extracted and analysed by two-dimensional thin-layer chromatography (TLC) according to Embley and Wait [30]. Ethanolic molybdophosphoric

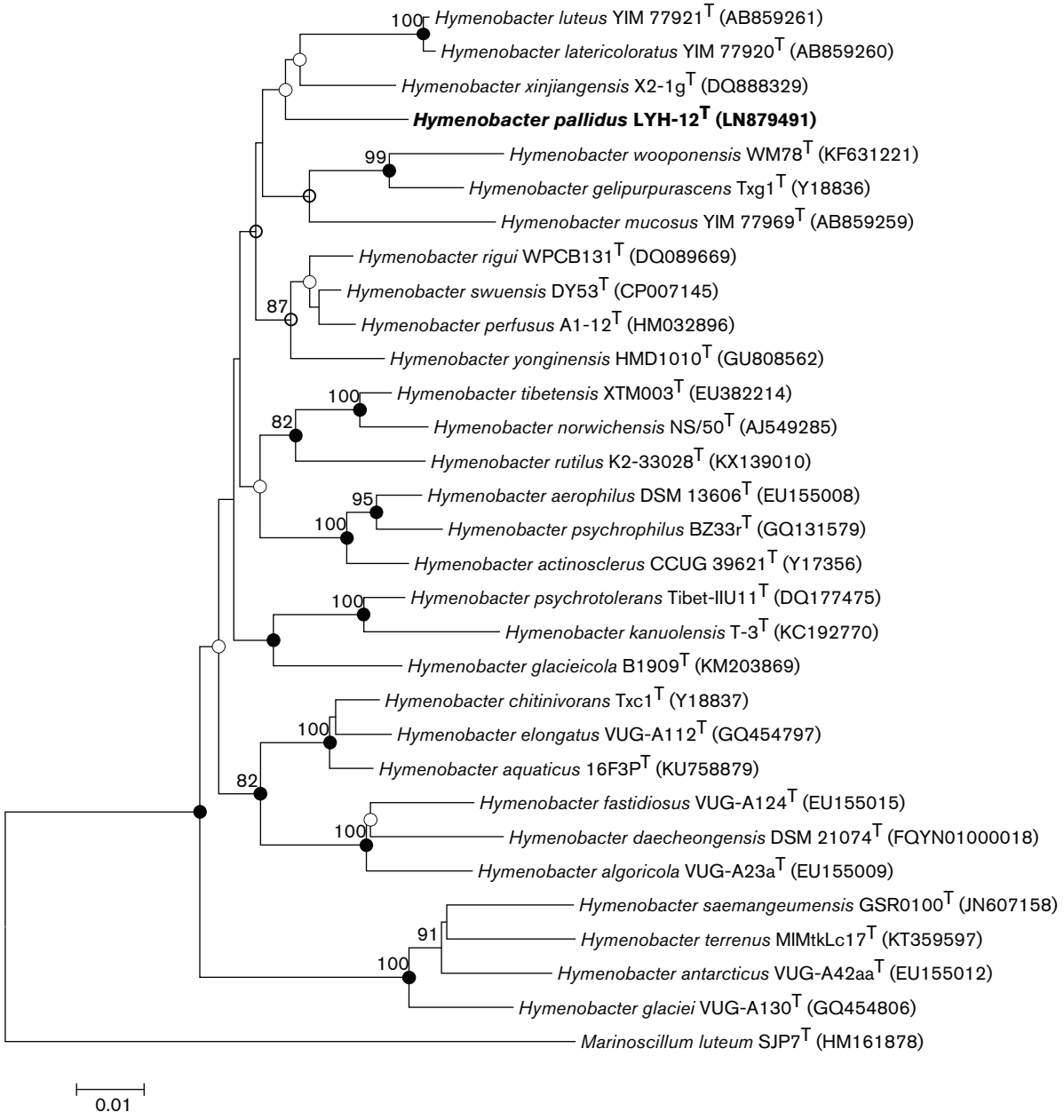

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of *Hymenobacter pallidus* LYH-12^T and closely related strains. Numbers at nodes are bootstrap percentages >70 % based on the neighbour-joining (above nodes) and maximum-parsimony (below nodes) tree-making algorithms. Filled circles indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony tree-making algorithms. Open circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. *Marinoscillum luteum* SJP7^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

acid (10 %) was used for the detection of the total polar lipids, ninhydrin for amino lipids, the Zinzadze reagent for phospholipids and the α-naphthol reagent for glycolipids. Strain LYH-12^T exhibited a complex polar lipid profile consisting of phosphatidylethanolamine (PE), one uncharacterized aminophospholipid (APL1), four uncharacterized aminolipids (AL1-AL4), two uncharacterized phospholipids (PL1-PL2) and three uncharacterized lipids (L1-L3) (see Figs S3 and S4).

Strain LYH-12^T 2^T, *H. xinjiangensis* X2-1g^T and *H. rigui* WPCB131^T contained PE as the predominant polar lipid (Fig. S3); the possession of PE as the common major polar lipid is consistent with previous descriptions of species of *Hymenobacter* [1–3, 31, 32]. Strain LYH-12^T exhibited a

very similar polar lipid profile to these two closest relatives and they all had PE, APL1, AL1, AL2, PL1, L1 and L2. However, AL3, AL4, PL1 and L3 were detected in strain LYH-12^T and *H. rigui* WPCB131^T, but not in *H. xinjiangensis* X2-1g^T. In addition, PL3 was only present in *H. rigui* WPCB131^T but absent in strain LYH-12^T and *H. xinjiangensis* X2-1g^T, and three unidentified lipids (L5-L7) were only present in *H. xinjiangensis* X2-1g^T but absent in strain LYH-12^T and *H. rigui* WPCB131^T. These results suggested that there are some differences in the polar lipid profiles among them, although they belong to the same genus and have very similar profiles.

The isoprenoid quinone of strain LYH-12^T was extracted and purified according to the method of Collins [33]. The only respiratory quinone detected was menaquinone (MK-7). The DNA G+C content of strain LYH-12^T determined by high performance liquid chromatography according to Mesbah *et al.* [34] was 64.3±1.0 mol%, a value within the range reported for *Hymenobacter* strains [2].

Table 1. Differential characteristics of *Hymenobacter pallidus* LYH-12^T and phylogenetically closely related *Hymenobacter* species

All data from this study except the G+C content of *H. xinjiangensis* X2-1g^T [37] and *H. rigui* WPCB131^T [38]. +, Positive reaction; −, negative reaction; w, weakly positive reaction. All strains grew well on R2A agar and were positive for catalase, alkaline phosphatase, C4 esterase, C8 esterase lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphophydrolase and *N*-acetyl-β-glucosaminidase activities, and hydrolysis of casein, starch, DNA, gelatin, and Tweens 40 and 60. All strains were negative for: Gram staining; gliding motility; nitrate reduction; indole production; D-glucose acidification; lipase, arginine dihydrolase, urease, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase and α-fucosidase activities; hydrolysis of chitin and Tween 80.

Characteristic	*H. pallidus* LYH-12^T	*H. xinjiangensis* X2-1g^T	*H. rigui* WPCB131^T
Isolation source	Fish culture pond	Desert sand	Wetland
Colony pigmentation	Light pink	Pink	Pinkish red
Temperature range for growth (°C) (optimum)	10–37 (20-30)	10–37 (20–25)	4–37 (30)
NaCl range for growth (%, w/v) (optimum)	0–1 (0.5)	0–0.5 (0)	0–2 (0)
pH range for growth (optimum)	6.5–7.5 (7)	5–7 (6)	5–9 (6)
Growth on:			
Nutrient agar	+	+	w
Trypticase soy agar	+	w	+
Hydrolysis of:			
Aesculin	+	+	−
CM-cellulose	+	+	−
Tween 20	+	−	−
Enzymatic activities:			
Oxidase	+	+	−
C14 lipase	+	−	−
Trypsin	+	−	−
α-Glucosidase	+	−	+
β-Glucosidase	+	−	−
α-Mannosidase	−	+	−
Carbon sources utilization:			
Dextrin	−	+	+
Tween 40	+	−	−
D-Glucose	+	−	+
Cellobiose	−	+	+
D-Fructose	−	+	+
D-Galactose	−	+	+
Maltose	−	+	+
D-Mannose	−	+	−
Raffinose	−	+	+
Trehalose	−	+	+
Sucrose	−	+	+
N-Acetylglucosamine	−	+	−
D-Sorbitol	−	+	−
D-Mannitol	−	+	−
DNA G+C content (mol%)	64.3	54	65

Table 2. Cellular fatty acid composition of *Hymenobacter pallidus* LYH-12^T and phylogenetically closely related *Hymenobacter* species

All data were obtained from this study. All strains were grown on R2A agar at 25 °C for 3 days. Only fatty acids with more than 1 % of the total acids in at least one of the strains are shown. –, not detected; TR, trace (<1 %). For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (*ω*) end of the carbon chain. *cis* isomer is indicated by the suffix *c*.

Fatty acid	*H. pallidus* LYH-12^T	*H. xinjiangensis* X2-1g^T	*H. rigui* WPCB131^T
Saturated			
$C_{12:0}$	1.3	2.8	1.1
$C_{14:0}$	1.1	1.7	TR
$C_{16:0}$	7.0	8.7	5.6
$C_{18:0}$	2.2	5.5	2.1
Unsaturated			
$C_{16:1}\omega5c$	12.8	5.2	13.1
$C_{18:1}\omega7c$	1.2	2.0	TR
$C_{18:1}\omega9c$	2.5	6.0	1.5
Branched			
iso-$C_{15:0}$	32.5	25.7	30.2
iso-$C_{16:0}$	1.1	TR	–
iso-$C_{17:0}$	3.9	4.1	4.4
anteiso-$C_{14:0}$	2.4	5.0	2.5
anteiso-$C_{15:0}$	1.0	4.4	5.1
anteiso-$C_{17:1}\omega9c$	5.3	–	–
Hydroxyl			
iso-$C_{15:0}$ 3-OH	2.4	1.8	TR
iso-$C_{17:0}$ 3-OH	5.8	4.3	2.7
Summed features*			
1	TR	1.3	TR
3	6.5	7.8	11.8
4	3.8	6.5	12.3

*Summed features are groups of two or three fatty acids that cannot be separated by gas–liquid chromatography by using the MIDI system. Summed feature 1 comprised iso-$C_{15:1}$ H and/or $C_{13:0}$ 3-OH; summed feature 3 comprised $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; and summed feature 4 comprised iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B.

Polyamines were extracted from strain LYH-12^T and analysis was carried out as described by Busse and Auling [35] and Busse *et al.* [36]. Strain LYH-12^T contained homospermidine (HSPD, 95.8 %) as the major polyamine, and spermidine (SPD, 2.7 %) and putrescine (PUT, 1.5 %) as minor components. The presence of homospermidine as the major polyamine in strain LYH-12^T is consistent with that reported for other species of the genus *Hymenobacter* [2, 31, 32].

Phenotypic examination revealed several common traits between the novel strain and its two closely related strains, *H. xinjiangensis* X2-1g^T and *H. rigui* WPCB131^T. However, strain LYH-12^T could be clearly differentiated from these two strains by: colony pigmentation; ability to optimally grow with higher NaCl concentration (0.5 %); ability to optimally grow at neutral condition (pH 7); ability to hydrolyse Tween 20; presence of C14 lipase, trypsin and β-glucosidase activities; ability to utilize Tween 40 as sole carbon sources; and inability to utilize dextrin, cellobiose, D-fructose, D-galactose, maltose, raffinose, trehalose and sucrose (Table 1).

Strain LYH-12^T is Gram-stain-negative, rod-shaped and aerobic. The predominant fatty acid contains iso-$C_{15:0}$ (32.5 %). The major polar lipid is phosphatidylethanolamine. The predominant polyamine is homospermidine. The major and only respiratory quinone is MK-7. The DNA G+C content is 64.3 mol%. These characteristics of strain LYH-12^T are consistent with the description of the genus *Hymenobacter* [1–3]. On the basis of phylogenetic inference, strain LYH-12^T occupies a distinct position within the genus *Hymenobacter* that is supported by a unique combination of chemotaxonomic and biochemical characteristics. Strain LYH-12^T is considered to represent a novel species of the genus *Hymenobacter*, for which we propose the name *Hymenobacter pallidus* sp. nov.

DESCRIPTION OF *HYMENOBACTER PALLIDUS* SP. NOV.

Hymenobacter pallidus (pal'li.dus. L. masc. adj. *pallidus* pale, the light pink colour of colonies).

Cells are Gram-stain-negative, aerobic, non-motile rods that are covered by large capsules. After 48 h of incubation on R2A agar at 25 °C, the mean cell size is 0.9–1.1 µm in diameter and 1.8–2.0 µm in length. Colonies on R2A agar are light pink, convex and circular with irregular, curled margins. The colony size is approximately 4.2–5.0 mm in diameter after 72 h at 25 °C. Growth occurs at 10–37 °C (optimum, 20–30 °C), at pH 6.5–7.5 (optimum, pH 7) and with 0–1 % NaCl (optimum, 0.5 %). Flexirubin-type pigments are not produced. Carotenoid pigments are present with maximum absorption at 482 nm with shoulders at 451 and 510 nm. Positive for poly-β-hydroxybutyrate accumulation. Positive for oxidase and catalase activities and hydrolysis of casein, starch, CM-cellulose, DNA, Tweens 20, 40 and 60. Negative for lipase and urease activities, and hydrolysis of lecithin, Tween 80 and chitin. In API 20NE tests, positive for aesculin and gelatin hydrolysis, and negative for nitrate reduction, indole production, D-glucose acidification, arginine dihydrolase, urease and β-galactosidase activities, and assimilation of glucose, arabinose, mannose, mannitol, *N*-acetyl-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate and phenyl-acetate. In the API ZYM kit, alkaline phosphatase, C4 esterase, C8 esterase lipase, C14 lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase,α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase activities are present, but not α-chymotrypsin, α-galactosidase,β-galactosidase, β-glucuronidase, α-mannosidase or α-fucosidase. Growth under aerobic

condition is positive on Tween 40 and α-D-glucose, but not on dextrin, Tween 80, cellobiose, D-fructose, D-galactose, maltose, D-mannose, raffinose, L-rhammose, trehalose, sucrose, *N*-acetylglucosamine, adonitol, D-mannitol or D-sorbitol. The major fatty acids (>5 % of the total fatty acids) are iso-$C_{15:0}$, $C_{16:1}\omega5c$, $C_{16:0}$, iso-$C_{17:0}$ 3-OH, summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and anteiso-$C_{17:1}\omega9c$. The only respiratory quinone is MK-7. The polar lipid profile consists of phosphatidylethanolamine, one uncharacterized aminophospholipid, four uncharacterized aminolipids, two uncharacterized phospholipids and three uncharacterized lipids. Homospermidine is the major polyamine, and spermidine and putrescine are minor components.

The type strain LYH-12^T (=BCRC 80919^T=LMG 29171^T =KCTC 42898^T) was isolated from a fish culture pond in the Sanyi Township in the vicinity of MiaoLi County, Taiwan. The DNA G+C content of the type strain is 64.3 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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