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Polynucleobacter difficilis sp. nov., a planktonic freshwater bacterium affiliated with subcluster B1 of the genus *Polynucleobacter*

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Abstract

Strain AM-8B5^T, isolated from Lake Sevan in Armenia, was characterized phenotypically, chemotaxonomically and phylogenetically. This chemo-organoheterotrophic, aerobic, facultatively anaerobic, catalase- and oxidase-positive, non-motile strain grew on NSY medium at NaCl concentrations of 0.0–0.2 % (w/v) and at 4–30 °C. Whole-cell fatty acids were dominated by summed feature 3 (including $C_{16:1}\omega7c$ and iso-$C_{15:0}$ 2-OH), $C_{16:0}$ and $C_{18:1}\omega7c$. $C_{12:0}$ 2-OH and $C_{16:1}$ 2-OH were the only hydroxylated fatty acids detected. Phylogenetic analysis as well as phenotypic and chemotaxonomic similarities indicated that the novel isolate was affiliated with the genus *Polynucleobacter*. 16S rRNA gene similarity values with the four previously described *Polynucleobacter* species ranged from 96.2 to 98.7 %. DNA–DNA hybridization experiments showed that the isolate did not belong to any of the previously described *Polynucleobacter* species. The isolate could be distinguished from all previously established *Polynucleobacter* species based on chemotaxonomic and phenotypic traits. The bacterium possessed a free-living lifestyle and represents a group of bacteria inhabiting the water column of many freshwater lakes. Based on the revealed phylogeny, and chemotaxonomic and phenotypic differences to previously described *Polynucleobacter* species, it is proposed that the isolate represents a novel species, *Polynucleobacter difficilis* sp. nov.; the type strain is AM-8B5^T (=DSM 22349^T=CIP 110078^T).

K. Heckmann and H.-J. Schmidt established the genus *Polynucleobacter* for obligately endosymbiotic bacteria living in cells of several species of ciliates affiliated with the genus *Euplotes* (Hypotrichia) and they described the species *Polynucleobacter necessarius* to

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accommodate obligate endosymbionts of *Euplotes aediculatus* (Heckmann & Schmidt, 1987). None of these obligate endosymbionts could be cultivated as pure cultures (Heckmann & Schmidt, 1987; Vannini *et al.*, 2007). However, Springer *et al.* (1996) cloned and sequenced the 16S rRNA gene of an endosymbiotic *P. necessarius* strain obligately associated with *E. aediculatus*. Their phylogenetic analyses of the obtained ribosomal sequence revealed the affiliation of the genus *Polynucleobacter* with the *Betaproteobacteria*. Investigations by cultivation-independent methods (e.g. Hiorns *et al.*, 1997; Zwart *et al.*, 2002; Burkert *et al.*, 2003; Crump & Hobbie, 2005) and cultivation methods (Hahn, 2003; Watanabe *et al.*, 2009) revealed a monophyletic genus-like group of bacteria including the previously characterized endosymbiotic *P. necessarius*. This large and diverse group of bacteria was subdivided in preliminary operational taxonomic units based on phylogenetic criteria (Hahn, 2003; Wu & Hahn, 2006a). The proposed operational taxonomic units were designated subclusters A (PnecA), B1 (PnecB1), B2 (PnecB2), C (PnecC) and D (PnecD). Furthermore, it was revealed that, in addition to obligate endosymbionts, this monophyletic group of bacteria also contains free-living strains (Hahn, 2003; Vannini *et al.*, 2007) that thrive in the water column of freshwater habitats as planktonic bacteria (Hahn *et al.*, 2005). Consequently, the descriptions of the genus *Polynucleobacter* and the species *P. necessarius* were emended by adding descriptions of free-living strains (Hahn *et al.*, 2009). Strains closely related to the endosymbionts of *E. aediculatus* but differing in lifestyle were separated into the two subspecies *P. necessarius* subsp. *necessarius* (obligate endosymbionts) and *P. necessarius* subsp. *asymbioticus* (obligately free-living strains) (Hahn *et al.*, 2009). Recently, strains affiliated with subclusters PnecD, PnecA and PnecB2 were described as the novel species *P. cosmopolitanus* (Hahn *et al.*, 2010), *P. rarus* (Hahn *et al.*, 2011a) and *P. acidiphobus* (Hahn *et al.*, 2011b), respectively, whereas subcluster PnecC is represented by the previously established species *P. necessarius* (Heckmann & Schmidt, 1987; Hahn *et al.*, 2009). Here, we have characterized isolate AM-8B5^T, which represents the only strain cultivated so far that is affiliated with subcluster PnecB1. This strain has a free-living planktonic lifestyle, in common with all other *Polynucleobacter* strains that have been cultivated in pure culture, but cultivation and maintenance of this strain were more difficult than for any previously isolated *Polynucleobacter* strain.

Isolation and characterization

Strain AM-8B5^T was isolated from the water column of eutrophic Lake Sevan located in Armenia. The lake possesses a surface area of 1416 km^2 and a maximum depth of 98.7 m, and is located at an altitude of 1915 m (Hovhannissian, 1994). The geographical coordinates of the sampling site (station #4) are 40° 29′ 35.50″ N 45° 11′ 31.34″ E. The strain was isolated from a water sample taken from a depth of 60 m close to the bottom of the lake on 13 November 2006. At the time of sampling, the lake was still thermally stratified and the thermocline was located at a depth of 25 m. The sampled hypolimnic water was characterized by a pH of 8.7, conductivity of 110 μS cm^{-1}, oxygen concentration of 7.17 mg l^{-1} (about 56 % saturation), and a temperature of 4.2 °C. The total number of bacteria in the water sample, determined by epifluorescent microscopy and direct counting, was 1.9×10^6 cells ml^{-1}.

The strain was isolated and cultivated by using the filtration acclimatization method and NSY medium (Hahn *et al.*, 2004). Strain AM-8B5^T could be maintained on R2A (Remel) or NSY medium with a strength of 3 g l^{-1}; however, better growth was observed on modified NSY medium enriched with 1 g L-cysteine l^{-1} and 1 g pyruvate l^{-1}. However, even when using optimized medium, cultivation and maintenance of strain AM-8B5^T remained delicate and more difficult than for any other of the >200 *Polynucleobacter* strain cultivated by us so far. Currently, it is not known which physiological trait of the strain makes cultivation more complicated than for the other *Polynucleobacter* strains. Growth at different temperatures and growth under anoxic conditions in an anaerobic chamber were examined on NSY agar or on NSY agar supplemented with nitrate (0.8 mM). NaCl tolerance was determined using NSY agar supplemented with various 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 supporting growth was tested on standard NSY agar plates exposed to different temperatures (4, 15, 20, 25, 30 and 35 °C). Utilization of various substrates was investigated in the same way as for previously described *Polynucleobacter* species (Hahn *et al.*, 2009, 2010, 2011a, b). Briefly, growth enabled by utilization of a specific substrate was determined by comparison of OD at 575 nm established in liquid one-tenth-strength NSY medium (0.3 g l^{-1}) with and without 0.5 g test substrate l^{-1}. Differences of <10 %, 10–50 % and >50 % of the OD with and without test substrate were scored after 10 days of growth as no utilization (−), weak utilization (w) and good utilization (+), respectively.

Sequencing of the 16S rRNA gene was performed as described previously (Hahn, 2003; Hahn *et al.*, 2005). Sequence similarity values were determined by using the software EzTaxon (Chun *et al.*, 2007). Phylogenetic analyses with neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) algorithms were performed by using the software MEGA versions 4 and 5 (Tamura *et al.*, 2007). For calculation of NJ trees, the Tamura–Nei substitution model and a gamma distribution with five categories were used. For ML tree calculation, the Tamura–Nei+G+I model was used as recommended by Model Test.

For analysis of the whole-cell fatty acid composition, the strain was grown on R2A agar plates for 7–9 days at 25 °C. During this time, the agar surface was kept moist with liquid R2A medium. The physiological age of the biomass harvested for fatty acid analysis was standardized as far as possible by observing growth development during incubation of the plates and choosing the moment of harvest according to growth state. Fatty acid methyl esters were obtained as described previously (Kämpfer & Kroppenstedt, 1996) and separated by GC (model 6890; Hewlett Packard). Peaks were computed automatically using the Microbial Identification systems MIDI 4.5 and 6.1 (Sasser, 1990). Chromatographic peaks were evaluated using the database TSBA 40. DNA for DNA–DNA reassociation experiments was obtained by disrupting cells by using a French pressure cell (Thermo Spectronic) and purifying DNA in the crude lysate by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-

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thermostatted 6×6 multicell changer and a temperature controller with *in situ* temperature probe (Varian).

Phenotypic and chemotaxonomic traits

The results of the phenotypic and chemotaxonomic characterization of strain AM-8B5^T are presented in Tables 1 and 2. The strain differed from *P. necessarius* subsp. *asymbioticus*, *P. cosmopolitanus*, *P. rarus* and *P. acidiphobus* strains in its inability to utilize acetate (Table 1).

The whole-cell fatty acid pattern of strain AM-8B5^T was typical for members of the genus *Polynucleobacter* isolated so far. The pattern was dominated by feature 3 of the MIDI system ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{18:1}\omega7c$ and $C_{16:0}$ (Table 2). As no other branched fatty acid components were detected, we concluded that the peak of feature 3 represented mainly $C_{16:1}\omega7c$ (Table 2). In comparison to other *Polynucleobacter* species, the novel isolate contained relatively high amounts of $C_{15:0}$ (1.4 %), summed features 1 (equivalent chain-length of 10.928; 2.4 %) and 7 ($C_{19:1}\omega6c$ and/or an as-yet undetermined compound; 1.8 %), and by the presence of $C_{17:0}$, $C_{17:1}\omega6c$ and $C_{12:0}$ 2-OH, whereas other hydroxylated compounds were lacking in other *Polynucleobacter* species. A peculiar marker of the novel species was the presence of $C_{16:1}$ 2-OH. The presence of relatively high amounts (2.7 %) of $C_{14:0}$, together with the presence of $C_{17:1}\omega6c$ and $C_{16:1}$ 2-OH, seem to be the most reliable feature to distinguish *P. difficilis* from *P. necessarius* subsp. *asymbioticus*, which also contains $C_{12:0}$ 2-OH.

Strain AM-8B5^T, like several strains affiliated with the other four *Polynucleobacter* species described so far, possessed the trait that at least some *in situ* grown cells can pass through membrane filters with pore sizes of 0.2 μm (Hahn, 2004).

Phylogeny

Phylogeny inference by NJ, MP and ML methods consistently resulted in clustering of the almost complete 16S rRNA gene sequence of strain AM-8B5^T within the genus *Polynucleobacter* (Fig. 1). All three methods indicated that strain AM-8B5^T represents a sibling taxon of *P. acidiphobus*. Analysis including 16S rRNA gene sequences of uncultivated bacteria (see below) revealed that strain AM-8B5^T is affiliated with subcluster PnecB1 (Wu & Hahn, 2006a) of the genus *Polynucleobacter*. These phylogenetic results are further supported by previous phylogeny inference based on 16S–23S ITS sequences (Hahn *et al.*, 2010). Furthermore, all three analyses of 16S rRNA sequences indicated that *P. necessarius* represents the closest relative of the subcluster (PnecB) represented by *P. acidiphobus* and AM-8B5^T. In contrast, trees constructed by the ML method differed from trees calculated by the other two methods regarding the branching order of *P. cosmopolitanus* and *P. rarus* (Fig. 1).

Genotypic traits

Analysis of >400 sequences affiliated with the genus *Polynucleobacter* revealed a diagnostic oligonucleotide sequence specific for the 16S rRNA gene of bacteria affiliated with

subcluster PnecB1. This sequence (5′-GCCGACTAGTTGTTGGGAATTTACATTCTCAG-3′; *Escherichia coli* positions 821–840) differs in a few base pairs from the sequences of strains affiliated with the other four *Polynucleobacter* subclusters. A BLAST search with this diagnostic sequence as query revealed 253 sequences with 100 % sequence identity. These sequences included many short partial 16S rRNA gene sequences. Therefore, a detailed phylogenetic analysis of these 253 sequences was omitted. Instead, a query with the reverse complement of the diagnostic sequence was performed by using the Probe Match service provided by the Ribosomal Database Project release 10 (Cole *et al.*, 2009). This resulted in 250 hits with 100 % sequence identity. Almost all of these hits (241) were classified by the Ribosomal Database Project as *Polynucleobacter* bacteria; the remaining nine sequences were classified as 'unclassified *Burkholderiaceae*', 'unclassified *Burkholderiales*', 'unclassified *Comamonadaceae*' or 'unclassified *Betaproteobacteria*'. BLAST searches with these nine sequences indicated that most of them are also affiliated with the genus *Polynucleobacter*, but two sequences seemed to represent uncultured bacteria not affiliated with this genus. In conclusion, the combined presence of this PnecB1-specific diagnostic sequence and a diagnostic sequence specific for the entire genus *Polynucleobacter* (5′-GAGCYGSTGTTTCTTCCC-3′; *E. coli* positions 445–463; Hahn *et al.*, 2005) (Supplementary Table S1, available in IJSEM Online) seems to be suitable for reliable identification of members of the *Polynucleobacter* subcluster PnecB1. An overview of diagnostic sequences for identification of *Polynucleobacter* taxa is presented in Supplementary Table S1.

DNA–DNA reassociation experiments

The sequence similarities between 16S rRNA genes of strain AM-8B5^T and the type strain of *P. necessarius* subsp. *asymbioticus*, a sequence representing the endosymbiotic *P. necessarius* subsp. *necessarius* 'E24', the type strain of *P. cosmopolitanus*, the type strain of *P. rarus* and the type strain of *P. acidiphobus* were 98.3, 97.9, 97.5, 96.2 and 98.7 %, respectively. Pairwise DNA–DNA reassociation experiments with strain AM-8B5^T and the type strains of the three *Polynucleobacter* species sharing 16S rRNA sequence similarity values >97 % were performed in order to reveal whether the novel isolate belonged to a previously described species (Stackebrandt & Goebel, 1994). Note that experiments with DNA of a representative of *P. necessarius* subsp. *necessarius* could not be performed due to the lack of pure cultures (Heckmann & Schmidt, 1987; Vannini *et al.*, 2007; Hahn *et al.*, 2009). In all cases, the duplicate reassociation experiments resulted in DNA–DNA hybridization values ≤37.8 %. If the threshold value for delineation of prokaryotic species recommended by the ad hoc committee of Wayne *et al.* (1987) of 70 % DNA–DNA similarity is considered, strain AM-8B5^T has to be viewed as a representative of a novel species that is distinct from all *Polynucleobacter* species described so far.

Proposal of a novel *Polynucleobacter* species

Results from the phylogenetic analysis and chemotaxonomic investigations demonstrated the affiliation of strain AM-8B5^T with the genus *Polynucleobacter* (Tables 1 and 2, Fig. 1) but also revealed pronounced differences between this strain and strains affiliated with the

four previously described *Polynucleobacter* species. DNA–DNA reassociation data indicated that the isolate does not belong to the previously described species *P. necessarius*, *P. cosmopolitanus* and *P. acidiphobus* and the 16S rRNA gene sequence similarity between strain AM-8B5^T and the type strain of *P. rarus* of <97 % clearly indicated that the isolate also does not belong to the latter species (Stackebrandt & Goebel, 1994). Strain AM-8B5^T can be discriminated from previously described *Polynucleobacter* species based on phenotypic, chemotaxonomic and genotypic traits. The strain differs from the *P. acidiphobus* type strain in its ability to utilize fumaric acid, D-galacturonic acid, and D-fucose, from the *P. rarus* type strain in its ability to utilize oxaloacetic acid, from all so far characterized *P. cosmopolitanus* strains in its ability to utilize D-lyxose and D-fucose, and from all so far characterized *P. necessarius* subsp. *asymbioticus* strains by the lack of acetate utilization (Table 2). The latter trait also discriminates strain AM-8B5^T from all other *Polynucleobacter* species characterized so far. Regarding the fatty acid composition, strain AM-8B5^T can be differentiated from each of the other *Polynucleobacter* species by differences in the relative occurrence of at least three fatty acid components (Table 2).

Based on these findings, we propose to establish the novel species, *Polynucleobacter difficilis* sp. nov., with strain AM-8B5^T as the type strain. Furthermore, we propose to preliminarily include all strains phylogenetically affiliated with subcluster PnecB1 of the *Polynucleobacter* lineage (Wu & Hahn, 2006a) in this novel species. Further taxonomic investigations will be needed to clarify whether all strains affiliated with subcluster PnecB1 belong to *P. difficilis* sp. nov.

A consequence of proposing *P. difficilis* sp. nov. is a split of the taxon *Polynucleobacter* subcluster PnecB (Hahn, 2003), which is synonymous with tribe PnecB recently proposed in a review paper on phylogeny and ecology of freshwater bacterioplankton (Newton *et al.*, 2011), into two separate species. Mainly for methodological reasons [i.e. the availability of a fluorescent *in situ* hybridization (FISH) probe; see Supplementary Table S1], this taxon was treated by ecologists as a taxonomic unit in several investigations on freshwater bacterioplankton (Wu & Hahn, 2006a, b; Salcher *et al.*, 2008; Alonso *et al.*, 2009; Newton *et al.*, 2011). If the two species *P. acidiphobus* (PnecB2) and *P. difficilis* (PnecB1) possess distinct ecological characteristics, new molecular tools to distinguish the two taxa would be required for appropriate investigations of their ecology.

Ecology and biogeography of *Polynucleobacter difficilis* sp. nov.

BLAST searches (7 December 2010) with the 16S rRNA gene sequence of strain AM-8B5^T resulted in 178 hits of almost full-length 16S rRNA genes with sequence similarities >99 %. Phylogenetic analyses of sequences of the top 250 BLAST hits and sequences representing the previously described *Polynucleobacter* species revealed that 182 sequences clustered together with AM-8B5^T within subcluster PnecB1 (Wu & Hahn, 2006a). These sequences deposited in GenBank/EMBL/DDBJ were obtained from two estuaries (Chesapeake Bay and Delaware Bay) located in the USA (Shaw *et al.*, 2008), an estuary (Weser) located in Europe (Selje *et al.*, 2005), a lake in China (Z. H. Li, Y. Q. Ding and G. Y. Wang, unpublished data) and four high-altitude lakes located in Tibet (R. Zhang and W.-T. Liu, unpublished data). Furthermore, a large number of partial 16S rRNA sequences of about

500–600 bp lengths affiliated with subcluster PnecB1 were obtained from an experimental system strongly influenced by growth of phytoplankton (Horner-Devine *et al.*, 2003) and deposited later in GenBank/EMBL/DDBJ (sequence accession numbers not mentioned by Horner-Devine *et al.*, 2003). The majority of almost full-length sequences clustering within PnecB1 originated from the four Tibetan lakes. In contrast, despite the enormous number of deposited bacterial sequences from these intensively investigated lakes, sequences affiliated with subcluster PnecB2 (*P. acidiphobus*) were not obtained in this interesting study. Only two habitats are represented by sequences in both subclusters PnecB1 and PnecB2. These are the estuaries of Chesapeake Bay and Delaware Bay (Shaw *et al.*, 2008), which potentially receive bacteria from ecologically distinct freshwater ponds and lakes drained by the large number of rivers and creeks discharging in these large bays. These differences in origin of sequences currently forming the two subclusters may indicate ecological differences between the two species representing the two subclusters. Therefore, generalization of results (Wu & Hahn, 2006a, b; Salcher *et al.*, 2008; Alonso *et al.*, 2009; Hahn *et al.*, 2011b) obtained previously by using a PnecB-specific FISH probe (Wu & Hahn, 2006a) not distinguishing between the two subclusters PnecB1 and PnecB2 could be problematic. On the other hand, isolation of strain AM-8A5^T from the water column of a lake and the exclusive origin of all other ribosomal sequences currently clustering within subcluster PnecB1 from the water columns of lakes and estuaries or experimental systems inoculated with water from lakes (Horner-Devine *et al.*, 2003) indicates that PnecB1 bacteria share a planktonic lifestyle with all other free-living *Polynucleobacter* strains investigated so far. Whether the distribution of PnecB1 bacteria is restricted to freshwater habitats as with other free-living *Polynucleobacter* or populations also persist in saline systems (e.g. saline Tibetan lakes) remains to be revealed. Investigations by using the PnecB-specific FISH probe did not indicate that bacteria either affiliated with subcluster PnecB1 or PnecB2 thrive in saline lakes (Wu *et al.*, 2006); however, the sequences deposited by R. Zhang and W.-T. Liu (unpublished data) in GenBank/EMBL/DDBJ seem to indicate that PnecB1 bacteria occur in some saline lakes.

Origination of PnecB1 sequences from habitats located in Asia, Europe and North America seems to indicate a cosmopolitan distribution of *P. difficilis* sp. nov. in freshwater habitats.

Description of *Polynucleobacter difficilis* sp. nov.

Polynucleobacter difficilis (dif.fi’ci.lis. L. masc. adj. *difficilis* difficult, troublesome, referring to difficulties in cultivating the type strain).

Curved, non-motile rods, 0.6–1.8 μm in length and 0.4–0.5 μm in width. Chemo-organotrophic, exhibits aerobic and anaerobic growth. Planktonic, free-living lifestyle. Inhabits various freshwater systems. Can be cultivated on NSY and R2A medium. Best growth is observed on modified NSY medium enriched with L-cysteine and pyruvate. Colonies grown on NSY agar are unpigmented, circular and convex with smooth surfaces. Mesophilic; growth occurs at 4 °C but not at 35 °C. Growth occurs without NaCl. Maximum NaCl concentration tolerated is 0.2 % (w/v). Oxidase- and catalase-positive. Utilizes formic acid, pyruvic acid, oxaloacetic acid, malic acid, succinic acid, fumaric acid, D-galacturonic acid, D-lyxose, D-fucose and L-cysteine when these substrates are provided in a medium

containing low levels of NSY. Does not utilize glyoxylic acid, glycolic acid, acetic acid, oxalic acid, propionic acid, malonic acid, levulinic acid, citric acid, D-mannose, D-glucose, D-galactose, D-fructose, D-sorbitol, L-glutamate, L-aspartate, L-alanine, L-serine, L-asparagine or betaine. Whole-cell fatty acids are dominated by summed feature 3 (including $C_{16:1}\omega7c$ and iso-$C_{15:0}$ 2-OH), $C_{16:0}$ and $C_{18:1}\omega7c$. The components $C_{12:0}$, $C_{15:0}$, $C_{17:0}$, $C_{17:1}\omega6c$, as well as $C_{12:0}$ 2-OH and $C_{16:1}$ 2-OH are present, but no other hydroxylated fatty acids. Tentatively, all strains affiliated with the genus *Polynucleobacter* and possessing the oligonucleotide sequence 5′-GCCGACTAGTTGTTGGGAATTTACATTCTCAG-3′ (*E. coli* positions 821–840) within the 16S rRNA gene shall be assigned to the proposed species.

The type strain is AM-8B5^T (=DSM 22349^T=CIP 110078^T), isolated from Lake Sevan, Armenia. The DNA G+C content of the type strain is 49.4 mol%.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FISH	fluorescent *in situ* hybridization
ML	maximum-likelihood
MP	maximum-parsimony
NJ	neighbour-joining

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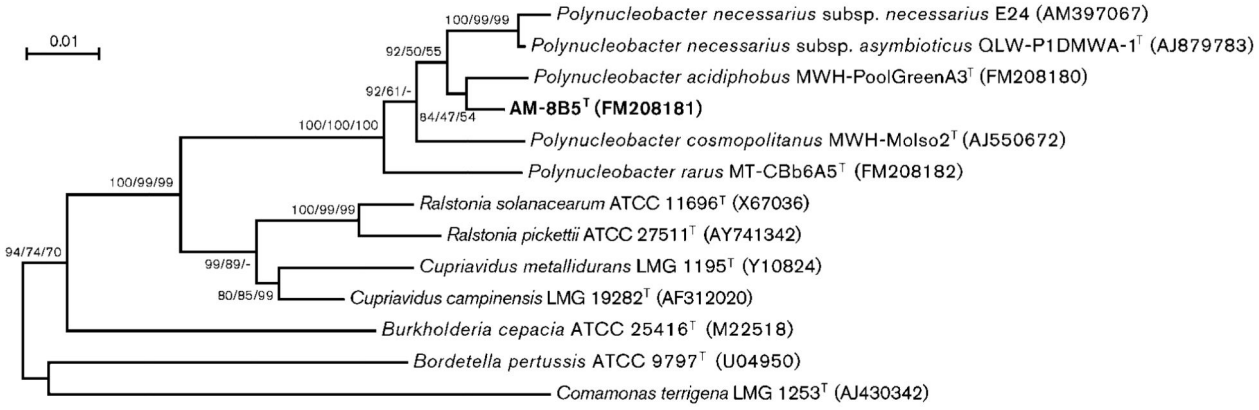

Fig. 1.

Neighbour-joining tree based on almost complete 16S rRNA gene sequences reconstructing the phylogenetic position of *Polynucleobacter difficilis* sp. nov. strain AM-8B5^T. Bootstrap percentages [1000 (NJ, MP) or 100 iterations (ML)] obtained by NJ/MP/ML methods are shown at nodes. Bar, 0.01 substitutions per nucleotide position.

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Table 1

Traits characterizing *P. difficilis* sp. nov. AM-8B5^T and previously described *Polynucleobacter* species

Taxa: 1, AM-8B5^T; 2, *P. acidiphobus* (Hahn *et al.*, 2011b); 3, *P. necessarius* subsp. *asymbioticus* (Hahn *et al.*, 2009); 4, *P. cosmopolitanus* (Hahn *et al.*, 2010); 5, *P. rarus* (Hahn *et al.*, 2011a). All taxa are non-motile. Note that sufficient phenotypic and chemotaxonomic characterizations are lacking for the obligately endosymbiotic strains of the subspecies *P. necessarius* subsp. *necessarius*. −, Negative; +, positive; W, weakly positive; +/−, some strains positive and some strains negative; +/W, some strains positive and some strains weakly positive; W/−, some strains weakly positive and some strains negative.

Characteristic	**1**	**2**	**3**	**4**	**5**
Cell morphology	Short curved rods	Short curved rods	Straight or curved rods	Curved rods	Straight rods
Nucleoids visible (DAPI)*	Rarely	Rarely	Rarely	Rarely	Frequently
Cell length (μm)	0.6–1.8	0.5–1.4	0.5–2.9	0.4–1.4	0.8–1.8
Cell width (μm)	0.4–0.5	0.4–0.5	0.3–0.5	0.3–0.5	0.6–0.8
Catalase	W	+	+	+	+
Oxidase	W	+	+	+	+
Growth at:					
5 °C	+	−	+	+/−	−
35 °C	−	+	+/−	+	−
Maximum NaCl tolerance (%, w/v)†	0.2 (W)	0.3	0.3–0.5	0.3–0.5	0.3 (W)
Anaerobic growth on:					
NSY medium	−	+	+/−	+/−	−
NSY+0.8 mM nitrate	W	+	+	+	+
Growth in mineral medium with acetic acid and B12	−	W	W/−	W/−	W
Assimilation of:					
Formic acid	W	−	W/−	−	−
Glyoxylic acid	−	−	W/−	W/−	+
Glycolic acid	−	−	−	−	W
Acetic acid	−	W	+	+	+
Oxalic acid	−	−	−	−	W
Propionic acid	−	−	+/−	+/W	−
Pyruvic acid	+	+	+	+	+
Malonic acid	−	W	+/−	+/−	−
Oxaloacetic acid	W	+	+/−	+	−
Malic acid	W	W	+/W	+	+
Succinic acid	+	W	+	+	+
Fumaric acid	W	−	+/W	+	+
Levulinic acid	−	−	W/−	W/−	+
Citric acid	−	−	−	+/−	−
D-Mannose	−	−	W/−	W/−	W
D-Glucose	−	W	W/−	W/−	−

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Characteristic	1	2	3	4	5
D-Galacturonic acid	W	−	W	+/W	+
D-Galactose	−	−	W/−	W	−
D-Lyxose	W	−	W/−	−	W
D-Fructose	−	−	W/−	−	W
D-Fucose	W	−	W/−	−	W
D-Sorbitol	−	−	W/−	−	−
L-Glutamate	−	−	+/−	W/−	−
L-Aspartate	−	−	+/−	−	−
L-Cysteine	+	+	+/W	+	W
L-Alanine	−	−	W/−	+/W	−
L-Serine	−	−	−	−	−
L-Asparagine	−	−	W/−	−	−
Betaine	−	−	W/−	−	−
DNA G+C content (mol%)	49.4	48.3	44–46	44.9	40.3

*DAPI, 4′,6-diamidino-2-phenylindole.

†Highest NaCl concentration (added to NSY medium) at which growth was observed.

Table 2

Whole-cell fatty acid compositions of *Polynucleobacter difficilis* strain AM-8B5^T and related strains
Taxa: 1, strain AM-8B5^T; 2, *P. acidiphobus* MWH-PoolGreenA3^T (Hahn *et al.*, 2011a); 3, *P. rarus* MT-CBb6A5^T (Hahn *et al.*, 2011b); 4, *P. cosmopolitanus* MWH-MoIso2^T (Hahn *et al.*, 2010); 5, five *P. cosmopolitanus* strains (Hahn *et al.*, 2010); 6, *P. necessarius* subsp. *asymbioticus* QLW-P1DMWA-1^T (Hahn *et al.*, 2009); 7, three *P. necessarius* subsp. *asymbioticus* strains (Hahn et al., 2009). Values are percentages of the summed fatty acids named in the peak library of the MIDI system (contents >0.2% are listed). Strains were grown on R2A agar plates for 7–9 days (strain AM-8B5^T) or 3–5 days at 28 °C. TR, Trace, presence is variable; −, not detected.

Fatty acid	1	2	3	4	5	6	7
Saturated							
$C_{12:0}$	3.6	3.8	3.0	−	−	3.4	3.4–5.5
$C_{14:0}$	3.0	0.9	0.4	0.7	0.6–2.3	0.9	0.3–1.2
$C_{15:0}$	1.4	−	−	−	−	0.3	0–0.3
$C_{16:0}$	16.4	24.2	19.8	15.4	11.0–15.4	22.2	15.5–29.6
$C_{17:0}$	0.8	−	0.3	−	−	−	0–0.5
$C_{18:0}$	0.8	0.5	0.5	0.8	0.5–1.1	1.2	0.5–2.4
10-Methyl-$C_{19:0}$	−	−	0.4	0.7	0–0.7	−	−
Unsaturated							
$C_{14:1}\omega5c$	−	−	−	0.6	0–1.1	−	0–0.6
$C_{15:1}\omega6c$	TR	−	0.3	−	−	−	0–0.6
$C_{16:1}\omega5c$	1.4	0.4	0.5	0.3	0–1.1	0.9	0–0.9
$C_{17:1}\omega6c$	1.1	−	−	0.5	0–0.7	−	−
$C_{18:1}\omega9c$	TR	−	−	0.3	0–2.0	−	0–0.4
$C_{18:1}\omega7c$	10.2	17.3	21.8	28.7	28.7–38.1	12.9	1.1–20.4
11-Methyl-$C_{18:1}\omega7c$	−	−	7.5	3.7	0.4–3.7	3.1	1.1–8.1
Hydroxylated							
$C_{12:0}$-2OH	1.3	−	−	−	−	2.5	1.3–2.5
$C_{12:0}$-3OH	−	−	−	11.1	7.1–11.2	−	−
$C_{14:0}$-2OH	−	2.9	−	−	−	−	−
$C_{16:1}$-2OH	0.7	−	−	−	−	−	−
$C_{16:0}$-3OH	−	0.5	0.3	−	−	−	−
Cyclic							
$C_{17:0}$ cyclo	−	3.0	−	−	−	−	−
Summed features*							
1	2.4	−	1.8	−	−	0.4	0.4–1.0
2	7.0	10.8	6.9	0.6	0.6–3.9	9.6	9.2–9.9
3	47.1	32.1	35.9	34.7	31.5–36.5	41.3	35.6–45.0
7	1.8	1.7	−	1.5	0–1.5	0.4	0.3–2.0

*Summed features contain components which cannot be separated or identified by the MIDI system. The MIDI suggestion for feature 1 (ECL of 10.928) is $C_{12:0}$ aldehyde; feature 2 comprises $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$; feature 3 comprises $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH; and feature 7 comprises $C_{19:1}\omega 6c$ and/or an as-yet undetermined component.

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