Limnohabitans curvus gen. nov., sp. nov., a planktonic bacterium isolated from a freshwater lake

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A chemo-organotrophic, aerobic, facultatively anaerobic, non-motile strain, MWH-C5^T, isolated from the water column of the oligomesotrophic Lake Mondsee (Austria), was characterized phenotypically, phylogenetically and chemotaxonomically. The predominant fatty acids of the strain were C_{16:1}ω7c/ω6c, C_{16:0}, C_{12:1} and C_{8:0}-3OH, the major quinone was ubiquinone Q-8 and the G + C content of the DNA of the strain was 55.5 mol%. 16S rRNA gene similarity to the closest related type strains was 96.6 % (*Curvibacter delicatus* LMG 4328^T) and 95.7 % (*Rhodoferrax fermentans* FR3^T). Phylogenetic analysis of 16S rRNA gene sequences revealed the affiliation of the strain with the family *Comamonadaceae* (*Betaproteobacteria*); however, the phylogenetic position of the strain did not support an affiliation to any previously described genus within this family. A family-wide comparison of traits revealed that the strain possesses a unique combination of DNA G + C content, major fatty acids and major 3-hydroxy fatty acid. Furthermore, the strain differs in several traits from the closest related genera. Based on the phylogeny of the strain and differences from closely related genera, we propose to establish the new genus and species *Limnohabitans curvus* gen. nov., sp. nov. to accommodate this strain. The type strain of *Limnohabitans curvus* is MWH-C5^T (=DSM 21645^T =CCUG 56720^T). The type strain is closely related to a large number of uncultured bacteria detected by cultivation-independent methods in various freshwater systems.

The bacterioplankton of freshwater habitats is composed mainly of phylogenetic groups absent from marine bacterioplankton and terrestrial habitats (Hahn, 2006; Zwart *et al.*, 2002). Investigations with cultivation-independent methods have demonstrated that the majority of taxa dwelling in the water column of freshwater lakes and ponds represent uncultured and undescribed taxa (Crump *et al.*, 1999; Eiler & Bertilsson, 2004; Zwart *et al.*, 2002). In this paper, we characterize a strain isolated from the pelagic zone of a freshwater lake that is closely related to uncultured bacteria numerously detected in freshwater samples and propose to establish a new genus and species within the family *Comamonadaceae* (*Betaproteobacteria*) to accommodate this strain.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MWH-C5^T is AJ938026.

An extended neighbour-joining tree including 16S rRNA gene sequences from uncultured clones, detailed 16S rRNA gene sequence similarity data and a family-wide comparison of phenotypic traits are available as supplementary material with the online version of this paper.

Strain MWH-C5^T was isolated from deep, oligomesotrophic Lake Mondsee (47° 50' 2.92" N 13° 22' 25.98" E) located in Austria. The strain was obtained by using the dilution-acclimatization method (DAM) and nutrient broth soytone yeast extract (NSY) medium (Hahn *et al.*, 2004, 2005). The isolated bacterium grows on a variety of solidified complex media, e.g. Luria–Bertani agar (Difco BD), casitone agar (Difco BD), R2A agar (Remel) and NSY agar (Hahn *et al.*, 2004), forming unpigmented, smooth, convex colonies.

Tests of growth of the strain on single carbon sources resulted only in weak growth. In order to avoid false-negative results in such tests caused by a too-weak growth response on single substrates, tests of substrate utilization were performed in the presence of complex substrate mixtures as described previously (Hahn *et al.*, 2009). Briefly, growth enabled by utilization of specific substrates was determined by comparison of the OD₅₇₅ established in liquid one-tenth-strength NSY medium (0.3 g l⁻¹) with and without 0.5 g test substance l⁻¹. Differences in OD₅₇₅

of <10, 10–50 and >50 % compared with growth on the medium without the test substance after 10 days of growth were respectively scored as no utilization, weak utilization and good utilization.

Analysis of the phylogenetic position of the strain was performed by means of 16S rRNA gene sequence analysis. Sequences were obtained, aligned and analysed by the neighbour-joining approach as described previously (Hahn *et al.*, 2009). In addition, trees were constructed by using the maximum-likelihood and Bayesian-inference tree-building methods. For the maximum-likelihood tree calculation and bootstrap analysis, we used the genetic algorithm GARLI (Zwickl, 2006) and a grid computing system (Cummings & Huskamp, 2005). A Bayesian inference tree was calculated by using the program MrBayes (Ronquist & Huelsenbeck, 2003), performing one million generations, and subsequently explored by the AWTY system (Nylander *et al.*, 2008). Determination of the G+C content of DNA and analyses of major respiratory lipoquinones were both carried out by the Identification Service and Dr B. J. Tindall, DSMZ, Braunschweig, Germany. Fatty acid profiles of strain MWH-C5^T and the type strains of *Curvibacter gracilis* and *Rhodoferrax fermentans* were characterized by using the MIS Sherlock automatic identification system (MIDI, Inc.) and the Sherlock Aerobic Bacterial Database (TSBA60) as described by Greenblatt *et al.* (1999). For each strain, biomass for the analysis came from replicated cultures obtained by growing the strains in NSY (3 g l⁻¹) for 2 days at 21 °C.

The results of the phenotypic and chemotaxonomic investigations are presented in Tables 1 and 2. Strain MWH-C5^T is an aerobic, chemo-organotrophic, facultatively anaerobic, non-motile bacterium; cells are small, curved rods (Fig. 1). The predominant fatty acids (>5 % of total) of the strain were C_{16:1}ω7clω6c and C_{16:0}, the sole 3-hydroxy fatty acid detected was C_{8:0} 3-OH, the major quinone was ubiquinone Q-8 and the G+C content of the DNA of the strain was 55.5 mol%.

Phylogenetic analyses with all three tree-building methods consistently revealed the affiliation of strain MWH-C5^T with the family *Comamonadaceae* (Fig. 2). The sequence of the strain did not cluster within any previously described genus in any tree generated with the three methods. The phylogenetic relationships reconstructed by using the three algorithms indicated in all three cases a close relationship of the strain with the genera *Rhodoferrax*, *Pseudorhodoferrax*, *Curvibacter*, *Polaromonas*, *Variovorax*, *Ramlibacter* and *Caenimonas*. Regarding the phylogenetic relationships of strain MWH-C5^T to these seven genera, the topologies and branching orders of the three trees were almost identical (not shown). The trees differed only in the position of the type species of the genus *Caenimonas*, which clustered with the genus *Ramlibacter* in the maximum-likelihood and Bayesian-inference trees and with the genus *Curvibacter* in the neighbour-joining tree. Pairwise sequence similarity of the 16S rRNA genes of strain MWH-C5^T and type strains

of the type species of these seven closely related genera was 94.4–96.2 % (mean 95.3 %), which is similar to the range of 93.0–96.5 % (mean 95.4 %) observed for pairwise comparisons among the type species of the seven genera (Supplementary Table S1, available in IJSEM Online).

A family-wide comparison was performed of traits of strain MWH-C5^T and the type strains of the species of all 30 genera (including *Pseudorhodoferrax* described by Bruland *et al.*, 2009) currently assigned to the family *Comamonadaceae*. This comparative analysis was limited by the heterogeneity of the datasets available for the different type species, because only a few traits have been described for the overwhelming majority of strains and virtually no test of substrate utilization has been performed for almost all strains. However, comparisons of chemotaxonomic traits, i.e. type of quinone, major fatty acids, major 3-hydroxy fatty acid and G+C content, revealed a unique combination of these traits for strain MWH-C5^T among all genera of the *Comamonadaceae* (Supplementary Table S2). Strain MWH-C5^T differs from all but two genera in its DNA G+C content of 55.5 mol%, which is >3 mol% lower than that of the other type strains. Among type strains of the *Comamonadaceae*, only those of *Gisbergeria* species (Grabovich *et al.*, 2006) and the type strain of the type species of the genus *Polaromonas* (*Polaromonas vacuolata*; Irgens *et al.*, 1996) possess G+C contents <59 mol%. Interestingly, *Polaromonas vacuolata* differs significantly from the type strains of three other *Polaromonas* species, which have G+C contents of 61.5–63.7 mol% (Weon *et al.*, 2008). The G+C content of the fifth described *Polaromonas* species has not been reported (Kämpfer *et al.*, 2006). Furthermore, the absence of C_{10:0} 3-OH is rare among bacteria of the *Comamonadaceae*, and all genera that share this trait with strain MWH-C5^T possess G+C contents >59 mol%.

A more detailed comparison with the seven most closely related genera (Fig. 2) revealed several differences from the type species of these genera that are suitable for discrimination of the new taxon from these previously described taxa (Table 3). The G+C content is again an important discriminative trait; however, the strain also differs in at least one additional trait from all other type species: for instance, from *Caenimonas*, *Pseudorhodoferrax* and *Variovorax* by the absence of C_{10:0} 3-OH, from the strictly aerobic *Polaromonas* by its ability to grow facultatively anaerobically, from *Curvibacter* type strains by a much lower salinity tolerance, from *Ramlibacter* by the ability to assimilate glucose and citrate and from *Rhodoferrax* type strains by the ability to utilize α-ketoglutarate as a carbon source and the lack of rhodoquinone.

BLAST searches with the 16S rRNA gene sequence of strain MWH-C5^T and subsequent phylogenetic analyses revealed that strain MWH-C5^T is closely related to a large number of uncultured strains (Supplementary Fig. S1) detected in cultivation-independent investigations on the diversity of

Table 1. Phenotypic traits of strain MWH-C5^T and related type strains

Strains: 1, MWH-C5^T; 2, *Curvibacter gracilis* CCUG 49445^T; 3, *Rhodoferrax fermentans* CCUG 45364^T. The reference strains represent the type strains of the type species of the genera *Curvibacter* and *Rhodoferrax*. Data were obtained in this study unless indicated. Substrate utilization tests were performed for all three strains under the same conditions. All three strains assimilated acetate, pyruvate, fumarate, succinate, citrate, gluconate and glucose and none of the strains assimilated L-carnitine. All three strains have curved rod-shaped cells and are oxidase- and catalase-positive. –, Negative; +, positive; w, weakly positive; RQ, rhodoquinone; ND, no data available.

Characteristic	1	2	3
Cell length (µm)	1.0–1.5	1.1–2.8 ^{a*}	1.5–3.0 ^b
Cell width (µm)	0.4–0.5	0.4–0.5 ^a	0.6–0.9 ^b
Temperature for growth (°C)	4–34	9–40†	ND
Maximum NaCl concentration (% w/v)	0.5	<3 ^a	ND
Anaerobic growth	+	ND	+ ^c
Utilization of:			
Ethanol	w	–	w
Glycerol	–	+	+
Glyoxylate	–	w	–
Gycolate	–	+	–
D-Glycerate	+	+	w
Oxalate	–	–	w
DL-Lactate	–	+	–
Propionate	w	+	w
DL-Malate	+	+	w
Malonate	–	w	–
α-Ketoglutarate	+	+	w
Oxaloacetate	–	+	w
L-Arginine	–	–	+
L-Glutamate	–	+	+
L-Glutamine	–	+	w
L-Histidine	–	+	–
L-Phenylalanine	–	+	–
L-Proline	–	+	+
L-Serine	–	+	+
L-Tryptophan	–	+	w
D-Ribose	w	w	+
L-Sorbose	–	w	–
D-Galactose	w	w	+
D-Mannose	+	w	+
Sucrose	w	+	w
N-Acetylglucosamine	–	w	+
Betaine	–	–	w
Spermidine	–	–	w
Quinone type	Q-8	Q-8 ^a	Q-8, RQ-8 ^c
DNA G + C content (mol%)	55.5	66.0 ^a	60 ^c

*Data taken from: *a*, Ding & Yokota (2004); *b*, Madigan *et al.* (2000); *c*, Hiraishi *et al.* (1991).

†Data presented by Ding & Yokota (2004) in the description of the genus *Curvibacter*.

freshwater bacteria (e.g. Crump & Hobbie, 2005; Shaw *et al.*, 2008; Zwart *et al.*, 2002). Some of these uncultured bacteria share 16S rRNA gene sequence similarities of 99.9% with strain MWH-C5^T. The phylogenetic cluster formed by these uncultured organisms was previously designated the '*Rhodoferrax* sp. BAL47' cluster (Zwart *et al.*, 2002). The large number of sequences affiliated with this

cluster, as well as their geographically widespread origins, indicates a significant contribution of bacteria affiliated with this cluster to bacterioplankton in many freshwater lakes and ponds. This is also indicated by an investigation of 15 diverse lakes in northern Europe for the presence of bacteria affiliated with the '*Rhodoferrax* sp. BAL47' cluster or 14 other clusters that also contain taxa frequently

Table 2. Whole-cell fatty acid compositions of strain MWH-C5^T and related type strains

Strains: 1, MWH-C5^T; 2, *Curvibacter gracilis* CCUG 49445^T; 3, *Rhodoferrax fermentans* CCUG 45364^T. The three strains were cultivated under identical conditions [NSY medium (3 g l⁻¹) at 21 °C for 2 days]. The presented data were obtained in this study from replicated cultures. Values are percentages of total fatty acids. ND, Not detected.

Fatty acid	1	2	3
C _{8:0} 3-OH	2.7	3.9	5.1
C _{9:0} 3-OH	ND	0.1	ND
C _{10:0} 3-OH	ND	ND	0.2
C _{12:0}	4.5	ND	5.6
C _{12:0} 3-OH	ND	ND	ND
C _{14:0}	1.0	0.2	1.2
C _{14:1} ω5c	0.4	ND	0.3
C _{15:0}	ND	2.0	ND
C _{15:1} ω6c	ND	0.1	0.1
C _{15:1} ω8c	ND	0.2	ND
C _{16:0}	14.0	46.3	15.2
C _{16:1} ω5c	0.2	0.3	0.8
C _{16:1} ω7c/ω6c	76.7	44.1	49.2
C _{17:0}	ND	0.4	ND
C _{17:0} cyclo	ND	ND	ND
C _{17:1} ω6c	ND	0.6	0.6
C _{17:1} ω8c	ND	0.2	ND
C _{18:0}	0.3	ND	ND
C _{18:1} ω7c 11-methyl	0.3	0.2	0.7
C _{18:1} ω7c/ω6c	1.8	1.3	20.7
C _{18:1} ω9c	0.2	ND	ND
C _{19:1} ω6c/C _{19:0} cyclo	ND	0.1	ND

detected in freshwater habitats by a cultivation-independent method (Lindström *et al.*, 2005; Zwart *et al.*, 2003). The ‘*Rhodoferrax* sp. BAL47’ cluster and an actinobacterial cluster were the only clusters detected in all 15 investigated habitats. A prominent and well-investigated subgroup of the ‘*Rhodoferrax* sp. BAL47’ cluster is the so-called R-BT065 group (Šimek *et al.*, 2001, 2005), which can be detected by a specific fluorescent *in situ* hybridization (FISH) probe. Investigations of several freshwater ponds and lakes by using this FISH probe revealed that the targeted cells possess a planktonic lifestyle and that this taxon typically comprises 5–30 % (maximum ~50 %) of the total bacterioplankton cells in non-acidic stagnant freshwater habitats (Kasalický *et al.*, 2010; Šimek *et al.*, 2001, 2005, 2010a). The origin of strain MWH-C5^T from the pelagic zone of a freshwater lake, as well as the close phylogenetic relationship with strains inhabiting the water column of freshwater systems, indicates that this strain shares a planktonic lifestyle with R-BT065 bacteria and other members of the ‘*Rhodoferrax* sp. BAL47’ cluster.

On the basis of the reconstructed phylogenetic position of strain MWH-C5^T (Fig. 2), the phylogenetic distances from type species of the closest related genera (Supplementary

Fig. 1. Electron microscope image illustrating cell morphology and size of strain MWH-C5^T. Cells of a liquid culture were concentrated by centrifugation and fixed with 2.5 % glutaraldehyde, post-fixed with OsO₄ and embedded with Spurr resin. Ultrathin sections were counterstained with uranyl acetate and lead citrate. The image was obtained by transmission electron microscopy at ×20 000 magnification.

Table S1) and the phenotypic and chemotaxonomic differences between the strain and type species of related genera (Table 3 and Supplementary Table S2), we propose to establish the new genus and species *Limnohabitans curvus* gen. nov., sp. nov. to accommodate strain MWH-C5^T. The genus *Limnohabitans* is a diverse taxon consisting of at least four taxonomically and ecologically well-separated species (Hahn *et al.*, 2010; Kasalický *et al.*, 2010; Šimek *et al.*, 2010b).

Description of *Limnohabitans* gen. nov.

Limnohabitans (Lim.no.ha'bi.tans. Gr. n. *limne* lake; L. part. adj. *habitans* inhabiting; N.L. part. adj. used as a masc. n. *Limnohabitans* lake dweller, referring to the type of ecosystem from which the type species was first isolated).

Aerobic, facultatively anaerobic, chemo-organotrophic, oxidase- and catalase-positive bacteria. Cells are non-pigmented, non-motile, curved rods. Not halotolerant; do not grow at NaCl concentrations >0.5 %. Mesophilic. Major fatty acids (constituting >5 % of total fatty acids) are C_{16:0} and C_{16:1}ω7c/ω6c. The major quinone is ubiquinone Q-8. The only cultured strain was isolated from the water column of freshwater habitats. The genus is affiliated to the class *Betaproteobacteria* and to the family *Comamonadaceae*. The type species is *Limnohabitans curvus*.

Fig. 2. Neighbour-joining tree (Kimura-2 correction) based on almost-complete 16S rRNA gene sequences showing the position of strain MWH-C5^T. The tree includes sequences of the type species of almost all genera currently affiliated with the family Comamonadaceae. Only *Caenibacterium thermophilum*, which was proposed by Lütke-Eversloh *et al.* (2004) to be a later heterotypic synonym of *Schlegelella thermodepolymerans* (the type species of the genus *Schlegelella*), is not shown. Members of the genera *Polynucleobacter*, *Duganella* and *Herbaspirillum* represent reference taxa not affiliated with the family Comamonadaceae. Bootstrap values $\geq 60\%$ (from 1000 iterations) are depicted. The tree was rooted by using archaeal sequences (not shown). Bar, 5 substitutions per 100 nucleotide positions.

Description of *Limnohabitans curvus* sp. nov.

Limnohabitans curvus (cur'vus. L. masc. adj. *curvus* curved or crooked).

Displays the following properties in addition to those described for the genus. Cells are 1.0–1.5 μm long and 0.4–

0.5 μm wide. Colonies grown on NSY agar are unpigmented, circular and convex with smooth surfaces. Growth occurs at 4–34 $^{\circ}\text{C}$ and with 0–0.5% (w/v) NaCl. Assimilates acetate, glycerate, α -ketoglutarate, pyruvate, fumarate, citrate, malate, succinate, gluconate, glucose and mannose. Weak assimilation of several substances is observed (Table 1).

Table 3. Phenotypic and chemotaxonomic characteristics that differentiate strain MWH-C5^T from related genera

The closest related genera were determined from the phylogenetic reconstruction presented in Fig. 2. Data for reference genera were obtained as follows: *Caenimonas* (Ryu *et al.*, 2008); *Curvibacter* (Ding & Yokota, 2004; this study); *Polaromonas* (Kämpfer *et al.*, 2006; Sizova & Panikov, 2007; Jeon *et al.*, 2004; Irgens *et al.*, 1996); *Pseudorhodoferrax* (Bruland *et al.*, 2009); *Rhodoferrax* (Madigan *et al.*, 2000; Hiraishi *et al.*, 1991; Finneran *et al.*, 2003; this study); *Variovorax* (Yoon *et al.*, 2006; Kim *et al.*, 2006; Miwa *et al.*, 2008); *Ramlibacter* (Heulin *et al.*, 2003; Ryu *et al.*, 2008). +, Positive; –, negative; NA, no data available; d, variable; w, weak; SF3, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH).

Character	MWH-C5 ^T	<i>Caenimonas</i>	<i>Curvibacter</i>	<i>Polaromonas</i>	<i>Pseudorhodoferrax</i>	<i>Ramlibacter</i>	<i>Rhodoferrax</i>	<i>Variovorax</i>
Cell morphology	Curved rods	Rods	Slightly curved rods	Rods	Short rods	Pleomorphic [rods to cocci (cysts)]	Curved rods	Oval or rod-shaped
Flagella	None	None	None or polar	None or single, polar	Single, polar	None	Single, polar	Peritrichous
Psychrotolerant growth	+	–	–	d	NA	–	–	–
Oxygen requirement	Facultatively anaerobic	Strictly aerobic	Aerobic or microaerophilic	Obligately aerobic	Aerobic	Aerobic	Facultatively aerobic	Strictly aerobic
Maximum salinity (%)	0.5	NA	3	6	NA	NA	1	7
Carbon source used for growth:								
α-Ketoglutarate	+	NA	+	+	NA	NA	–	NA
Citrate	+	–	+	+	d	–	+	NA
D-Gluconate	+	–	d	d	+	–	d	+
D-Glucose	+	–	d	d	–	–	d	+
Glutamate	–	NA	+	+	NA	NA	d	NA
Glutamine	–	NA	+	NA	NA	NA	–	NA
Glycerol	–	–	+	d	NA	NA	–	+
Glycolate	–	NA	+	NA	NA	NA	–	NA
L-Histidine	–	NA	+	–	NA	NA	–	NA
DL-Lactate	–	NA	+	d	NA	+	+	NA
DL-Malate	+	–	+	d	+	–	+	NA
Malonate	–	+	w	d	NA	NA	–	d
D-Mannose	+	–	d	–	–	–	d	+
N-Acetylglucosamine	–	–	w	NA	NA	–	+	NA
Oxaloacetate	–	NA	+	d	NA	NA	w	NA
L-Phenylalanine	–	NA	+	NA	NA	NA	–	NA
L-Proline	–	NA	+	+	NA	NA	+	NA
Pyruvate	+	NA	+	+	NA	+	+	NA
L-Serine	–	NA	+	d	NA	NA	+	NA
L-Tryptophan	–	–	+	–	NA	NA	w	d
Quinone(s)	Q-8	Q-8	Q-8	Q-8, Q-9	NA	NA	Q-8, RQ-8	Q-8
Major fatty acids	16:0, 16:1	16:0, 18:1, SF3	16:0, 16:1, 18:1	16:0, 16:1, 18:1	16:0, 18:1, SF3	NA	16:0, 16:1	16:0, 16:1, 18:1
Major 3-OH acid(s)	8:0	10:0	8:0	8:0, 10:0*	10:0	NA	8:0	10:0
DNA G + C content (mol%)	55.5	63	62–66	52–63†	69–70	67–70	59–62	66–69
Isolation source(s)	Fresh water	Activated sludge	Well water	Fresh water, Activated sludge or marine	Activated sludge or soil	Soil	Fresh water, activated sludge, marine	Soil

*Only *Polaromonas hydrogenivorans* DSM 17735^T and *Polaromonas naphthalenivorans* CJ2^T contain C_{10:0} 3-OH as the major hydroxylated fatty acid (Jeon *et al.*, 2004; Sizova & Panikov, 2007).

†Only the type strain of *Polaromonas vacuolata* has a G + C content of 52 mol%; the other type strains of *Polaromonas* species for which data are available have G + C contents of 62–64 mol%.

No assimilation of glycerol, glyoxylate, glycolate, oxalate, lactate, malonate, oxaloacetate, arginine, glutamate, glutamine, histidine, phenylalanine, proline, serine, tryptophan,

sorbose, *N*-acetylglucosamine, betaine, spermidine or carnitine. The DNA G + C content of the type strain is 55.5 mol%.

The type strain is MWH-C5^T (=DSM 21645^T =CCUG 56720^T), isolated from Lake Mondsee, Austria.

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