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Polynucleobacter meluiroseus sp. nov. a bacterium isolated from a lake located in the mountains of the Mediterranean island of Corsica

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Abstract

Strain AP-Melu-1000-B4 was isolated from a lake, located in the mountains of the Mediterranean island of Corsica (France). Phenotypic, chemotaxonomic and genomic traits were investigated. Phylogenetic analyses based on 16S rRNA gene sequencing referred the strain to the cryptic species complex PnecC within the genus *Polynucleobacter*. The strain encoded genes for biosynthesis of proteorhodopsin and retinal. When pelleted by centrifugation the strain showed an intense rose colouring. Major fatty acids were $C_{16:1}$ ω7c, $C_{16:0}$, $C_{18:1}$ ω7c and feature 2 ($C_{16:1}$ isoI and $C_{14:0}$-3OH). The sequence of the 16S rRNA gene contained an indel which was not present in any previously described *Polynucleobacter* species. Genome sequencing revealed a genome size of 1.89 Mbp and a G+C content of 46.6 mol %. In order to resolve the phylogenetic position of the new strain within subcluster PnecC, its phylogeny was reconstructed from sequences of 319 shared genes. To represent all currently described *Polynucleobacter* species by whole genome sequences, three type strains were additionally sequenced. Our phylogenetic analysis revealed that strain AP-Melu-100-B4 occupied a basal position compared with previously described PnecC strains. Pairwise determined whole genome average nucleotide identity (gANI) values suggested that strain AP-Melu-1000-B4 represents a new species, for which we propose the name *Polynucleobacter meluiroseus* sp. nov. with the type strain AP-Melu-1000-B4^T (=DSM 103591^T= CIP 111329^T)

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The GenBank/EMBL/DDBJ accession number for the genome and 16S rRNA sequences of strain AP-Melu-1000-B4^T are OANS00000000 and MF872602

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

Ethical statement
The presented study does not include any experimental work with humans or vertebrates.

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Keywords

Polynucleobacter; cryptic species complex; *Burkholderiaceae*; genome; whole genome average nucleotide identity (gANI); freshwater lake

The genus *Polynucleobacter* was established by Heckmann and Schmidt [1] for obligately endosymbiotic bacteria living in the cytoplasm of some freshwater ciliates belonging to the genus *Euplotes*. They described the species *Polynucleobacter necessarius,* a bacterial endosymbiont of *Euplotes aediculatus,* which is essential for the host. Some years later it became apparent that many important or abundant free-living freshwater bacteria are closely related to *P. necessarius* [2–5]. The genus *Polynucleobacter* is affiliated with the family *Burkholderiaceae* but is distinguished from all other genera in the family by small genome size (1.6 – 2.3 Mbp) and relatively low G+C contents (<50 mol %). Comparative genome analyses of a planktonic [6] and an endosymbiotic strain [7] revealed a degenerative genome evolution in the endosymbiotic strain. The type material representing *P. necessarius* is not any more available, however it was shown that the complete genome sequence of the endosymbiotic strain STIR1 well represents the type species of the genus *Polynucleobacter* [8]. Analyses of 16S rRNA gene sequences suggested that the genus *Polynucleobacter* can be subdivided in subclusters [4]. Within the subcluster PnecC, which currently includes the endosymbiotic *P. necessarius* and seven free-living species, the 16S rRNA gene sequences are very similar (≥99 %). Nevertheless, it seems that this group represents a cryptic species complex containing a lot of different species [8, 9].

The free-living *Polynucleobacter* bacteria are ubiquitous and abundant in lakes, ponds and streams and were also detected in groundwater systems. In many freshwater systems they represented >10 % of the bacterioplankton [10]. Cultivated strains represent delicate bacteria not suitable for many standard methods of examination. The weak performance of *Polynucleobacter* strains in experiments utilizing artificial media may be related to their relatively small genome size.

Here we describe strain AP-Melu-1000-B4, which was isolated from a mountain lake located on the Mediterranean island of Corsica. The strain is affiliated with subcluster PnecC but differs in some unusual genetic and phenotypic features from the other *Polynucleobacter* type strains. We propose to establish for this strain the species name *Polynucleobacter meluiroseus* sp. nov.

Home habitat and isolation

Strain AP-Melu-1000-B4^T was isolated from Lake Melu (Lac de Melu / Lac de Melo) (Fig. 1), a small lake located in the mountains (Restonica valley) of the Mediterranean island of Corsica (France). The approximate geographic coordinates are 42.213 N 9.023 E and the lake is located at 1710 m above sea level. It has an area of approximately 6.5 hectare and a maximum depth of 20 m. Water sampled from the lakeside in July 2015 at circa 0.2 m depth (Fig. 1) had a pH of 6.8, a conductivity of 15.3 µS cm^{-1} and a temperature of 20.8 °C, oxygen was saturating. While the content of ions like sodium, magnesium, calcium, chloride

was within the range for similar lakes, the content of nitrate was relatively high (0.3 mg/l). The strain was isolated by the filtration-acclimatization method with NSY medium [11, 12].

Phenotypic and chemotaxonomic characterization

Cells of strain Melu-1000-B4^T were rods of small size (Table 1). The strain formed small circular colonies with shiny surface on NSY agar plates which appeared rose-coloured in older cultures. Growing on liquid NSY medium in 100 ml Erlenmeyer flasks for seven days it did not appear obviously coloured, but the pellet obtained by centrifugation had an intense rose colouring (Fig. 2). Such pigmentation is unusual in *Polynucleobacter* bacteria and was not shown by any type strain of subcluster PnecC (Fig. 2). Growth at different temperatures and growth under anoxic conditions in an anaerobic chamber were examined by using NSY agar plates as described previously [13]. Salinity (NaCl) tolerance was determined using NSY agar supplemented with various NaCl concentrations [13]. The strain showed no anaerobic growth on NSY plates with or without added nitrate. It grew at temperatures up to 28°C and tolerated salt concentrations up to 0.4% (Table 1).

Utilization of various substrates was investigated in the same way as for previously described *Polynucleobacter* species [13–17]. Briefly, growth enabled by utilization of a specific substrate was determined by comparison of the optical density (OD) at 575 nm following growth in one tenth-strength NSY broth (0.3 g l^{-1}) with and without 0.5 g l^{-1} test substrate. Increases of < 10 %, 10–50% and >50% of the OD obtained with the test substrates were scored after 10 days of growth as no utilization (-), weak utilization (w) and good utilization (+) (Table 1).

The analysis of the whole-cell fatty acid composition (Table 2) was carried out as described previously [14] except that the cell masses were cultivated on R2A agar slants which were filled up with 1.5 ml liquid R2A medium. The slants were incubated at 28°C and inspected for growth daily. Once biomass was easily visible, the cell mass was harvested. The composition of fatty acid was similar to that of other members of the PnecC subgroup and comprised $C_{16:1}$ ω7c, $C_{16:0}$, $C_{18:1}$ ω7c and feature 2 ($C_{16:1}$ isoI and $C_{14:0}$-3OH) as the major components and small amounts of 2-hydroxylated fatty acids (Table 2).

Genomic characterization

The genome of strain AP-Melu-1000-B4^T was sequenced and annotated as described below. In order to enable a whole genome-based reconstruction of its phylogeny, genomes of three *Polynucleobacter* type strains not available so far, were also sequenced and annotated. DNA used for genome sequencing of all four strains was extracted from cultures grown in liquid NSY medium as described previously [6].

Strain AP-Melu-1000-B4^T was sequenced at the DOE-Joint Genome Institute as part of the Genomic Encyclopedia of Type Strains, Phase III (KMG-III) study [18] using the Illumina HiSeq–2000 1TB platform. Paired-end sequencing (2 x 150 bp) of a fragment library resulted in about 8.2 x 10^6 quality filtered reads. Assembly of reads resulted in eleven contigs with a total sequence length of 1.89 Mbp and a sequencing coverage of about 650-fold. The obtained genome sequence was annotated using the IMG/ER annotation pipeline

[19] and subsequently deposited at DDBJ/EMBL/GenBank. The IMG Genome ID and the GenBank accession number of the genome of strain AP-Melu-1000-B4^T are 2710724120 and OANS00000000, respectively.

The genomes of type strains *P. rarus* MT-CBb6A5^T [16], *P. difficilis* AM-8B5^T [14], and *P. acidiphobus* MWH-PoolGreenA3^T [17] were sequenced as described previously for the *Silvanigrella aquatica* type strain [20]. For each strain, two libraries were constructed. One library of each strain had an insert size of 8 kb and was paired-end sequenced by an Illumina MiSeq instrument. In addition, shotgun libraries were mate-pair sequenced by a GS FLX instrument by using Titanium chemistry. A hybrid assembly approach combining reads obtained by the two sequencing methods was used to construct the genome sequences [20]. The sequences were annotated by the IMG/ER pipeline and made available in the IMG system and in DDBJ/EMBL/GenBank. An overview of these and other genome sequences from the genus *Polynucleobacter* that were used for comparison with strain AP-Melu-1000-B4^T is given in Table 3.

The size of the genome of strain AP-Melu-1000-B4^T was 1.89 Mbp and in the range of the sizes of the other seven free-living type strains representing subcluster PnecC, but the smallest one (Table 3). The mol % G+C of the genome of strain AP-Melu-1000-B4^T was 46.6 mol % and in the range of the other strains, but the highest one (Table 3).

The gene composition of strain AP-Melu-1000-B4^T differed from the other seven free living PnecC strains, encoding some gene clusters absent in most of the other strains and lacking some genes common in the seven other strains of subcluster PnecC (Table 4). For instance, strain AP-Melu-1000-B4^T possessed genes putatively encoding a proteorhodopsin (bacteriorhodopsin-like protein), the biosynthesis of 7,8 dihydro-ß-carotene and a beta-carotene 15,15'-monooxygenase. The latter gene encodes the last step of retinal biosynthesis the cofactor for the light driven proton pump proteorhodopsin [21]. Among the seven other type strains affiliated with subcluster PnecC only *P. aenigmaticus* strain MWH-K35W1^T possessed a gene putatively encoding a proteorhodopsin. Of additional interest, strain AP-Melu-1000-B4^T shared a gene cluster encoding flagella only with type strains *P. duraquae* MWH-Mok4^T and *P. aenigmaticus* MWH-K35W1^T.

Among undescribed and previously genome sequenced *Polynucleobacter* strains belonging to the subcluster PnecC genes for proteorhodopsin were rare. An on-going survey of over 100 isolates of a species-like subgroup of the PnecC subcluster found genes for proteorhodopsin in only in a few strains (Hahn et al., unpublished genome data). Moreover, the amino acid sequence of strain AP-Melu-1000-B4^T proteorhodopsin contained features typical for green proteorhodopsin (GPR) (γ-proteobacterium SAR86) [22], i.e.. the amino acids GPR L105 and GPR A178 responsible for the green absorption maximum at 525 nm [23]. This corresponds with the fact, that the strain was isolated from surface water, where more green light is available than in deeper layers.

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Phylogengy

Previous phylogenetic reconstructions for *Polynucleobacter* species were based on a single gene (16S rRNA gene) [15, 17] or eight protein-encoding housekeeping genes [24, 25]. With the availability of genome sequences of all *Polynucleobacter* type strains, it was possible to construct a phylogeny based on a large number of protein-encoding genes. Shared genes of *Polynucleobacter* strains were identified by > 60% nucleotide sequence identity, > 80% query coverage, and E values < $1e^{-10}$ in BLAST searches of the genomes of 59 *Polynucleobacter* strains. Genes of strain *Cupriavidus metallidurans* CH34^T were added to the sequence collection to serve as an outgroup in the phylogenetic reconstruction. Genes of that strain were selected based on amino acid sequence comparisons (>60% AA sequence identity, E values < $1e^{-10}$, alignment coverage > 60%). Nucleotide sequences of the 319 shared genes (Table S1) were extracted from genome sequences, concatenated and aligned by using the software MAFT [26]. This resulted in a total alignment length of 344,288 bp. The software GBlocks Masking 3.9.17 [27] was used to select conserved blocks from the alignment for the further analyses. This resulted in 305,399 alignment positions in 695 selected blocks. The CIPRES Science Gateway V. 3.3 [28] was used to calculate a RAxML tree [29] (Fig. 3). In addition, neighbour joining and maximum parsimony trees were calculated with MEGA7 [30]. For comparison, phylogenetic reconstructions based on 16S rRNA genes (Fig. 4) were performed.

The phylogenetic reconstructions based on the 319 shared genes performed with three different algorithms differed only in nodes related to the two species *P. difficilis* AM-8B5^T and *P. acidiphobus* MWH-PoolGreenA3^T (Fig. 3) previously shown to form subcluster PnecB in 16S rRNA gene trees. All other nodes were present in all three shared gene trees (Fig. 3) and supported by bootstrap values of 100%. Although the three trees differed in the branching order of the four subclusters, in all cases subcluster PnecC was well separated from the other subclusters. Strain AP-Melu-1000-B4^T was affiliated in a basal position within the PnecC subcluster.

While phylogenies based on 16S rRNA gene sequences are not able to resolve branching within this subcluster (Fig. 4), the data set based on the 319 shared genes provide insights into phylogenies within this cryptic species complex. All three algorithms yielded identical branching orders for all PnecC subcluster species. Compared to phylogenetic reconstructions in previous publications with eight protein encoding housekeeping genes [25, 31] utilization of 319 shared genes more fully resolved the phylogenetic relationships among the PnecC species.

The phylogenetic reconstruction based on 16S rRNA gene sequences (Fig. 4) and the presence of the signature sequence 5′-GAGCCGGTGTTTCTTCCC-3′ in the 16S rRNA gene (*E. coli*. position 445-463) [11] confirmed placement of AP-Melu-1000-B4^T in the subcluster PnecC. However, the sequence of the 16S rRNA gene revealed an exceptional indel. The 16S rRNA gene sequence from *E. coli* position 1132-1142 is 5’-CATTTAGTTG -3’ while all other strains presented in Fig. 3 show the sequence 5’-CGCAAG-3’. According to the web server RNA structure [32] the indel is predicted to form an additional bulge loop within the stem of a hairpin structure and a reduction of the loop (Fig. 5). In order to

estimate the frequency of this indel in the 16S rRNA gene among *Polynucleobacter* strains, 1000 sequences were retrieved from Genbank by using the search term '*Polynucleobacter* AND 16S' and additional filtering for sequence length > 400 bp. The retrieved sequences were aligned by MUSCLE [30] and sequences not covering *E. coli* positions 1132-1142 were discarded. All the remaining 776 16S rRNA gene sequences possessed > 96% sequence similarity and appeared to represent *Polynucleobacter* species. Surprisingly, not one of these sequences contained the indel found in strain AP-Melu-1000-B4^T. In addition, BLAST searches of Genbank with the indel of strain AP-Melu-1000-B4^T and flanking sequences of various lengths were performed. These searches retrieved 31 partial 16S rRNA gene sequences of uncultured bacteria, which all contained the sequence 5'-CATTTAGTTG -3' at the same position as found in AP-Melu-1000-B4^T. Thirteen of these sequences, all with length < 920 bp, were obtained from Lake Aixeus, a mountain lake (altitude 2366 m) located in the Central Pyrenees (Spain) [33]. Lake Aixeus revealed similarities to Lake Melu the home habitat of strain AP-Melu-1000-B4^T. Both lakes are mountain lakes located at the same latitude, but 600 km apart and also share some chemical characteristics including pH < 7, low conductivity and rather high nitrate concentrations. Eight sequences with indel originated from a study at Lake Mizugaki, a stratified lake in Japan [34]. In contrast to the origin of the strain AP-Melu-1000-B4^T these sequences were obtained from greater depths (25 – 43 m). One sequence with the indel originated from Adirondack Lakes (New York, United States) [5]. These 22 sequences from uncultured clones (Fig. 6) showed a 16S rRNA gene similarity of > 99% with strain AP-Melu-1000-B4^T as well as the other PnecC *Polynucleobacter* strains (Fig. 4). An alignment performed with MEGA 7 [30] with the 22 clone sequences and the sequence of AP-Melu-1000-B4^T confirmed the identity of the indel and the flanking positions (Fig. 6). The rest of the 31 partial 16S rRNA gene sequences showed lower similarities (98 % to 94 %,) with strain AP-Melu-1000-B4^T. Step-by-step BLAST analyses with consecutive portions of individual 16S rRNA gene sequences revealed that they might be chimeras and they were not further considered. These analyses suggested that the indel was quite rare among *Polynucleobacter* and probably only found in strains closely related to strain AP-Melu-1000-B4^T.

Ecology

The isolation of strain AP-Melu-100-B4^T from the water of a small lake and its ability to grow in artificial medium in absence of a potential host [35] suggested that this strain represented a free-living planktonic bacterium. The presence of flagella genes supported this conclusion. The predicted ability to utilize sunlight via proteorhodopsin as energy source and the origin of the sample suggested its adaption to growth in upper water layers.

A cultivation-independent investigation based on a protein-encoding marker (priB gene) on 56 European freshwater systems (Huemer, A., Schmidt, J. & M.W. Hahn, unpublished data) suggested that strain AP-Melu-1000-B4^T represented a large fraction of the PnecC community in Lake Melu at the day of sampling. Surprisingly no further detection of the taxon represented by the strain was observed in the other of the 55 investigation habitats. This corresponds with the observation that the indel found in the 16S rRNA gene sequence (see above) and distinctive for this taxon, is extremely rare in sequences representing

Polynucleobacter strains. Both results suggested that strain AP-Melu-100-B4^T represents a rare *Polynucleobacter* species, which is restricted to specific habitats.

Proposal of the new species *Polynucleobacter meluiroseus* sp. nov.

Strain AP-Melu-1000-B4^T can be discriminated from the type strains of *Polynucleobacter* species not affiliated with subcluster PnecC by chemotaxonomic traits. As for other species affiliated with subcluster PnecC, strain AP-Melu-1000-B4^T can be distinguished from the type strains of *P. rarus* MT-CBb6A5^T [16], *P. acidiphobus* MWH-PoolGreenA3^T [17] and *P. difficilis* AM-8B5^T [14] based on the G+C content of their DNA [8]. The discrimination of the strain from *P. cosmopolitanus* MWH-MoIso2^T and *P. victoriensis* MWH-VicM1^T (both affiliated with subcluster PnecD) is possible by the absence of the fatty acid $C_{12:0}$3-OH [15].

To test if strain AP-Melu-1000-B4^T was affiliated with one of the seven previously described free-living species affiliated with subcluster PnecC, average nucleotide identity (gANI) analyses with whole genome sequences by using the IMG/ER system were performed [19]. Pairwise ANI values of 75.6 – 76.4% (Table 5) suggested that the strain is not affiliated with any of these seven species [36–40]. It has been previously proposed [8] that the genome sequence of the endosymbiont STIR1 surrogate *P. necessarius* and can be used for gANI comparison. The gANI value with strain AP-Melu-1000-B4^T of 75.9% was very similar to the values obtained for the seven free-living type strains (Table 5). Consequently, strain AP-Melu-1000-B4^T has to be considered to represent a new species affiliated with subcluster PnecC of the genus *Polynucleobacter*.

Some features distinguish strain AP-Melu-1000-B4^T from all previously described type strains affiliated with subcluster PnecC. The intense rose colouring shown by colonies grown on NSY agar plates and in liquid NSY medium after centrifugation (Fig. 2) was so far only found in strain AP-Melu-1000-B4^T. This may indicate that genes putatively encoding for a proteorhodopsin and the complete synthesis pathway of the cofactor retinal were (constitutively) expressed. Similar genes were also present in *P. aenigmaticus* MWH-K35W1^T, which never showed a rose colouring.

As the exceptional indel within the 16S rRNA gene sequence of strain AP-Melu-1000-B4^T is not present in other cultivated *Polynucleobacter* strains with available 16S rRNA gene sequences, it might be a marker for this species within the PnecC subcluster.

Furthermore, features distinguishing strain AP-Melu-1000-B4^T from all previously described type strains of species affiliated with the subcluster PnecC are the absence of growth in assimilation tests on D-galacturonic acid and the combination of absence of growth with D-fructose and a weak growth with D-sorbitole and L-aspartate (Table 1).

Description of *Polynucleobacter meluiroseus* sp. nov.

Polynucleobacter meluiroseus sp. nov. (me.lu.i.ro'se.us. L. masc. adj. *roseus* rose-coloured; N.L. masc. adj. *meluiroseus,* a rose-coloured (bacterium) from Lake Melu)

Cells form rods, 0.8-2.1 μm in length and 0.3-0.6 μm in width, depending on growth stage. They grow chemo-organotrophically and aerobically. Colonies grown on NSY agar are rose pigmented, circular and convex with smooth surface. Growth occurs up to 28 °C and in 0-0.4 % (w) NaCl. Cells assimilate acetic acid, pyruvic acid, succinic acid, propionic acid, malonic acid, oxaloacetic acid, malic acid, fumaric acid, levulinic acid, D-sorbitole, L-glutamate, L-aspartate, L-cysteine and do not assimilate glycolic acid, citric acid, glyoxylic acid, oxalic acid, D-galacturonic acid, D-mannose, D-glucose, D-galactose, D-lyxose, D-fructose, L-fucose, L-histidine, L-alanine, L-asparagine, L-leucine L-serine or betaine. Major fatty acids are $C_{16:1}$ ω7c, $C_{16:0}$, $C_{18:1}$ ω7c and feature 2 ($C_{16:1}$ isoI and $C_{14:0}$-3OH).

The type strain is AP-Melu-1000-B4^T (=DSM 103591^T= CIP 111329^T), which was isolated from a small lake with low conductivity and nearly neutral pH located in the mountains of the island of Corsica (France). The genome of the type strain is characterized by a size of 1.89 Mbp and a G+C content of 46.6 mol%. Genome and 16S rRNA gene sequences characterizing the type strain are available under the accessions OANS00000000 and MF872602.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

gANI	whole genome average nucleotide identity
IMG/ER	Integrated Microbial Genomes/Expert Review
ML	maximum likelihood
NSY medium	nutrient broth soyatone yeast extract medium
OD	optical density
PnecC	cryptic species complex PnecC

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Fig. 1.
Lac de Melu, home habitat of strain AP-Melu-1000-B4^{T}: View from the lakeside.

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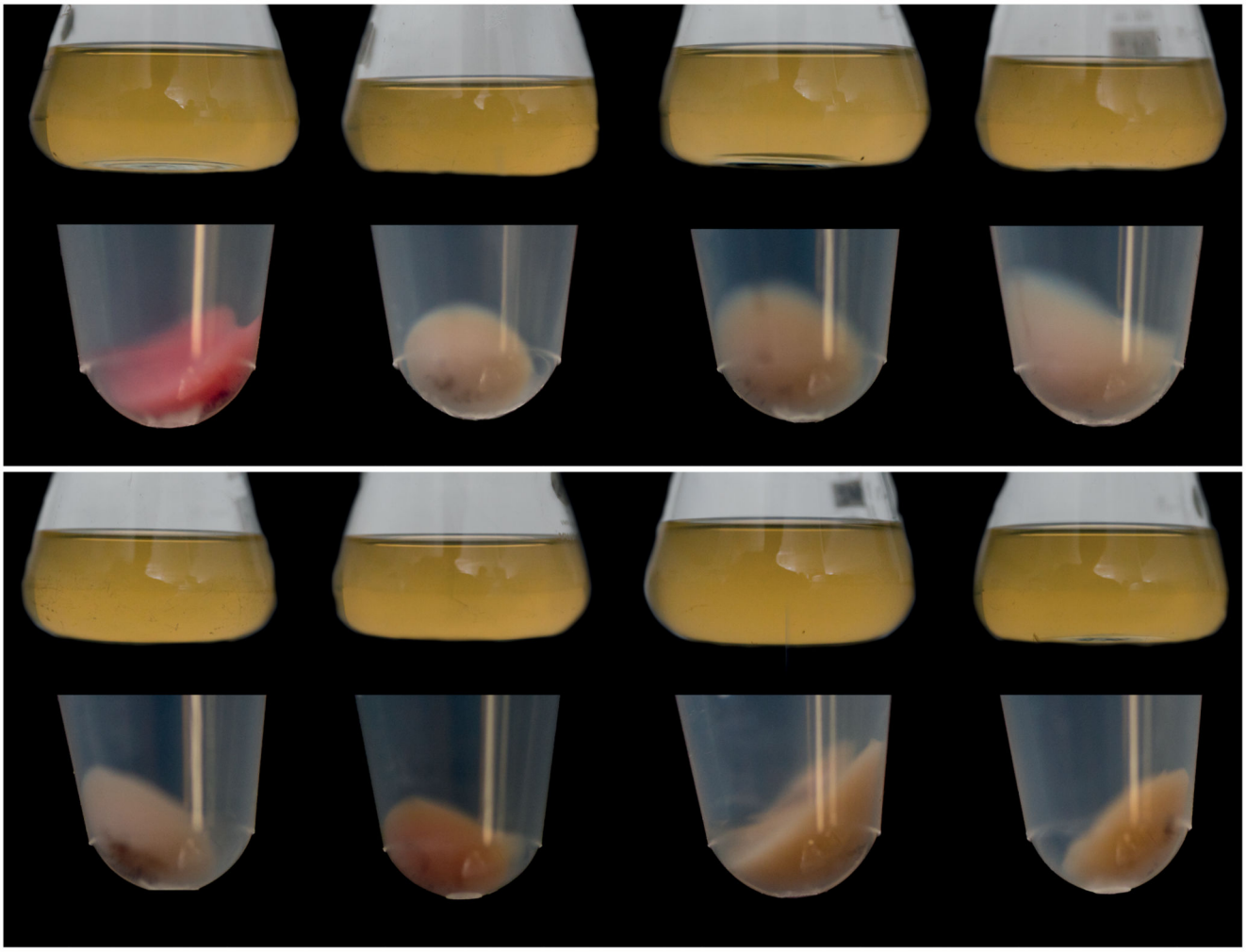

Fig. 2.

Pigmentation of strain AP-Melu-1000-B4^{T} and the seven free-living *Polynucleobacter* type strains affiliated with subcluster PnecC. First line: Erlenmeyer flask after 7 days cultivation in liquid NSY-medium. Below: pellet after centrifugation. From left to right: *Polynucleobacter meluiroseus* sp. nov. AP-Melu-1000-B4^{T}, *P. aenigmaticus* MWH-K35W1^{T}, *P. sphagniphilus* MWH-Weng1-1^{T}, *P. wuianus* QLW-P1FAT50C-4^{T}, *P. asymbioticus* QLW-P1DMWA-1^{T}, *P. duraquae* MWH-MoK4^{T}, *P. sinensis* MWH-HuW1^{T}, *P. yangtzensis* MWH-JaK3^{T}

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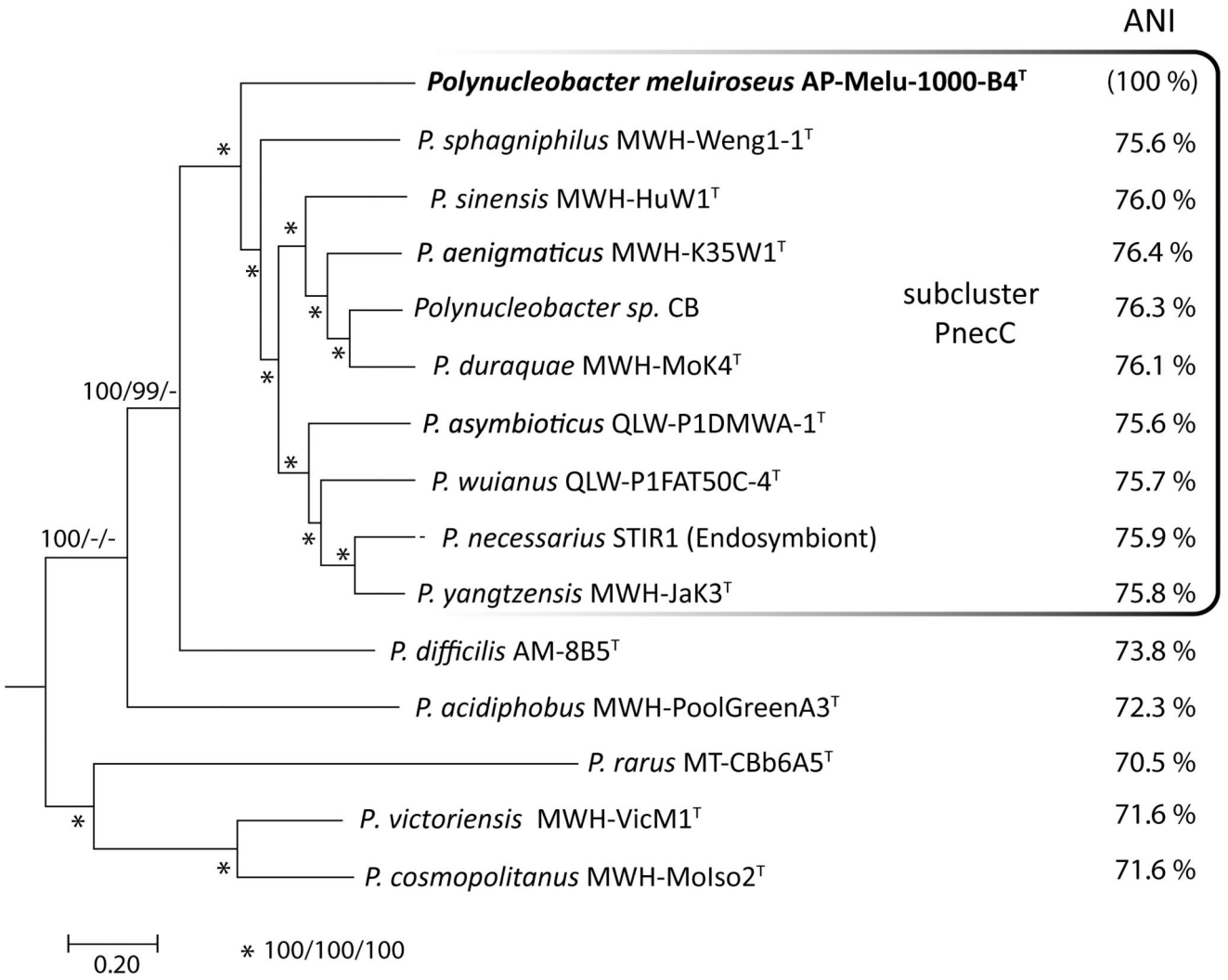

Fig. 3.

Phylogenetic analyses of strain AP-Melu-1000-B4^T. RAxML tree calculated with gene sequences of 319 shared genes. Bootstrap values are shown from left to right for maximum likelihood, neighbour joining, and maximum parsimony trees calculated with the same sequence set. Percentage values behind the strain names indicate gANI values obtained in comparison with strain AP-Melu-1000-B4^T. The tree was rooted with *Cupriavidus metallidurans* CH34^T (not shown, accession number: CP000352-CP000355 [43]). Bar, 0.2 substitutions per nucleotide position; Asterisk, bootstrap values 100/100/100

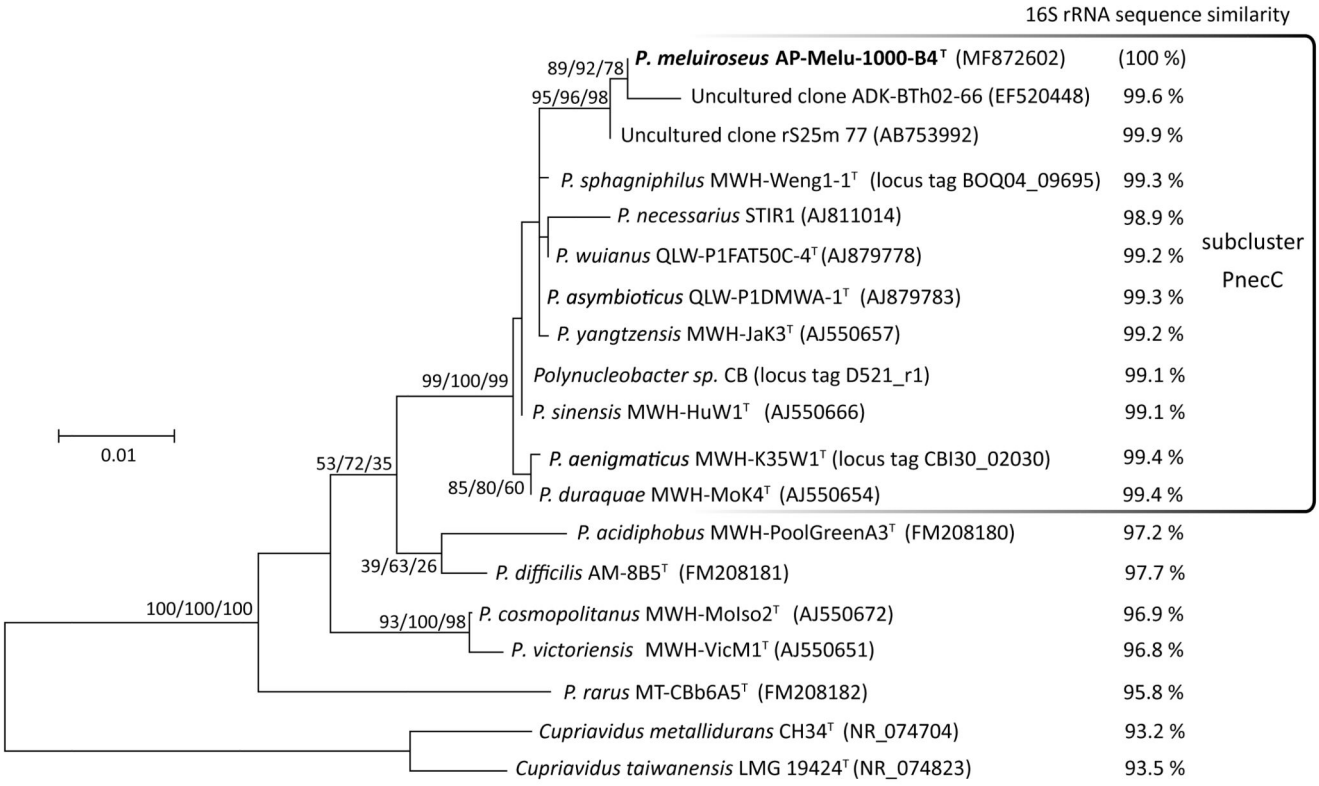

Fig. 4.

Reconstruction of the phylogenetic position of strain AP-Melu-100-B4^T based on almost full length 16S rRNA gene sequences (1407 alignment positions). Shown is a maximum likelihood tree. Bootstrap values are shown from left to right for ML, neighbour joining, and maximum parsimony trees calculated with the same sequence set. Percentage values behind the strain names indicate 16S rRNA gene sequence similarity values obtained in comparison with strain AP-Melu-1000-B4^T. Bar, 0.01 substitutions per nucleotide position.

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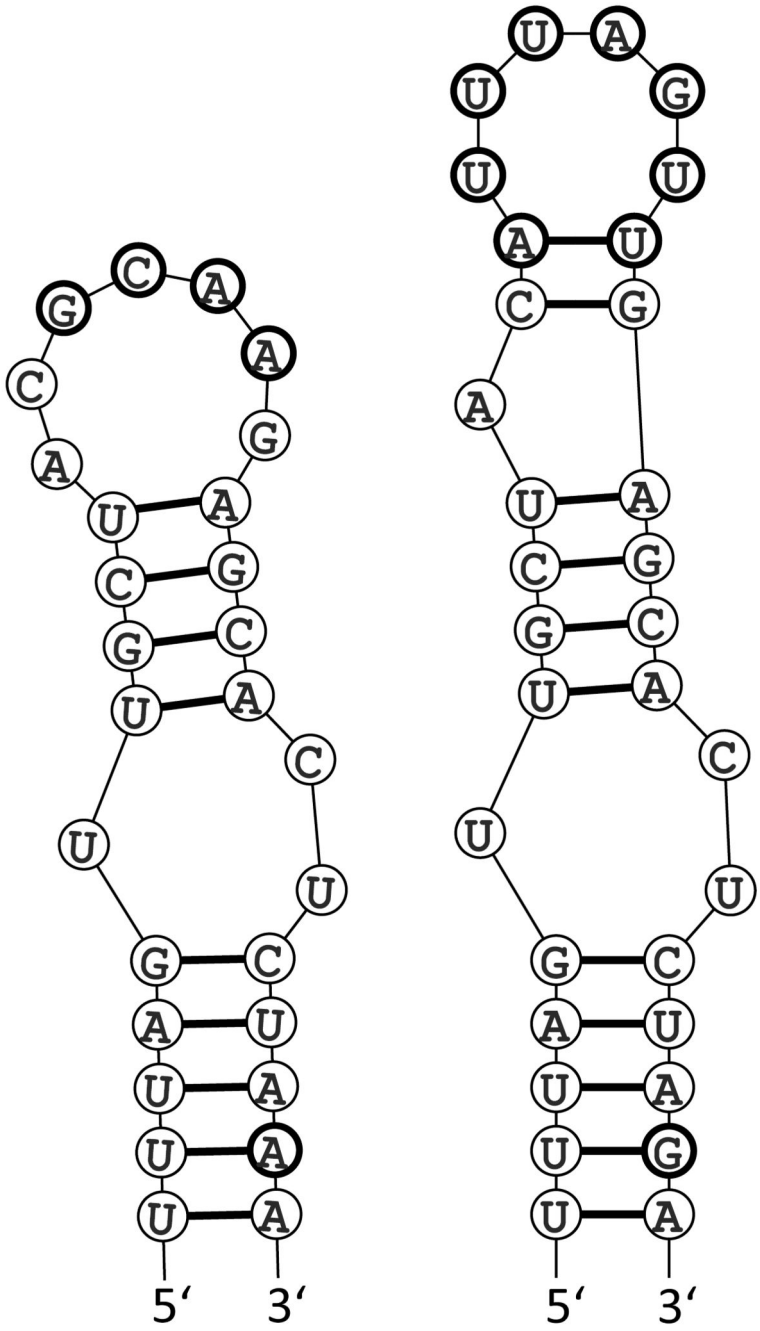

Fig. 5.

Part of the reconstructed secondary structure of 16S rRNA molecules of the seven free-living *Polynucleobacter* type strains affiliated with subcluster PnecC (left) and AP-Melu-1000-B4^{T} (*E. coli* position 1120-1153). Probability 90-100 %. Differing nucleotides with bold border. An alignment of the depicted region is presented in Fig. 6.

Clone rS25m 85 (AB753990)	Clone rS25m 77 (AB753992)	Clone rS25m 65 (AB753994)
Clone rS25m 13 (AB753991)	Clone rS35m 41 (AB754103)	Clone rS35m 65 (AB754104)
Clone rS35m 29 (AB754105)	Clone rS43m 90 (AB754176)	Clone ADK-BTh02-66 (EF520448)
Clone Aixeus_E5 (HE856857)	Clone Aixeus A7 (HE856827)	Clone Aixeus A9 (HE856829)
Clone Aixeus C10 (HE856838)	Clone Aixeus C7 (HE856843)	Clone Aixeus C8 (HE856844)
Clone Aixeus D12 (HE856847)	Clone Aixeus D4 (HE856848)	Clone Aixeus E7 (HE856859)
Clone Aixeus F10 (HE856862)	Clone Aixeus F4 (HE856864)	Clone Aixeus G11 (HE856871)
Clone Aixeus H4 (HE856881)		

Fig. 6.

Alignment of partial 16S rRNA gene sequences (*E. coli* 1120-1153) First line: Sequence of the seven free-living *Polynucleobacter* type strains affiliated with subcluster PnecC, second line: strain AP-Melu-1000-B4^{T} and sequences from 22 uncultured clones (list below) containing the same indel.

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Table 1

Traits characterizing strain AP-Melu-1000-B4^T and the seven free-living *Polynucleobacter* type strains affiliated with subcluster PnecC. All strains have the following characteristics in common: assimilation of acetic acid, pyruvic acid and succinic acid; no assimilation of glycolic acid and citric acid. 1, *Polynucleobacter meluiroseus* sp. nov. AP-Melu-1000-B4^T, 2, *P. aenigmaticus* MWH-K35W1^T, 3, *P. sphagniphilus* MWH-Weng1-1^T, 4, *P. wuianus* QLW-P1FAT50C-4^T, 5, *P. asymbioticus* QLW-P1DMWA-1^T, 6, *P. duraquae* MWH-MoK4^T, 7, *P. sinensis* MWH-HuW1^T, 8, *P. yangtzensis* MWH-JaK3^T. +, increase in optical density (OD); w, weak increase in OD; –, no significant increase in OD. All data were investigated by the authors under the same laboratory conditions, some were published previously: 2 [31], 3 [25], 4 [24], 5, 6, 7, 8 [13].

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Cell morphology	rods	short rods	short rods	short rods	short rods	curved rods	short curved rods	short rods
Cell length (µm)	0.8-2.1	0.5-1.0	0.6-1.0	0.6-1.7	0.7-1.2	0.9-2.9	0.6-1.4	0.5-1.5
Cell width (µm)	0.3-0.6	0.3-0.5	0.3-0.5	0.3-0.6	0.4-0.5	0.4-0.5	0.4-0.5	0.3-0.5
Temperature range of growth (°C)	5-28 (w)	5-32 (w)	5 - 31	5 - 34	5 - 34(w)	5 - 30	5 - 35	5 - 35
NaCl tolerance (%NaCl, w/v)	0-0.4 (w)	0-0.3	0 - 0.4	0 - 0.5	0 - 0.5(w)	0 - 0.3	0 - 0.5	0 - 0.3(w)
Anaerobic growth	-	-	-	-	+	-	-	+
Assimilation of:								
Glyoxylic acid	-	-	-	w	w	-	-	-
Propionic acid	+	w	w	+	+	-	+	+
Malonic acid	w	+	+	w	w	-	+	w
Oxaloacetic acid	+	-	-	+	-	+	+	+
Malic acid	+	+	+	+	+	w	+	+
Fumaric acid	+	+	+	+	+	w	+	+
Oxalic Acid	-	w	-	-	-	-	-	-
Levulinic acid	w	-	w	w	w	-	-	-
D-Galacturonic acid	-	w	+	w	w	w	w	w
D-Mannose	-	-	w	-	w	-	-	-
D-Glucose	-	w	-	-	w	w	-	-
D-Galactose	-	-	-	-	w	-	-	-
D-Lyxose	-	w	-	-	w	w	-	w
D-Fructose	-	-	w	w	w	w	-	w
L-Fucose	-	-	w	-	w	-	-	w
D-Sorbitole	w	-	w	-	w	-	-	-

	1	2	3	4	5	6	7	8
L-Glutamate	+	+	+	+	+	-	+	-
L-Histidine	-	-	-	+	-	w	-	-
L-Aspartate	w	-	-	+	+	-	-	-
L-Cysteine	+	+	+	w	+	+	w	+
L-Alanine	-	+	+	+	w	-	-	-
L-Asparagine	-	-	-	w	w	-	-	-
L-Leucine	-	-	-	-	-	w	-	w
L-Serine	-	w	-	-	-	-	-	-
Betaine	-	-	-	-	w	-	-	-

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Table 2

Major fatty acid compositions of *P. meluiroseus* and *Polynucleobacter* type strains representing subcluster PnecC. 1, *Polynucleobacter meluiroseus* sp. nov. AP-Melu-1000-B4^T, 2, *P. aenigmaticus* MWH-K35W1^T, 3, *P. sphagniphilus* MWH-Weng1-1^T; 4, *P. wuianus* QLW-P1FAT50C-4^T; 5, *P. asymbioticus* QLW-P1DMWA-1^T; 6, *P. duraquae* MWH-MoK4^T; 7, *P. sinensis* MWH-HuW1^T; 8, *P. yangtzensis* MWH-JaK3^T. Incubation times were eight days for 2, seven days for 1, 4, 6, 8, five days for 7, four days for 3, 5. Compounds at a percentage of 0.2 or higher are listed. All data were investigated by the authors in a single lab under the same laboratory conditions, and only data of strain 2 [31] and 3 [25] were published previously.

Fatty acid	1	2	3	4	5	6	7	8
$C_{12:0}$	4.8	3.4	4.4	4.0	3.9	4.3	4.7	4.1
$C_{14:0}$	-	0.5	1.4	0.2	0.6	0.5	0.4	1.0
$C_{15:0}$	-	-	-	-	0.3	-	-	-
$C_{16:0}$	30.0	18.0	26.2	18.5	25.5	16.8	27.5	19.9
$C_{17:0}$	-	0.6	-	-	-	-	-	-
$C_{18:0}$	1.0	1.8	0.9	0.9	0.7	0.4	1.3	0.6
$C_{20:0}$	-	-	-	-	0.7	-	-	-
$C_{14:1}$ ω5c	-	-	0.5	-	-	-	-	-
$C_{16:1}$ ω5c	-	-	0.4	-	-	-	-	0.4
$C_{16:1}$ ω7c (feature 3)	36.9	35.9	35.8	39.1	39.6	41.3	36.9	35.6
$C_{18:1}$ ω7c	10.5	19.2	15.1	27.8	15.6	18.9	14.1	20.6
11-methyl $C_{18:1}$ ω7c	4.2	7.9	4.3	2.7	2.6	4.5	3.1	6.4
$C_{12:0}$ 2-OH	1.3	-	1.9	1.0	1.8	0.6	1.3	1.9
$C_{16:1}$ 2-OH	-	2.2	0.5	0.5	-	2.0	-	0.3
Feature 2	10.2	9.8	8.7	4.7	8.6	9.8	10.9	8.0
Feature 7	1.0	0.3	-	0.4	-	0.8	-	1.0

Summed features represent groups of two fatty acids which could not be separated by GLC and the MIDI system, such as summed feature 2 containing $C_{16:1}$ isoI and $C_{14:0}$-3OH and summed feature 7 containing $C_{19:1}$ ω6c and an unknown compound with an ECL of 18.846.

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Table 3

Genome characteristics of strain AP-Melu-1000-B4^T, the seven free-living type strains of species affiliated with subcluster PnecC and other *Polynucleobacter* strains used for comparison and phylogenetic reconstructions.

Species	Strain	Lifestyle	Genome size (Mbp)	Scaffolds	G+C content (mol%)	DDBJ/EMBL/GenBank accession number	Reference
PnecC							
P. meluiroseus sp. nov.	AP-Melu-1000-B4^T (=DSM 103591T)	FL	1.89	11	46.6	OANS00000000	This study
P. aenigmaticus	MWH-K35K1^T (=DSM 24006^T)	FL	2.14	37	46.0	NGUO00000000	[31]
P. sphagniphilus	MWH-Weng1-1^T (=DSM 24018^T)	FL	2.04	17	45.6	MPIY01000000	[25]
P. wuianus	QLW-P1FAT50C-4^T (=DSM 24008^T)	FL	2.23	1	44.9	CP015922	[24]
P. asymbioticus	QLW-P1DMWA-1^T (=DSM 18221^T)	FL	2.16	1	44.8	CP000655	[6]
P. duraquae	MWH-MoK4^T (=DSM 21495^T)	FL	2.03	1	45.2	CP007501	[8]
P. sinensis	MWH-HuW1^T (=DSM 21492^T)	FL	2.32	19	45.5	LOJJ01000000	[8]
P. yangtzensis	MWH-JaK3^T (=DSM 21493^T)	FL	2.05	42	45.4	LOJI01000000	[8]
P. necessarius	STIR1 [host, *Euplotes aediculatus*]	E	1.56	1	45.6	CP001010	
Polynucleobacter sp.	CB	FL	2.04	1	46.1	CP004348	[41]
Others							
P. difficilis	AM-8B5^T (= DSM 22349^T)	FL	2.00	1	49.5	CP023276	This study
P. cosmopolitanus	MWH-MoIso2^T (= DSM 21490^T)	FL	1.77	6	44.1	NJGG00000000	[42]
P. rarus	MT-CBb6A5^T (= DSM 21648^T)	FL	3.16	2	39.9	NTGB00000000	This study
P. acidiphobus	MWH-PoolGreenA3^T (= DSM 21994^T)	FL	1.85	1	48.2	CP023277	This study
P. victoriensis	MWH-VicM1^T (=DSM 21486^T)	FL	1.63	3	43.1	FYEX00000000	[42]

FL, free-living; E, endosymbiotic

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Table 4

Comparison of the presence and absence of selected genes of strain AP-Melu-1000-B4^T and the seven free-living type strains of species affiliated with subcluster PnecC (references and accession numbers see table 3).

Genes putatively encoding	*P. meluiroseus* sp. nov. AP-Melu-1000-B4^T	*P. aenigmaticus* MWH-K35W1^T	*P. sphagniphilus* MWH-Weng1-1^T	*P. wuianus* QLW-P1FAT50C-4^T	*P. asymbioticus* QLW-P1DMWA-1^T	*P. duraquae* MWH-MoK4^T	*P. sinensis* MWH-HuW1^T	*P. yangtzensis* MWH-JaK3^T
Inorganic nutrients								
ABC-type Fe^{3+} transport system	-	+	-	-	-	+	+	+
feoAB genes (uptake of Fe^{2+})	+	+	+	+	+	-	+	+
ABC-type iron complex transport system	+	+	-	-	-	+	+	-
ABC-type Nitrate/Nitrite/Cyanate transporter	-	+	+	+	+	-	-	+
Nitrate reductase (assimilatory)	-	+	+	+	+	-	-	+
Nitrite reductase (assimilatory)	-	+	+	+	+	-	-	+
Cyanate lyase (releases NH_3 + CO_2 from cyanate)	-	+	+	+	+	-	-	+
Urease and ABC-type urea transporter	-	-	+	+	+	-	-	-
Oxidative phosphorylation/ Energy metabolism								
Cytochrome bd-I terminal oxidase (CydAB)	-	-	+	+	+	-	+	-
Fumarate reductase	-	+	+	+	-	+	-	+
Carbon monoxide dehydrogenase	-	+	+	+	-	2 clusters	-	+
Acetate permease actP	-	+	+	-	+	-	-	-
Anoxygenic photosynthesis								
Photosynthesis gene cluster	-	-	-	+	-	+	-	-
Motility								
Flagella genes	+	+	-	-	-	+	-	-
Oxidative stress								
Catalase	1 gene	1 gene	1 gene	-	2 genes	-	-	1 gene
Other								
Cellulose synthase operon protein C	-	-	-	-	+	-	-	-
Cellulose synthase catalytic subunit	-	-	-	-	+	-	-	-
Proteorhodopsin	+	+	-	-	-	-	-	-
Biosynthesis of 7,8 dihydro-ß-carotene	+	+	-	-	-	-	-	-
Beta-carotene 15,15'-monooxygenase	+	+	-	-	-	-	-	-

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Table 5

Whole genome average nucleotide identity (gANI) values of strain AP-Melu-1000-B4^T with the genomes of the seven free-living type strains of species affiliated with subcluster PnecC and the endosymbiont *P. necessarius* STIR1, which is also affiliated with this subcluster. Analyses were performed by using the IMG/ER system [19]. Exchanging of subject and query genome resulted in all pairwise calculations in identical gANI values, however the obtained alignment fractions (AFs) differed when query and reference genomes were exchanged.

Strain	gANI (%)	AF (%)
P. aenigmaticus MWH-K35W1^T	76,4	60^a/67^b
P. sphagniphilus MWH-Weng1-1^T	75,6	61^a/66^b
P. asymbioticus QLW-P1DMWA-1^T	75,6	58^a/66^b
P. yangtzensis MWH-JaK3^T	75,8	60^a/65^b
P. sinensis MWH-HuW1^T	76,0	53^a/65^b
P. duraquae MWH-MoK4^T	76,1	60^a/65^b
P. wuianus QLW-P1FAT50C-4^T	75,7	55^a/65^b
P. necessarius STIR1 (Endosymbiont)	75,9	66^a/54^b

^{a}Genome of AP-Melu-1000-B4^T used as the subject genome

^{b}Genome of AP-Melu-1000-B4^T used as the query genome