Polynucleobacter cosmopolitanus sp. nov., free-living planktonic bacteria inhabiting freshwater lakes and rivers

Martin W. Hahn,¹ Elke Lang,² Ulrike Brandt,¹ Heinrich Lünsdorf,³ Qinglong L. Wu^{1,4} and Erko Stackebrandt²

Correspondence

Martin W. Hahn
martin.hahn@oeaw.ac.at

¹Institute for Limnology, Austrian Academy of Sciences, Mondseestrasse 9, 5310 Mondsee, Austria

²DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7B, D-38124 Braunschweig, Germany

³Helmholtz Center for Infection Research (formerly GBF), Electron Microscopy Laboratory, Inhoffenstraße 7B, D-38124 Braunschweig, Germany

⁴State Key Laboratory of Lake Science and Environment, Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences, East Beijing Road 73, Nanjing 210008, PR China

Five heterotrophic, aerobic, catalase- and oxidase-positive, non-motile strains were characterized from freshwater habitats located in Austria, France, Uganda, P. R. China and New Zealand. The strains shared 16S rRNA gene similarities of $\geq 99.3\%$. The novel strains grew on NSY medium over a temperature range of 10–35 °C (two strains also grew at 5 °C and one strain grew at 38 °C) and a NaCl tolerance range of 0.0–0.3 % (four strains grew up to 0.5 % NaCl). The predominant fatty acids were C_{16:0}, C_{18:1ω7c}, C_{12:0} 3-OH, and summed feature 3 (including C_{16:1ω7c}). The DNA G+C content of strain MWH-Molso2^T was 44.9 mol%. Phylogenetic analysis of 16S rRNA gene sequences demonstrated that the five new strains formed a monophyletic cluster closely related to *Polynucleobacter necessarius* (96–97 % sequence similarity). This cluster also harboured other isolates as well as environmental sequences which have been obtained from several habitats. Investigations with taxon-specific FISH probes demonstrated that the novel bacteria dwell as free-living, planktonic cells in freshwater systems. Based on the revealed phylogeny and pronounced chemotaxonomic differences to *P. necessarius* (presence of $>7\%$ C_{12:0} 3-OH and absence of C_{12:0} and C_{12:0} 2-OH), the new strains are suggested to represent a novel species, for which the name *Polynucleobacter cosmopolitanus* sp. nov. is proposed. The type strain is MWH-Molso2^T (=DSM 21490^T=CIP 109840^T=LMG 25212^T). The novel species belongs to the minority of described species of free-living bacteria for which both *in situ* data from their natural environments and culture-based knowledge are available.

The genus *Polynucleobacter* was described by K. Heckmann and H.-J. Schmidt for bacterial endosymbionts of freshwater ciliates within the genus *Euplotes*. The species *Polynucleobacter necessarius* was erected for obligate endosymbionts living in the cytoplasm of *Euplotes aediculatus* (Heckmann & Schmidt, 1987). Phylogenetic analysis of the 16S rRNA gene sequence of an endosym-

biotic *P. necessarius* strain in a culture of *E. aediculatus* revealed the affiliation of the strain to the class *Betaproteobacteria* (Springer *et al.*, 1996). Recently, it was demonstrated that the obligate endosymbionts representing the species *P. necessarius* are closely related to obligately free-living strains (Hahn, 2003; Vannini *et al.*, 2007). Therefore, the description of the genus *Polynucleobacter* and its sole species, *P. necessarius*, was emended by the addition of descriptions of free-living strains (Hahn *et al.*, 2009). Due to the very pronounced differences in lifestyle of the obligately endosymbiotic and obligately free-living strains, the placement of these organisms in two subspecies *P. necessarius* subsp. *necessarius* (for endosymbionts of *E. aediculatus* and *Euplotes harpa*) and *P. necessarius* subsp. *asymbioticus* (for obligately free-living strains) was proposed (Hahn *et al.*, 2009). The obligately free-living

Abbreviations: FAMES, fatty acid methyl esters; FISH, fluorescent *in situ* hybridization; Mbp, mega base pairs; ML, maximum-likelihood; NJ, neighbour-joining; OD, optical density; PFGE, pulsed field gel electrophoresis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains MWH-Molso2^T, MWH-CaK1, MWH-VicM1, MWH-TaW3 and MWH-NZ8W13 are AJ550672, AJ550667, AJ550651, AJ550661 and AM110084, respectively.

P. necessarius strains represent aerobic chemo-organotrophic, non-motile bacteria. Cultivation-independent investigations by fluorescent *in situ* hybridization (FISH) with *P. necessarius*-specific probes have demonstrated that the free-living strains possess a planktonic lifestyle and inhabit a large variety of freshwater habitats (Hahn *et al.*, 2005; Wu & Hahn, 2006; Salcher *et al.*, 2008; Alonso *et al.*, 2009).

In previous studies, strains have been isolated or sequences obtained by cultivation independent approaches. Such strains were phylogenetically closely related to *P. necessarius* but did not cluster in trees together with strains of *P. necessarius* (Crump *et al.*, 1999; Zwart *et al.*, 2002; Hahn, 2003; Watanabe *et al.*, 2009). Therefore, these isolates were grouped in separate species-like operational taxonomic units (designated subclusters A, B, C and D), which were defined by phylogenetic reconstructions (Hahn, 2003). In the present study, five strains affiliated to the so-called subcluster D (PnecD) of the *Polynucleobacter* cluster (Hahn, 2003; Wu & Hahn, 2006) were characterized. These strains share many phenotypic, chemotaxonomic and ecological traits with strains of *P. necessarius* subsp. *asymbioticus*, but can be distinguished from them by phylogenetic and chemotaxonomic traits. Based on these

differences, a second species within the genus *Polynucleobacter* is proposed.

Isolation and characterization

All five strains investigated were isolated by using the filtration-acclimatization method (Hahn, 2003; Hahn *et al.*, 2004) with samples obtained from freshwater habitats located in Austria, France, New Zealand, China, and Uganda (Table 1). These habitats were located in temperate, subtropical and tropical climatic zones. Four strains were isolated from the surface waters of lakes, but one strain was isolated from a navigable channel fed with water from the River Loire, France. A recent investigation has demonstrated that strains closely related to the five strains characterized in this study could be directly isolated from agar plates with modified R2A medium (Watanabe *et al.*, 2009).

All strains investigated were routinely grown on NSY medium (Hahn *et al.*, 2004) at a strength of 3 g l^{-1} . All strains also grew on R2A medium and Bacto peptone, at least when concentrations were lowered to 3 g l^{-1} (Hahn, 2003). Growth at different temperatures and growth under anoxic conditions in an anaerobic chamber were examined

Table 1. Characteristic traits of the five novel strains

Strains: 1, MWH-MoIso2^T (=DSM 21490^T); 2, MWH-CaK1 (=DSM 21494); 3, MWH-VicM1 (=DSM 21486); 4, MWH-TaW3 (=DSM 21487); 5, MWH-NZ8W13 (=DSM 21488); 6, *P. necessarius* subsp. *asymbioticus* (QLW-PIDMWA-1^T, MWH-JaK3, MWH-MoK4, MWH-HuW1). All strains show the following characteristics: DAPI stained cells only rarely show nucleoid-like structures, non-motile, catalase- (strain MWH-TaW3 only weakly positive) and oxidase-positive. –, Negative; +, positive; w, weakly positive; ND, not determined.

Characteristic	1	2	3	4	5	6
Origin						
Country	Austria	France	Uganda	PR China	New Zealand	–
Habitat	Lake Mondsee	Canal Roanne à Digoïn	Lake Victoria	Lake Taihu	Lake near Aviemore	–
Climatic zone	Temperate	Temperate	Tropical	Subtropical	Temperate	–
Morphological characteristics						
Cell morphology	Short curved rods	Curved rods	Short curved rods	Short curved rods	Short curved rods	Straight or curved rods
Cell length (µm)	0.5–1.2	0.5–1.4	0.4–1.1	0.5–1.2	0.5–1.2	0.5–2.9
Cell width (µm)	0.3–0.5	0.3–0.5	0.3–0.5	0.3–0.5	0.3–0.5	0.3–0.5
Physiological characteristics						
Min. temp. for growth (°C)*	5 (w)	5	10	5 (w)	5	5
Max. temp. for growth (°C)*	35 (w)	35 (w)	38	38	35	30–35
Max. NaCl concentration (% w/v)	0.5 (w)	0.5 (w)	0.5	0.5	0.3	0.3–0.5
Anaerobic growth	+	+	+	–	w	+ / –
Growth in mineral medium with acetic acid and B12	–	w	w	–	ND	w / –
Other traits						
Genome size (Mbp)	ND†	ND†	ND†	ND†	ND†	2.1–2.5
DNA G + C content (mol%)	44.9	ND	ND	ND	ND	44–46

*Temperature range investigated, 5–38 °C.

†Genome sizes of 1.9–2.1 Mbp have been determined for other strains presumed to belong to *P. cosmopolitanus* sp. nov. (Vannini *et al.*, 2007).

on NSY agar. NaCl tolerance was determined using NSY agar supplemented with different NaCl concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 1.0, 1.25, 1.5, 1.75 and 2.0 %, w/v). The temperature range for growth was tested on NSY agar plates exposed to different temperatures (5, 10, 15, 20, 30, 33, 34, 35, 36 and 38 °C). Two of the four strains tested grew weakly on an inorganic mineral medium (Hahn *et al.*, 2004) supplemented with acetate as the sole carbon and energy source and vitamin B12 (Table 1). However, of three strains tested, all utilized acetate (Table 2) when this substrate was offered in combination with the complex NSY medium (see below). The novel strains shared weak growth performance on medium with a sole carbon source with previously investigated strains of *P. necessarius* subsp.

Table 2. Utilization patterns of three novel strains compared with data reported for strains of free-living *P. necessarius* (Hahn *et al.*, 2009)

Strains: 1, MWH-MoIso2^T; 2, MWH-CaK1; 3, MWH-VicM1; 4, *P. necessarius* subsp. *asymbioticus* (four strains). All strains tested were similar to *P. necessarius* subsp. *asymbioticus* in that they did not utilize glycolate, oxalate or L-serine and that they were able to utilize acetate, pyruvate and succinate. The results for *P. necessarius* subsp. *asymbioticus* are from four strains. Only one symbol is given if all four strains showed a uniform response, otherwise the different responses are presented separated by a slash. –, Negative; +, positive; w, weakly positive.

Assimilation of	1	2	3	4
L-Alanine	+	w	+	w/–
L-Asparagine	–	–	–	w/–
L-Aspartate	–	–	–	+/–
Betaine	–	–	–	w/–
Citric acid	–	+	–	–
L-Cysteine	+	+	+	+/w
Formic acid	–	–	–	w/–
D-Fructose	–	–	–	w/–
D-Fucose	–	–	–	w/–
Fumaric acid	+	+	+	+/w
D-Galactose	w	w	w	w/–
D-Galacturonic acid	+	+	w	w
D-Glucose	–	w	–	w/–
L-Glutamate	–	w	–	+/–
Glyoxylic acid	–	w	–	w/–
Levulinic acid	w	–	–	w/–
D-Lyxose	–	–	–	w/–
Malic acid	+	+	+	+/w
Malonic acid	–	+	+	+/–
D-Mannose	–	w	–	w/–
Oxaloacetic acid	+	+	+	+/–
Propionic acid	w	+	+	+/–
D-Sorbitol	–	–	–	w/–
Thiosulfate	+	–	+	+/–
Urea	+	–	+	+/–

asymbioticus (Hahn *et al.*, 2009). Due to this weak performance and so that the strains could be compared with the previously investigated strains of the genus *Polynucleobacter*, tests on the utilization of various organic compounds were not performed with sole substrate media or by using commercially available test kits. Instead, experiments were performed by applying the method previously used for strains of *P. necessarius* (Hahn *et al.*, 2009). Briefly, growth enabled by the utilization of a specific substrate was determined by comparison of the optical density (OD) established in liquid one-tenth-strength NSY medium (0.3 g l^{–1}) with and without 0.5 g l^{–1} test substance. OD differences of <10 %, of 10–50 % and of >50 % of the OD established on the medium without the test substance were scored after 10 days of growth as no utilization (–), weak utilization (w) and good utilization (+), respectively.

Sequencing and phylogenetic analyses of the 16S rRNA genes were performed as described previously (Hahn, 2003; Hahn *et al.*, 2005). Neighbour-joining trees were calculated by using MEGA4 software (Tamura *et al.*, 2007) and maximum-likelihood trees were generated by using the RaxML web server (Stamatakis *et al.*, 2008). Morphological characterization of the proposed type strain, strain MWH-MoIso2^T, by electron microscopy was performed as described previously (Yakimov *et al.*, 1998). The G+C content of DNA was determined according to the method of Tóth *et al.* (2008). Fatty acid methyl esters (FAMES) were obtained as described previously (Kämpfer & Kroppenstedt, 1996) and separated by a gas chromatograph (model 6890, Hewlett Packard). Peaks were automatically computed using the Microbial Identification standard software package (Sasser, 1990).

The five strains investigated were similar to strains of *P. necessarius* subsp. *necessarius* in that they produced only low yields of biomass in relatively rich media (Hahn *et al.*, 2009). Biomass yields were usually one order of magnitude lower than found for other members of the family *Burkholderiaceae*. The results of the chemotaxonomic and phenotypic characterization of the novel strains are presented in Tables 1, 2 and 3. Experiments on the utilization capabilities of three of the novel strains (MWH-MoIso2^T, MWH-CaK1 and MWH-VicM1) resulted only in 68 % of the 37 tested substrates giving identical results (three categories; i.e. no, weak and good utilization) (Table 2). Testing of four strains of *P. necessarius* subsp. *asymbioticus* with the same substrates yielded identical results for only 25 % of the tested substrates (Hahn *et al.*, 2009). Despite the relatively large number of substrates tested, no discriminating traits were found between the three novel strains and the four strains of *P. necessarius* subsp. *asymbioticus* (Table 2). Therefore, the utilization patterns of the remaining two novel strains, i.e. MWH-TaW3 and MWH-NZ8W13, were not examined. All of the novel strains grew in NSY medium as curved rods with small cell sizes (Fig. 1). Doubling times for growth in liquid NSY medium at 20 °C were 4.5, 5.7, and 5.9 h for strains

Table 3. Whole-cell fatty acid contents of the novel strains and *P. necessarius* subsp. *asymbioticus* QLW-P1DMWA-1^T

Strains: 1, MWH-MoIso2^T; 2, MWH-CaK1; 3, MWH-VicM1; 4, MWH-TaW3; 5, MWH-NZ8W13; 6, *P. necessarius* subsp. *asymbioticus* QLW-P1DMWA-1^T (Hahn *et al.*, 2009). Values are percentages of the summed fatty acids named in the peak library of the MIDI system (contents >0.2 %). Strains were grown on R2A agar plates for 3–5 days at 28 °C.

Fatty acid	1	2	3	4	5	6
Saturated						
C _{12:0}	—	—	—	—	—	3.4
C _{14:0}	0.7	0.6	0.9	2.3	1.3	0.9
C _{15:0}	0.2	—	0.2	—	—	0.3
C _{16:0}	15.4	11.0	11.1	14.9	13.5	22.2
C _{18:0}	0.8	0.7	0.5	1.1	1.1	1.2
Unsaturated						
C _{14:1} ω5c	0.6	0.4	0.6	—	1.1	—
C _{16:1} ω5c	0.3	0.5	0.5	1.1	—	0.9
C _{17:1} ω6c	0.5	0.3	0.7	—	—	—
C _{18:1} ω9c	0.3	—	0.2	2.0	—	—
C _{18:1} ω7c	28.7	37.5	35.6	33.7	38.1	12.9
C _{18:1} ω5c	0.2	0.2	0.2	—	—	—
11 Methyl-C _{18:1} ω7c	3.7	2.2	0.4	1.0	2.3	3.1
Hydroxylated						
C _{12:0} 2-OH	—	—	—	—	—	2.5
C _{12:0} 3-OH	11.1	9.9	7.1	11.2	10.5	—
Summed features						
1 (C _{12:0} ALDE?)	0.1	—	0.2	—	—	0.4
2 (including C _{14:0} 3-OH)	0.6	0.7	3.9	0.8	0.6	9.6
3 (including C _{16:1} ω7c)	34.7	35.4	36.5	32.0	31.5	41.3
7 (including C _{19:1} ω6c)	1.5	0.7	0.6	—	—	0.4

MWH-VicM1, MWH-MoIso2^T, and MWH-CaK1, respectively. All three novel strains grew under these conditions with mean cell volumes of <0.1 μm³ (Hahn, 2003).

The major components of the whole-cell fatty acids were C_{16:1}ω7c, C_{18:1}ω7c, C_{16:0} and C_{12:0} 3-OH (Table 3). The fatty acid pattern of strains of *P. necessarius* was dominated by the same non-hydroxylated acids (Table 3 and Hahn *et al.*, 2009). The occurrence of several other fatty acids in minor amounts was shared by *P. necessarius* and all of the novel strains studied. Minor fatty acids were C_{14:0}, C_{18:0}, and 11 methyl-C_{18:1}ω7c. The novel strains could be clearly distinguished from strains of *P. necessarius* by the absence of C_{12:0} and C_{12:0} 2-OH and the presence of high amounts (>7 %) of C_{12:0} 3-OH. In all *P. necessarius* strains studied so far, no C_{12:0} 3-OH has been detected, and the C_{12:0} and C_{12:0} 3-OH contents were higher than 3 % and 1 %, respectively (Hahn *et al.*, 2009). Furthermore, MIDI summed feature 2, including C_{14:0} 3-OH was present in the novel strains in much lower amounts (<4 %) than in strains of *P. necessarius* (>9 %). The content of fatty acid C_{18:1}ω7c was >28 % in the novel strains but was <21 % in strains of *P. necessarius*.

Fig. 1. Transmission electron microscope survey view of Pt-C shadow-cast cells of strain MWH-MoIso2^T. The arrowheads indicate the direction of shadow casting. Bar, 500 nm.

The genome sizes of the five novel strains were not determined, however, the genome sizes of strains tentatively assigned to the proposed taxon range from 1.9 to 2.1 Mbp (Vannini *et al.*, 2007), which may indicate that the novel strains possess smaller genome sizes than strains of *P. necessarius* subsp. *asymbioticus*.

Phylogeny

Phylogenetic analyses of the 16S rRNA genes of the five novel strains revealed clustering of the strains in a monophyletic clade representing a sibling taxon of *P. necessarius* (Fig. 2). The minimum 16S rRNA sequence similarity of the five strains was 99.3 % (sequence stretches ranging from *Escherichia coli* positions 28–1542 were analysed). The sequence similarity between the 16S rRNA genes of strain MWH-MoIso2^T and the type strain of *P. necessarius* subsp. *asymbioticus* and a sequence representing *P. necessarius* subsp. *necessarius* E24 were 97.3 % and 96.8 %, respectively. BLAST searches with the 16S rRNA gene sequences of the novel strains and subsequent phylogenetic analyses revealed that the five novel strains formed a tight monophyletic cluster together with 99 cultured and uncultured strains (data not shown). This cluster was phylogenetically identical with the previously characterized ‘Polynucleobacter subcluster D’ (Hahn, 2003) for which a minimum within-cluster sequence similarity of 99.1 % has been reported (Wu & Hahn, 2006). Phylogenetic analysis of the 16S–23S ITS sequences confirmed the separate clustering of the five novel strains and previously investigated strains of *P. necessarius* and demonstrated tight clustering of the sequences within both groups (Fig. 3).

Genotypic traits

All members of the ‘Polynucleobacter subcluster D’ possessed the oligonucleotide sequence 5′-AA(T/G)CCCT(A/T)AGGGGGAAA-3′ within the 16S rRNA gene (*E. coli* positions 181–197). In almost all cases, the

Fig. 2. Neighbour-joining (NJ) phylogenetic tree based on almost complete 16S rRNA gene sequences, showing the phylogenetic position of the five novel strains within the family *Burkholderiaceae*. A maximum-likelihood (ML) tree (not shown) calculated with the same sequence set differed from the NJ tree in the positions of *Bordetella pertussis* and *Limnobacter thiooxidans*. Bootstrap values obtained by the NJ (first value) and the ML algorithm (second value) are shown if at least one value was >60%. PnecA, 'Polynucleobacter subcluster A'; PnecB, 'Polynucleobacter subcluster B'. Bar, 0.02 substitutions per nucleotide position.

presence of this sequence could be determined by fluorescent *in situ* hybridization of whole cells by using a mixture of the fluorescently labelled oligonucleotide probes PnecD1-181 and PnecD2-181 (Hahn *et al.*, 2005). The sequences of these two probes differ in the second variable position (*E. coli* position 188) of the diagnostic sequence, but not in the first variable position (*E. coli* position 183). Analysis of 104 sequences forming the phylogenetic cluster

representing the proposed new taxon, *Polynucleobacter cosmopolitanus* sp. nov., demonstrated that only one of the novel strains differed from the probe sequences in the first variable position of the diagnostic sequence. Besides the FISH probe, a reverse line blot hybridization probe (PolynucD123-144) for the detection of the proposed new taxon in environmental samples has been developed and applied successfully (Wu *et al.*, 2007).

Fig. 3. Neighbour-joining (NJ) phylogenetic tree based on complete 16S-23S ITS sequences (approx. length 500 bp). The five novel strains are shown in bold type. Sequences of strains affiliated with the PnecA and PnecB groups were included in this analysis. A ML tree (not shown) calculated with the same sequence set confirmed the tight clustering of the *P. cosmopolitanus* sp. nov. strains, as well as the tight clustering of the *P. necessarius* strains (bootstrap value 100%). Bootstrap values obtained by the NJ (first value) and the ML algorithm (second value) are shown if at least one value was >60%. Bar, 0.05 substitutions per nucleotide position.

Biogeography of *P. cosmopolitanus* sp. nov.

Strains affiliated to the phylogenetic cluster represented by *P. cosmopolitanus* sp. nov. were isolated from freshwater habitats located in Eurasia, South America, Africa and Oceania (Hahn, 2003; M. W. Hahn, Q. L. Wu, M. Pöckl, K. Simek & U. Brandt, unpublished data). A strain has even been isolated from a freshwater habitat located on the Hawaiian Archipelago (strain MWH-Haw2W10a, GenBank accession no. AM110098). Recently, many strains have been isolated from various lakes located in Japan (Watanabe *et al.*, 2009). Furthermore, cultivation-independent investigations have indicated the presence of this novel taxon at various locations, including Northern America (Crump *et al.*, 1999; Zwart *et al.*, 2002; Simpson *et al.*, 2004; Crump & Hobbie, 2005), a river estuary of northern Taiwan (Liao *et al.*, 2007) and in running water in Germany (Beier *et al.*, 2008). The phylogenetic group representing the proposed novel species has even been detected by cultivation-independent methods in a pond located at the Tibetan Plateau at an altitude of 3210 m above sea level (Wu *et al.*, 2006). The geographical distribution of habitats colonized by strains phylogenetically belonging to the proposed novel species clearly indicates a cosmopolitan distribution of the new taxon over all continents and all climatic zones.

Ecology of *P. cosmopolitanus* sp. nov.

Members of the phylogenetic cluster represented by the proposed novel species have been isolated and detected in the water columns of running and stagnant freshwater habitats such as ponds, lakes and streams. A recent investigation compared the salinity tolerance determined by ecophysiological experiments with six pure culture strains of the proposed new taxon with the occurrence of the taxon along a salinity gradient of sixteen lakes (Wu *et al.*, 2006). This gradient ranged from 0.2 g l⁻¹ (freshwater) to 222 g l⁻¹ salinity (hypersaline). While the novel strains tested grew in the laboratory up to maximum salinities of 3.6–5.6 g NaCl l⁻¹ (note that the salinity tolerance considers the total amount of dissolved salt, while the NaCl tolerance mentioned above considers exclusively the NaCl added to the medium but not the other salts contained in the medium), the taxon was detected *in situ* by cultivation-independent methods exclusively in habitats with salinities <1 g l⁻¹. However, the new taxon has also been detected in a metagenomic study of two estuaries and a salinity of 3.5 g l⁻¹ (oligosaline) was reported for one of these estuaries (Chesapeake Bay, MD, USA) (Shaw *et al.*, 2008). The detection of the novel taxon has never been reported at terrestrial or offshore marine sites. All these observations indicate that the new taxon is mainly found in the water column of freshwater habitats, but may also occur in oligosaline habitats, and that it is absent from eusaline (oceans) and hypersaline environments.

The new taxon could be detected by the FISH probes PnecD1-181/PnecD2-181 in several habitats (Wu & Hahn, 2006; Q. L. Wu and M. W. Hahn, unpublished data). In a

survey of 60 freshwater lakes, the proposed new taxon was detected by FISH in 15 habitats. Usually, the level of detection ranged from 0% (below the detection limit by FISH) to 0.9% of the total prokaryotic numbers. The highest recorded relative abundance of 8.1% of total prokaryotic cells was observed in a freshwater pond located on the Tibetan Plateau (see above). All cells detected by the probes in the various habitats investigated possessed a curved rod morphology. In a seasonal study on the shallow eutrophic Lake Taihu located in subtropical China, two population peaks, with 0.3% relative abundance (equalling 1 × 10⁴ cells ml⁻¹) could be observed in winter and late spring (Wu & Hahn, 2006).

In a study focusing on the vulnerability of strains affiliated with the new taxon to predation by bacterivorous flagellates, a relatively low vulnerability was observed (Boenigk *et al.*, 2004). Predation by such flagellates in general represents a major mortality factor for planktonic freshwater bacteria (Hahn & Höfle, 2001). The low vulnerability found was explained by the small cell sizes of the strains (<0.1 µm³, ultramicrobacteria); however, a complete resistance to predation was not observed. Thus, factors determining the occurrence and relative abundance of the novel taxon in various freshwater habitats remain to be revealed.

The results from the phylogenetic analysis and chemotaxonomic investigations demonstrated pronounced differences between the five novel strains and strains of *P. necessarius* (Figs 2 and 3, Table 4). Furthermore, the 16S rRNA gene sequence similarities of ≤97.3% between the type strain of the proposed novel species and the strains

Table 4. Characteristics that discriminate *P. cosmopolitanus* sp. nov. and *P. necessarius* subsp. *asymbioticus*

Taxa: 1, *P. cosmopolitanus* sp. nov.; 2, *P. necessarius* subsp. *asymbioticus*. Note that only an incomplete phenotypic and chemotaxonomic description of *P. necessarius* subsp. *necessarius* is available because of its obligately endosymbiotic lifestyle. ND, Not detected.

Characteristic	1	2
Fatty acid		
C _{12:0}	ND	>3%*
C _{18:1ω7c}	>28%*	<21%*
C _{12:0} 2-OH	ND	>1%*
C _{12:0} 3-OH	>7%*	ND
Summed feature 2 (including C _{14:0} 3-OH)	<4%*	>9%*
Probe PnecC-16S-445†	—	+
Probe PnecD-181‡	+	—

*Percentages of the summed fatty acids named in the peak library of the MIDI system.

†Hahn *et al.* (2005).

‡Equal mixture of probes PnecD1-181 and PnecD2-181 (Hahn *et al.*, 2005).

representing the two subspecies of *P. necessarius* suggest that strain MWH-MoIso2^T represents a novel species (Stackebrandt & Ebers, 2006). The coherence of the taxon represented by the five novel strains could not be checked by DNA–DNA hybridization experiments due to the limitations in the production of sufficient amounts of DNA. However, the phylogenetic analysis of the 16S rRNA gene sequences and of the much less conserved 16S–23S ITS sequences of *Polynucleobacter* spp. strains (Fig. 3) strongly indicate that the five novel strains represent a coherent taxon. The latter phylogenetic marker is much less conserved than 16S rRNA gene sequences, thus significant intra-taxon heterogeneity would be expected to be indicated by this marker. It is suggested that strain MWH-MoIso2^T and the four other new strains represent a novel species for which the name *Polynucleobacter cosmopolitanus* sp. nov. is proposed.

Description of *Polynucleobacter cosmopolitanus* sp. nov.

Polynucleobacter cosmopolitanus (cos.mo.po.li'ta.nus. Gr. adj. *kosmopolites* an inhabitant of the world, a cosmopolitan; N.L. masc. adj. *cosmopolitanus* cosmopolitan).

Curved, non-motile rods, 0.5–1.4 µm in length and 0.3–0.5 µm in width. Chemo-organotrophic, aerobic strains, at least some of the strains are able to grow anaerobically. Can be cultivated on NSY, R2A, and Bacto peptone media, and some other complex media at concentrations of ≤3 g l⁻¹. Colonies grown on NSY agar are unpigmented, circular and convex with smooth surfaces and reach a diameter of 1 mm after 5–10 days at 20–24 °C. Growth occurs at 10–35 °C, four strains also grow at 5 °C and two strains are able to grow at 38 °C. Grows without NaCl. Maximum NaCl concentration tolerated is strain dependent and ranges from 0.3 to 0.5 % (w/v). Utilizes acetate, pyruvate, propionate, oxaloacetate, malate, succinate, fumarate, D-galacturonic acid, D-galactose, L-cysteine and L-alanine when these substrates are provided in a medium containing low amounts of NSY. None of the strains utilize formate, glycolate, oxalate, D-lyxose, D-fructose, D-fucose, D-sorbitol, L-aspartate, L-serine, L-asparagine or betaine. Major cellular fatty acids are C_{18:1ω7c}, C_{16:0} and C_{12:0} 3-OH. No 2-hydroxylated compounds are detected. Fractions of natural populations pass through 0.2 µm filters. Can be distinguished from *P. necessarius* by using the *P. necessarius*-specific FISH probe PnecC-16S-445 (Hahn *et al.*, 2009), as well as by differences in fatty acid profiles (Table 3). In contrast to *P. necessarius*, exclusively free-living strains and no endosymbiotic strains are reported, however, the existence of endosymbiotic strains cannot be completely excluded at present. Tentatively, all strains possessing the oligonucleotide sequence 5'-AA(T/G)CCCT(A/T)AGG-GGAAA-3' (*E. coli* positions 181–197) within the 16S rRNA gene shall be assigned to the proposed species. In almost all cases (with one known exception), the presence of this diagnostic sequence can be determined by FISH

with a mixture of the two probes PnecD1-181 and PnecD2-181 (Hahn *et al.*, 2005).

The type strain, MWH-MoIso2^T (=DSM 21490^T=CIP 109840^T=LMG 25212^T), was isolated from the water column (pelagic zone) of Lake Mondsee located in the Salzkammergut Lake District in Austria (Hahn, 2003). The DNA G + C content of the type strain is 44.9 %. Four other reference strains are available: MWH-CaK1 (=DSM 21494), MWH-VicM1 (=DSM 21486); MWH-TaW3 (=DSM 21487) and MWH-NZ8W13 (=DSM 21488).

Acknowledgements

We are grateful to R. M. Kroppenstedt for performing the fatty acid analyses, to P. Schumann for determining the G + C content and to M. Kopitz and M. Pöckl for technical assistance. This study was supported by the Austrian Science Fund (Projects P15655 and P19853 granted to M. W. H.).

References

- Alonso, C., Zeder, M., Piccini, C., Conde, D. & Pernthaler, J. (2009). Ecophysiological differences of betaproteobacterial populations in two hydrochemically distinct compartments of a subtropical lagoon. *Environ Microbiol* **11**, 867–876.
- Beier, S., Witzel, K. P. & Marxsen, J. (2008). Bacterial community composition in central European running waters examined by temperature gradient gel electrophoresis and sequence analysis of 16S rRNA genes. *Appl Environ Microbiol* **74**, 188–199.
- Boenigk, J., Stadler, P., Wiedroither, A. & Hahn, M. W. (2004). Strain-specific differences in the grazing sensitivities of closely related ultramicrobacteria affiliated with the *Polynucleobacter* cluster. *Appl Environ Microbiol* **70**, 5787–5793.
- Crump, B. C. & Hobbie, J. E. (2005). Synchrony and seasonality in bacterioplankton communities of two temperate rivers. *Limnol Oceanogr* **50**, 1718–1729.
- Crump, B. C., Armbrust, E. V. & Baross, J. A. (1999). Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean. *Appl Environ Microbiol* **65**, 3192–3204.
- Hahn, M. W. (2003). Isolation of strains belonging to the cosmopolitan *Polynucleobacter necessarius* cluster from freshwater habitats located in three climatic zones. *Appl Environ Microbiol* **69**, 5248–5254.
- Hahn, M. W. & Höfle, M. G. (2001). Grazing of protozoa and its effect on populations of aquatic bacteria. *FEMS Microbiol Ecol* **35**, 113–121.
- Hahn, M. W., Stadler, P., Wu, Q. L. & Pöckl, M. (2004). The filtration-acclimatization method for isolation of an important fraction of the not readily cultivable bacteria. *J Microbiol Methods* **57**, 379–390.
- Hahn, M. W., Pöckl, M. & Wu, Q. L. (2005). Low intraspecific diversity in a *Polynucleobacter* subcluster population numerically dominating bacterioplankton of a freshwater pond. *Appl Environ Microbiol* **71**, 4539–4547.
- Hahn, M. W., Lang, E., Brandt, U., Wu, Q. L. & Scheuerl, T. (2009). Emended description of the genus *Polynucleobacter* and the species *Polynucleobacter necessarius* and proposal of two subspecies, *P. necessarius* subsp. nov. and *P. necessarius* subsp. *asymbioticus* subsp. nov. *Int J Syst Evol Microbiol* **59**, 2002–2009.
- Heckmann, K. & Schmidt, H. J. (1987). *Polynucleobacter necessarius* gen. nov., sp. nov., an obligately endosymbiotic bacterium living in the cytoplasm of *Euplotes aediculatus*. *Int J Syst Bacteriol* **37**, 456–457.

- Kämpfer, P. & Kroppenstedt, R. M. (1996).** Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.
- Liao, P. C., Huang, B. H. & Huang, S. (2007).** Microbial community composition of the Danshui river estuary of northern Taiwan and the practicality of the phylogenetic method in microbial barcoding. *Microb Ecol* **54**, 497–507.
- Salcher, M. M., Pernthaler, J., Zeder, M., Psenner, R. & Posch, T. (2008).** Spatio-temporal niche separation of planktonic *Betaproteobacteria* in an oligo-mesotrophic lake. *Environ Microbiol* **10**, 2074–2086.
- Sasser, M. (1990).** *Identification of bacteria by gas chromatography of cellular fatty acids*, MIDI Technical Note 101. Newark, DE: MIDI Inc.
- Shaw, A. K., Halpern, A. L., Beeson, K., Tran, B., Venter, J. C. & Martiny, J. B. (2008).** It's all relative: ranking the diversity of aquatic bacterial communities. *Environ Microbiol* **10**, 2200–2210.
- Simpson, J. M., Santo Domingo, J. W. & Reasoner, D. J. (2004).** Assessment of equine fecal contamination: the search for alternative bacterial source-tracking targets. *FEMS Microbiol Ecol* **47**, 65–75.
- Springer, N., Amann, R., Ludwig, W., Schleifer, K. H. & Schmidt, H. (1996).** *Polynucleobacter necessarius*, an obligate bacterial endosymbiont of the hypotrichous ciliate *Euplotes aediculatus*, is a member of the beta-subclass of *Proteobacteria*. *FEMS Microbiol Lett* **135**, 333–336.
- Stackebrandt, E. & Ebers, J. (2006).** Taxonomic parameters revisited: tarnished gold standard. *Microbiol Today* **33**, 152–155.
- Stamatakis, A., Hoover, P. & Rougemont, J. A. (2008).** A rapid bootstrap algorithm for the RAxML web servers. *Syst Biol* **57**, 758–771.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007).** MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Tóth, E. M., Kéki, Z., Homonnay, Z. G., Borsodi, A. K., Márialigeti, K. & Schumann, P. (2008).** *Nocardioides daphniae* sp. nov., isolated from *Daphnia cucullata* (Crustacea: Cladocera). *Int J Syst Evol Microbiol* **58**, 78–83.
- Vannini, C., Pöckl, M., Petroni, G., Wu, Q. L., Lang, E., Stackebrandt, E., Schrällhammer, M., Richardson, P. M. & Hahn, M. W. (2007).** Endosymbiosis in statu nascendi: close phylogenetic relationship between obligately endosymbiotic and obligately free-living *Polynucleobacter* strains (*Betaproteobacteria*). *Environ Microbiol* **9**, 347–359.
- Watanabe, K., Komatsu, N., Ishii, Y. & Negishi, M. (2009).** Effective isolation of bacterioplankton genus *Polynucleobacter* from freshwater environments grown on photochemically degraded dissolved organic matter. *FEMS Microbiol Ecol* **67**, 57–68.
- Wu, Q. L. & Hahn, M. W. (2006).** Differences in structure and dynamics of *Polynucleobacter* communities in a temperate and a subtropical lake revealed at three phylogenetic levels. *FEMS Microbiol Ecol* **57**, 67–79.
- Wu, Q. L., Schauer, M., Kamst-Van Agterveld, M. P., Zwart, G. & Hahn, M. W. (2006).** Bacterioplankton community composition along a salinity gradient of sixteen high-mountain lakes located on the Tibetan Plateau, China. *Appl Environ Microbiol* **72**, 5478–5485.
- Wu, Q. L., Zwart, G., Wu, J., Kamst-van Agterveld, M. P., Liu, S. & Hahn, M. W. (2007).** Submersed macrophytes play a key role in structuring bacterioplankton community composition in the large, shallow, subtropical Taihu Lake, China. *Environ Microbiol* **9**, 2765–2774.
- Yakimov, M. M., Golyshin, P. N., Lang, S., Moore, E. R. W., Abraham, W.-R., Lünsdorf, H. & Timmis, K. N. (1998).** *Alcanivorax borkumensis* gen. nov., sp. nov., a new hydrocarbon-degrading and surfactant producing marine bacterium. *Int J Syst Bacteriol* **48**, 339–348.
- Zwart, G., Crump, B. C., Kamst-van Agterveld, M. P., Hagen, F. & Han, S.-K. (2002).** Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquat Microb Ecol* **28**, 141–155.