Published in final edited form as:
Int J Syst Evol Microbiol. 2017 November ; 67(11): 4646–4654. doi:10.1099/ijsem.0.002347.

Polynucleobacter aenigmaticus sp. nov. isolated from the permanently anoxic monimolimnion of a temperate meromictic lake

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Abstract

The bacterial strain MWH-K35W1^T was isolated from a permanently anoxic water layer of a meromictic lake located in the Austrian Salzkammergut area. The basically chemorganoheterotrophic strain was isolated and is maintained under aerobic conditions. Phylogenetic analyses of the 16S rRNA and the glutamine synthetase gene (glnA) of the strain suggested an affiliation to the genus *Polynucleobacter* and the cryptic species complex PnecC. The type strains of the six free-living *Polynucleobacter* species affiliated with this species complex share 16S rRNA gene sequence similarities of 99.6-99.9%, while the type material of the obligate endosymbiont *P. necessarius*, which is also affiliated with this complex, shares a gene sequence similarity of 99.1%. Genome sequencing resulted in a genome size of 2.14 Mbp and a G+C content of 45.98 mol%. Major fatty acids were $C_{16:1}$ ω7c, $C_{18:1}$ ω7c and $C_{16:0}$. This strain is the first *Polynucleobacter* strain found to encode a proteorhodopsin-like protein, but in contrast to some other strains affiliated to this genus, does not encode a putative anoxygenic photosynthesis system. Multilocus sequence analysis based on partial sequences of eight housekeeping genes, as well as average nucleotide identity (ANI) analyses, did not suggest that strain MWH-K35W1^T belongs to a previously described species. We propose the name *Polynucleobacter aenigmaticus* for the novel species and strain MWH-K35W1^T (=DSM 24006^T, = LMG 29706^T) as the type strain.

The genus *Polynucleobacter* was established by Heckmann and Schmidt [1] to accommodate obligate endosymbionts of ciliates affiliated with the genus *Euplotes*. Later it was discovered that a substantial fraction of freshwater bacterioplankton is represented by strains closely related to these obligate endosymbionts [2–5]. Investigations by fluorescent *in situ*

DDBJ/EMBL/GenBank accession numbers
Polynucleobacter aenigmaticus sp. nov. MWH-K35W1T: NGUO00000000 (genome) and CBI30_02030 (Locus Tag of 16S rRNA gene)

Conflicts of Interest
The authors declare the absence of any conflict of interest.

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

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hybridization (FISH) with taxon-specific probes demonstrated that these bacteria possess a free-living planktonic lifestyle [3, 6, 7] thus differ in this trait from the related endosymbionts. Comparative genome analyses of a planktonic [8, 9] and an endosymbiotic strain [10] revealed that the endosymbiotic strain represents an evolutionary derivative form undergoing reductive genome evolution. Isolation of free-living strains from various freshwater systems [11] enabled the description of ten new *Polynucleobacter* species recently [12–18]. The majority of these species are affiliated with subcluster PnecC [11], which represents a large cryptic species complex characterized by 16S rRNA gene sequence similarity values ≥ 99% [16, 19].

Here we characterize strain MWH-K35W1^T, which is also affiliated with subcluster PnecC but differs in some interesting aspects from all other so far investigated *Polynucleobacter* strains, and propose for this strain the novel species name *Polynucleobacter aenigmaticus* sp. nov.

Home habitat and isolation

In contrast to all previously investigated *Polynucleobacter* strains, strain MWH-K35W1^T was isolated from an anoxic water sample. This strain was isolated from meromictic Lake Krottensee [5] located in the Salzkammergut lake district in Austria. The water body of meromictic lakes is lacking a complete mixing resulting in a permanently anoxic deep water zone called monimolimnion. Meromictic lakes represent a rather rare lake type. Lakes located in the temperate climatic zones, which develop a temporarily anoxic deep water zone (hypolimnion) usually mix completely (down to the maximum depth) at least once or twice a year. This complete mixing is resulting in reestablishment of oxic conditions in the deeper water layers. Previous investigations [5, 20] and the observed increase of conductivity with depth in investigations of 2003 (Supplementary Materials Fig. S1) and 2007 [5] suggest that water layers of Lake Krottensee deeper than about 15-20 meters down to the maximum depth of 46 m were most likely permanently anoxic since centuries. Interestingly, an abundance peak of PnecC bacteria of about 10^5 cells per mL was found in the monimolimnion of Lake Krottensee at a depth of 23 meters previously [5]. Actually, the depth profile presented by Jezberova and colleagues suggests that PnecC abundance was at the day of investigation by average higher in anoxic than in oxic water layers. Strain MWH-K35W1^T was isolated from a water sample taken from a depth of 35 meters (Supplementary Materials Fig. S1). This sample was characterised by a temperature of 6.0 °C, a pH of 7.6, conductivity of 431 µS cm^{-1} and lack of dissolved oxygen. The strain was isolated by using the filtration-acclimatization method and Nutrient-Broth-Soytone-Yeast-Extract (NSY) medium [21] under aerobic conditions.

Phenotypic and chemotaxonomic characterization

Cells of strain MWH-K35W1^T are short rods of small size (Table 1). The strain forms small circular, convex, colourless colonies with shiny surface on NSY agar plates. Growth at different temperatures and growth under anoxic conditions in an anaerobic chamber were examined by using NSY agar plates as described previously [22]. Anaerobic growth was tested by using standard NSY agar plates and NSY plates with increased nitrate

concentrations (2 g l^{-1} $NaNO_3$). Salinity (NaCl) tolerance was determined using NSY agar supplemented with various NaCl concentrations as described previously [22]. The strain showed with both media no anaerobic growth, grew well at temperatures up to 31°C, but only weakly at 32°C, and tolerated salt concentrations up to 0.3% (Table 1).

Utilization of various substrates was investigated in the same way as for previously described *Polynucleobacter* species [12–18]. Briefly, growth enabled by utilization of a specific substrate was determined by comparison of optical densities (OD) at 575 nm established in liquid one tenth-strength NSY medium (0.3 g l^{-1}) with and without 0.5 g l^{-1} test substrate, respectively. Differences of < 10 %, 10–50% and >50% of the OD_{575nm} obtained in the test treatments compared to the OD_{575nm} obtained without test substrate (i.e. in 0.3 g l^{-1} NSY medium) were scored after 8 - 12 days of growth as no utilization (-), weak utilization (w) and good utilization (+), respectively. Results of the substrate assimilation experiments are presented in Table 1.

The analysis of the whole-cell fatty acid composition (Table 2) was carried out as described previously [18]. Biomass was grown on R2A medium at 28°C. The cultures had been inspected for growth daily, starting the third day after inoculation. Once biomass was well visible, the cell mass was collected. As its phylogenetic neighbours, strain MWH-K35W1^T contained as the main components $C_{16:1}$ ω7c, $C_{18:1}$ ω7c, $C_{16:0}$ and feature 2 comprising $C_{16:1}$-isoI and $C_{14:0}$-3OH. Conspicuous is the lack of the hydroxylated compound $C_{12:0}$ 2-OH, which is also absent in members of the subcluster PnecD, namely all investigated strains currently classified as *P. cosmopolitanus* [12].

Genomic characterization

Extraction of DNA of strain MWH-K35W1^T, whole genome sequencing, assembly and annotation was performed as described previously [18]. The genome of strain MWH-K35W1^T does not differ in basic characteristics from genomes of other PnecC type strains (Table 3). All PnecC genomes investigated so far share a small genome size of about 2 Mbp and a G+C content of 45 - 46 mol%. By contrast, the new type strain differed pronouncedly in gene content from all PnecC genomes analysed so far (Table 4). An unusual feature of the genome of MWH-K35W1^T is the presence of a putative proteorhodopsin gene (Genbank Accession WP_088526317, IMG Gene ID 2676729459). While gene clusters putatively encoding anoxygenic photosynthesis were found in some *Polynucleobacter* strains previously [17, 19, 23], proteorhodopsin genes were not reported from strains representing this genus so far.

Gene content suggests several adaptations to growth under anoxic conditions which were partly not found in other *Polynucleobacter* strains previously. The genome of strain MWH-K35W1^T encodes a putative fumarate reductase possibly enabling fumarate respiration (Table 4). Furthermore, genes putatively encoding a respiratory membrane-bound nitrate reductase (NarGHIJ) and a NADH-dependent nitrite reductase (NirBD) were found. This suggests a potential for denitrification with ammonia as final product. In contrast to the respiratory nitrate reductase NrfA found in some other organisms performing incomplete denitrification, nitrite reduction by NirBD is coupled to oxidation of NADH and not to an

electron transport chain (quinone pool) involved in energy conversion [24]. This could either suggest that NirBD has only a detoxification function in the incomplete nitrate respiration of strain MWH-K35W1^T, or that the main function of the gene is assimilatory nitrite reduction. The genes for the respiratory nitrate reductase NarGHIJ belong to a gene cluster of ten genes also including a two-component histidine kinase system (NarL family) for sensing nitrate and nitrite, a nitrate/nitrite transporter of the major facilitator superfamily, a carbonic anhydrase and nitroreductase putatively enabling utilization of nitro residues as nitrogen source. The two component nitrate/nitrite sensor could be involved in upregulation of the putative dissimilatory nitrate reduction and down regulation of the putative fumarate reductase. Interestingly, the genes encoding the putative NADH-dependent nitrite reductase (NirBD) are not included in this gene cluster but group together with genes encoding the putative assimilatory nitrate reductase NasAB, which could suggest that NirBD has indeed primarily an assimilatory function. Interestingly, no sensor for oxygen concentrations/availability were found in the genome of the strain. Strain MWH-K35W1^T shares with *P. sinensis* MWH-HuW1^T the gene cluster containing the respiratory nitrate reductase. Both gene clusters share the same gene content and are fully syntenic, but share only sequence similarities of about 80%. In contrast to strain MWH-K35W1^T strain MWH-HuW1^T does not encode a NirBD nitrite reductase (Table 4). Besides the mentioned nitrate/nitrite sensor, the genome encodes three putative two component sensors for nitrogen availability (putatively regulating expression of the glutamine synthetase), a K^+ limitation sensor (but seem to lack a potassium transporter to be regulated), and a phosphate limitation sensor. Interestingly, the genome encodes many genes for flagella synthesis and a putative chemotaxis system, however it is unknown which chemical gradients the strain may be tracking with this system. The putative flagella and chemotaxis genes are located in a 60 kbp cluster of about 70 genes.

Phylogeny

The genome of strain MWH-K35W1^T encodes only a single copy of the ribosomal operon. The partial (1361 bp) 16S rRNA gene sequence obtained by Sanger sequencing previously is identical with the full-length sequence of the gene obtained by the genome sequencing (Locus Tag CBI30_02030; IMG Gene ID 2676729854). The gene shares sequence similarities between 99.1% (*P. necessarius*) and 99.9% (*P. duraquae*) with type strains or type material of other species affiliated with the species complex PnecC. These similarity values correspond to 12 and 1 nucleotides sequence differences, respectively. Analysis of the 16S rRNA gene of strain MWH-K35W1^T confirmed the expected affiliation of the strain to subcluster PnecC of the genus *Polynucleobacter* (Fig. 1), however, as repeatedly demonstrated previously [16, 17, 19, 25], this phylogenetic marker is too conserved to resolve phylogenetic relationships among species of this subcluster. The affiliation of strain MWH-K35W1^T to subcluster PnecC was also confirmed by previous phylogenetic analyses with partial glutamine synthetase gene (glnA) sequences [26]. Due to lack of phylogenetic resolution of 16S rRNA gene sequences of PnecC strains at the species level, phylogenetic analyses of eight housekeeping genes (Table 5, Suppl. Material M1) were performed. These previously selected genes [16] are expected to belong to the core genome of *Polynucleobacter* bacteria and their horizontal transfer by genomic islands seems to be

unlikely [27]. The performed multilocus sequence analysis confirmed the affiliation of the strain to subcluster PnecC (Fig. 2) and further suggested that the next known relatives are *P. sinensis* [16] and *P. duraquae* [16]. The type strain of *P. duraque* was isolated from oxic surface water layers of Lake Mondsee which is a neighbouring habitat of Lake Krottensee. The affiliation of strain MWH-K35W1^T to a previously described species is not suggested by the reconstructed phylogeny.

Average nucleotide identity (ANI) analyses [28, 29] were performed in order to test if the new strain should be considered to belong to a previously described *Polynucleobacter* species. The type strains of all previously described PnecC species but *P. necessarius* [1] are represented by whole genome sequences. Due to it's obligatory endosymbiotic lifestyle [30] the species *P. necessarius* lacks a type strain and a genome sequence representing the type material [16]. However, a whole genome sequence is available for the *P. necessarius* strain STIR1, and multilocus sequences are available for strain Ammermann, which should be identical with the strain contained in the type material of the species [16]. Pairwise ANI comparisons with the type strains of all described species resulted in values ≤ 82% (Table 6) clearly suggesting that strain MWH-K35W1^T represents a new species [29].

Ecology

Strain MWH-K35W1^T differs from all previously investigated *Polynucleobacter* strains by its origin from a permanently anoxic water body. The phenotypic characterization did not reveal any adaptations to anaerobiosis. On the other hand, the performed genome analysis suggested that this strain is much better adapted to anaerobiosis than any previously investigated *Polynucleobacter* strain. Because of the presence of genes for an incomplete nitrate respiration and for fumarate respiration it is conceivable that the strain really dwells under permanently anoxic conditions. Whether the detected proteorhodopsin gene also plays a role in anaerobiosis of the strain is unknown. This is at least unlikely in the depths of 35 meters, from which the strain was isolated, however, the putative proteorhodopsin gene could play a role in growth of the strain in the upper anoxic layers of the monimolimnion. It is known that these layers of the lake are also inhabited by a dense layer of anoxygenic phototrophic bacteria [20].

While it is conceivable to assume that the strains dwells at least temporarily in anoxic water bodies, it is not known if the strain prefers these conditions and is competitive under such environmental conditions, or if it represents only a facultative anaerobe usually dwelling in oxic water layers. Further investigations will be required for revealing the enigmatic ecology of the strain.

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

The performed ANI analyses and the phylogenetic reconstruction clearly demonstrate that strain MWH-K35W1^T is not affiliated with any previously described *Polynucleobacter* species. Analyses of partial *glnA* sequences suggest, that strain MWH-K35W1^T is the only strain representing this new species in our collection of > 300 *Polynucleobacter* strains. This was suggested by *glnA* sequence comparison across the strains of the culture collection. The

highest found *glnA* sequence similarity between strain MWH-K35W1^T and any other strain of the collection was 93% nucleotide identity. Among the eight strains sharing about 93% *glnA* similarity, two were whole genome sequenced previously (Hahn et al., unpublished data). These two strains share only ANI values of 82% with MWH-K35W1^T, respectively, clearly suggesting that all these strains are not affiliated to the new species represented by the latter strain. Thus, the new species proposed here is only represented by a single isolate, which makes conclusions on typical species-specific traits of the new taxon preliminary.

Strain MWH-K35W1^T can be discriminated from *Polynucleobacter* species not affiliated with subcluster PnecC based on 16S rRNA phylogenies or sequence similarity values [11]. Among the type strains of so far described species of subcluster PnecC, strain MWH-K35W1^T is the only strain able to assimilate oxalic acid and L-serine (Table 1). The strain showed for these substrates an increase in OD_{575nm} of 35.4 and 15.3%, respectively, while most other strains investigated so far, responded to both substrates by growth inhibition resulting in negative OD_{575nm} values compared to the reference medium without test substance. Due to these pronounced differences in response to both test substrates, the discrimination of strains based on these tests should yield clear and objective results (Table 1).

We propose to establish for the investigated strain the new species *Polynucleobacter aenigmaticus* sp. nov. and to designate strain MWH-K35W1^T (= DSM 24006^T, = LMG 29706^T) as the type strain. The proposed species epithet indicates that important aspects of the ecology of the type strain are still enigmatic.

Description of *Polynucleobacter aenigmaticus* sp. nov.

Polynucleobacter aenigmaticus (ae.nig.ma'ti.cus. L. masc. adj. *aenigmaticus*, enigmatic, as it is unclear if the type strains is really able to inhabit permanently anoxic water layers).

Contains free-living *Polynucleobacter* strains dwelling in the water body of alkaline and circum-neutral freshwater systems, probably able to thrive in anoxic water layers.

Cells are short rods, 0.5 - 1.0 μm in length and 0.3 – 0.5 μm in width, depending on cultivation conditions. Chemoorganoheterotrophic, aerobic, no anaerobic growth detected, but gene content of the type strain suggests an ability for anaerobic respiration. Colonies grown on NSY agar are non-pigmented, circular and convex with smooth surface. Growth occurs up to 32 °C and in 0 – 0.3% (w/v) NaCl. Assimilates acetate, pyruvate, malonate, malate, fumarate, succinate, L-glutamate, L-cysteine, and L-alanine. Does not assimilate glycolate, glyoxylate, oxaloacetate, citrate, levulinic acid, D-mannose, D-galactose, D-fructose, L-fucose, D-sorbitol, L-leucine, L-histidine, L-aspartate, L-asparagine, or betaine. Weak assimilation of certain substrates was observed. Major fatty acids of the strain are $C_{16:1}$ ω7c, $C_{18:1}$ ω7c and $C_{16:0}$. The type strain is MWH-K35W1^T (= DSM 24006^T = LMG 29706^T), which was isolated from the monimolimnion of Lake Krottensee located in Austria. The genome of the type strain is characterized by a size of 2.14 Mbp and a G+C content of 45.98 mol%. Genome and 16S rRNA gene sequences characterizing the type strain are available under the accession NGUO00000000.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Gabriele Pötter for carrying out the fatty acid measurements, and the owner of Lake Krottensee for the permission to sample the lake in September 2003.

Funding Information

This study was supported by the Austrian Science Fund (FWF) project I482-B09 and the European Science Foundation (ESF) project FREDI.

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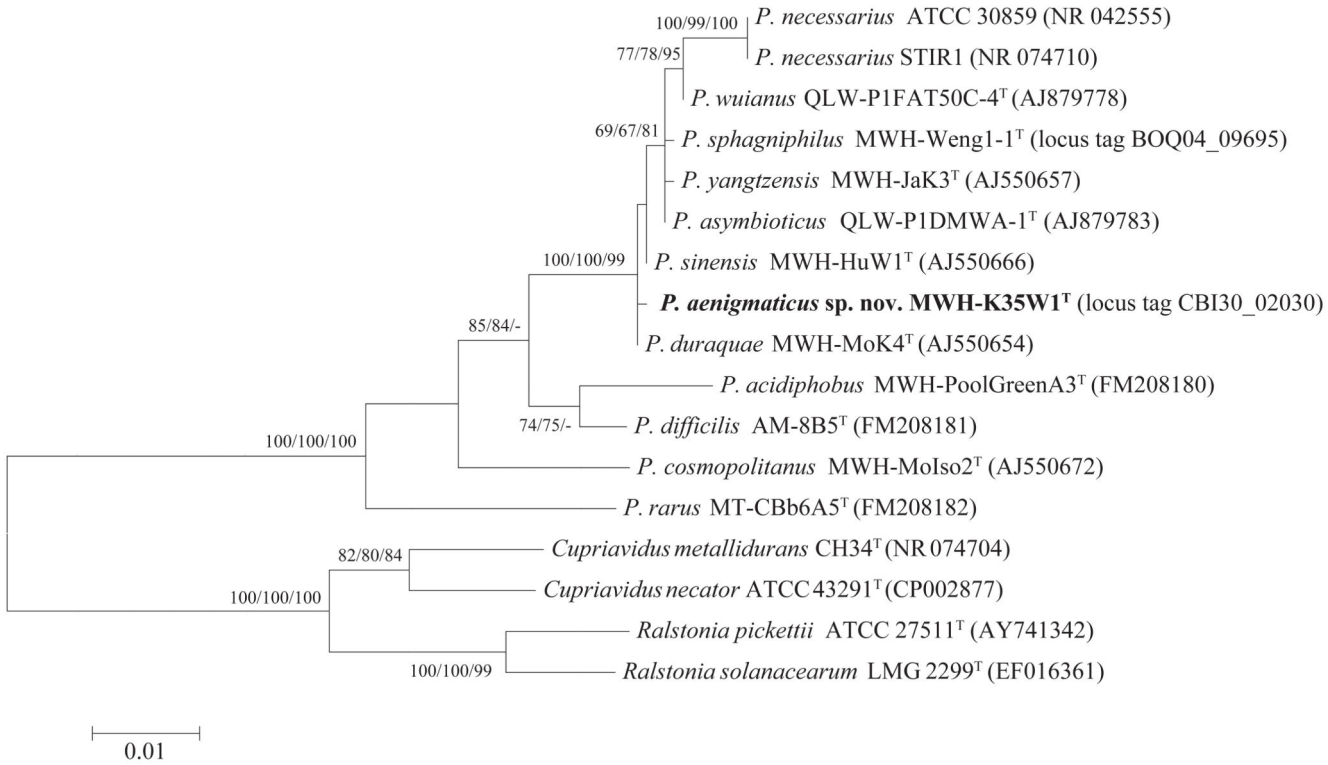

Fig. 1.

Reconstruction of the phylogenetic position of strain MWH-K35W1^T based on almost full length 16S rRNA gene sequences (1366 alignment positions). A maximum likelihood (ML) tree is shown, which includes bootstrap values of a neighbour-joining (NJ) and a maximum parsimony (MP) tree calculated with the same sequence alignment. Bootstrap values for 1000 iterations, respectively, refer from left to right to the NJ, the ML and the MP tree. Bar, 0.01 substitutions per nucleotide position.

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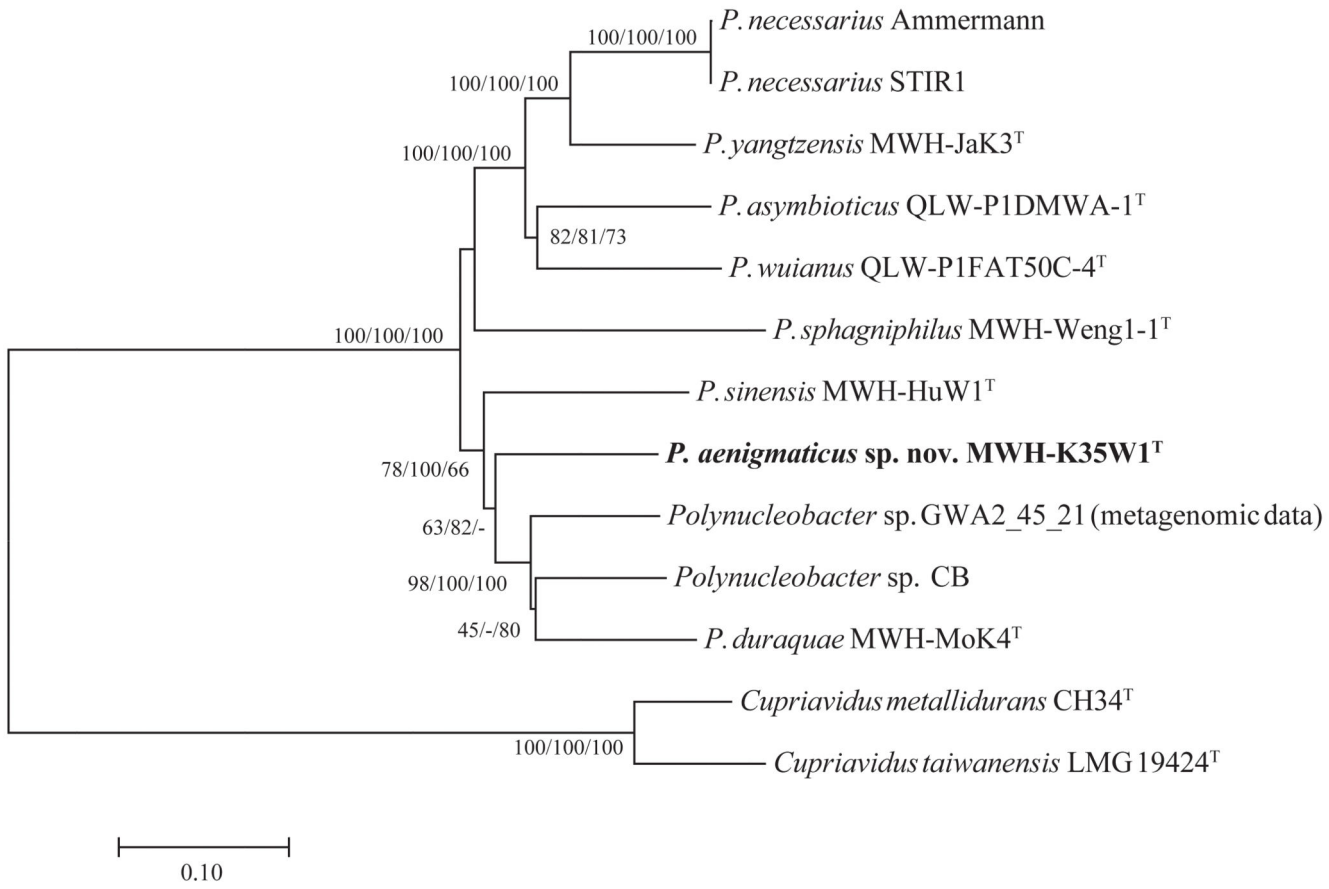

Fig. 2.

Reconstruction of the phylogenetic position of strain MWH-K35W1^T based on concatenated multilocus sequences of eight protein-encoding genes. A neighbour-joining (NJ) tree is shown, which includes bootstrap values of a maximum likelihood (ML) and a maximum parsimony (MP) tree calculated with the same sequence set. Bootstrap values refer from left to right to the NJ (1000 iteration), the ML (100 iteration) and the MP tree (1000 iteration). The utilized sequences were extracted from whole genome sequences (Suppl. Material Table S1). The alignment used for construction of the three trees had a length of 6249 alignment positions. Bar, 0.1 substitutions per nucleotide position.

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Table 1

Phenotypic characteristics of *Polynucleobacter* type strains. 1, strain MWH-K35W1^T; 2, *P. sphagniphilus* MWH-Weng1-1^T [18]; 3, *P. wuianus* QLW-P1FAT50C-4^T [17]; 4, *P. asymbioticus* QLW-P1DMWA-1^T [16]; 5, *P. duraquae* MWH-MoK4^T [16]; 6, *P. sinensis* MWH-HuW1^T [16]; 7, *P. yangtzensis* MWH-JaK3^T [16]. All strains have the following traits in common. DAPI stained cells show only rarely nucleoid-like structures; assimilation of acetic, succinic, and pyruvic acid; no assimilation of glycolic, and citric acid. +, increase in optical density (OD); w, weak increase in OD; -, no significant increase in OD.

	1	2	3	4	5	6	7
Cell morphology	short rods	short rods	short rods	short rods	curved rods	short curved rods	short rods
Cell length (µm)	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 - 0.5	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 – 32(w)	5 - 31	5 - 34	5 - 34(w)	5 - 30	5 - 35	5 - 35
NaCl tolerance (%NaCl, w/v)	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
Oxaloacetic acid	-	-	+	-	+	+	+
Malic acid	+	+	+	+	w	+	+
Fumaric acid	+	+	+	+	w	+	+
Oxalic acid	w	-	-	-	-	-	-
Levulinic acid	-	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	-	-	-
L-Glutamate	+	+	+	+	-	+	-
L-Histidine	-	-	+	-	w	-	-
L-Aspartate	-	-	+	+	-	-	-
L-Cysteine	+	+	w	+	+	w	+
L-Alanine	+	+	+	w	-	-	-
L-Asparagine	-	-	w	w	-	-	-
L-Leucine	-	-	-	-	w	-	w
L-Serine	w	-	-	-	-	-	-
Betaine	-	-	-	w	-	-	-

Table 2

Fatty acid composition of *Polynucleobacter aenigmaticus* MWH-K35W1^T and phylogenetic relatives. Compounds at a percentage of 0.2 or higher are listed. Strains were grown on R2A agar slants filled with 2 ml liquid R2A medium. Data for *P. duraquae* MWH-Mok4^T and *P. sinensis* MWH-HuW1^T were taken from Hahn *et al.* (2016) [16], and those for *P. sphagniphilus* MWH-Weng1-1^T were taken from Hahn *et al.* (in press) [18]. Incubation times were, from left to right column, 8, 4, 6 and 5 days. Data for more distantly related *Polynucleobacter* species can be found elsewhere [12–17].

Fatty acid	MWH-K35W1^T DSM 24006^T	MWH-Weng1-1 ^{T} DSM 24018 ^{T}	MHW-MoK4 ^{T} DSM 21495 ^{T}	MWH-HuW1 ^{T} DSM 21492 ^{T}
$C_{12:0}$	3.4	4.4	3.8	4.7
$C_{14:0}$	0.5	1.4	0.3	0.3
$C_{16:0}$	18.0	26.2	15.9	27.5
$C_{17:0}$	0.6	-	-	-
$C_{18:0}$	1.8	0.9	0.5	1.3
$C_{14:1}$ ω5c	-	0.5	-	-
$C_{16:1}$ ω5c	-	0.4	0.4	-
$C_{16:1}$ ω7c (feature 3)	35.9	35.8	38.6	36.9
$C_{18:1}$ ω9c	-	-	0.3	-
$C_{18:1}$ ω7c	19.2	15.1	19.8	14.1
11-methyl $C_{18:1}$ ω7c	7.9	4.3	4.2	3.1
$C_{12:0}$ 2-OH	-	1.9	1.3	1.3
$C_{16:1}$ 2-OH	2.2	0.5	1.8	-
Feature 2	9.8	8.7	11.9	10.9
Feature 7	0.3	-	-	-

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, including its presumptive derivative, $C_{12:0}$ aldehyde, and summed feature 7 (ECL of 18.848) containing $C_{19:1}$ ω6c and an unknown compound.

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Table 3

Genome characteristics of the investigated *Polynucleobacter* strains. All *Polynucleobacter* genomes investigated so far have in common that they encode only one copy of the ribosomal operon.

Species	Strain	Life style	Genome size (Mbp)	Scaffolds	G+C content (mol%)	DDBJ/EMBL/GenBank accession number	Reference
P. aenigmaticus sp. nov.	MWH-K35W1^T (=DSM 24006^T)	FL	2.14	37	46.0	NGUO00000000	This study
P. sphagniphilus	MWH-Weng1-1^T (=DSM 24018^T)	FL	2.04	17	45.6	MPIY01000000	Hahn *et al.*, in press
P. wuianus	QLW-P1FAT50C-4^T (=DSM 24008^T)	FL	2.23	1	44.9	CP015922	Hahn *et al.*, 2017
P. asymbioticus	QLW-P1DMWA-1^T (=DSM 18221^T)	FL	2.16	1	44.8	CP000655	Meincke *et al.*, 2012
P. duraquae	MWH-MoK4^T (=DSM 21495^T)	FL	2.03	1	45.2	CP007501	Hahn *et al.*, 2016b
P. sinensis	MWH-HuW1^T (=DSM 21492^T)	FL	2.32	19	45.5	LOJJ01000000	Hahn *et al.*, 2016a
P. yangtzensis	MWH-JaK3^T (=DSM 21493^T)	FL	2.05	42	45.4	LOJI01000000	Hahn *et al.*, 2016a
P. necessarius	STIR1 [host, *Euplotes aediculatus*]	E	1.56	1	45.6	CP001010	Boscaro *et al.*, 2013

FL, free-living; E, endosymbiotic

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Table 4

Comparison of the presence and absence of selected genes in strain MWH-K35W1^T and the type strains of the six species of free-living bacteria affiliated with subcluster PnecC of the genus *Polynucleobacter*.

Genes putatively encoding	*P. aenigmaticus* sp. nov. 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 Nitrate/Nitrite/Cyanate transporter	+	+	+	+	-	-	+
Nitrate reductase NasAB (assimilatory)	+	+	+	+	-	-	+
Nitrite reductase NirBD (assimilatory?)	+	+	+	+	-	-	+
Cyanate lyase	+	+	+	+	-	-	+
Urease and ABC-type urease transporter	-	+	+	+	-	-	-
Oxidative phosphorylation/energy metabolism							
Cytochrome bd-I terminal oxidase (CydAB)	-	+	+	+	-	+	-
Fumarate reductase	+	+	+	-	+	-	+
Nitrate reductase NarGHIJ (respiratory)	+	-	-	-	-	+	-
Carbon monoxide dehydrogenase	+	+	+	-	2 clusters	-	+
Anoxygenic photosynthesis							
Photosynthesis gene cluster	-	-	+	-	+	-	-
Proteorhodopsin gene	+	-	-	-	-	-	-
Motility and chemotaxis							
Flagella genes	+	-	-	-	+	-	-
Chemotaxis genes	+	-	-	-	-	-	-
Oxidative stress							
Catalase	1 gene	1 gene	-	2 genes	-	-	1 gene

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Table 5

Loci used for the multilocus sequence analysis presented in Fig. 2. The table lists the loci of two strains, the not genome sequenced endosymbiont *P. necessarius* strain Ammermann and the genome sequenced type strain of *P. asymbioticus* [8]. For the latter, the old and new Genbank locus tags and the Integrated Microbial Genomes (IMG) Gene ID [31] are provided. A complete alignment of the eight genes is provided as Supplementary Materials.

Locus	Gene product	*P. necessarius* strain Ammermann genes (GenBank Accessions)	*P. asymbioticus* QLW-P1DMWA-1^T (locus tag)[§]	*P. asymbioticus* QLW-P1DMWA-1^T (locus tag)[§§]	*P. asymbioticus* QLW-P1DMWA-1^T (IMG Gene ID)
glnA	Glutamine synthase	LN998990	PNUC_RS06585	Pnuc_1255	640471426
gyrA	DNA gyrase subunit A	LN998991	PNUC_RS02575	Pnuc_0493	640470647
icdA	Isocitrate dehydrogenase [NADP] (EC 1.1.1.42)	LN998992	PNUC_RS01920	Pnuc_0366	640470517
mdh	Malate dehydrogenase (EC 1.1.1.37)	LN998993	PNUC_RS03945	Pnuc_0756	640470913
msbA	Lipid A export ATP-binding/permease protein	LN998994	PNUC_RS03185	Pnuc_0610	640470763
pepN (*fbp*)	Fructose-1,6-bisphosphatase, type I (EC 3.1.3.11)	LN998995	PNUC_RS03485	Pnuc_0669	640470823
rpoB	DNA polymerase III beta subunit	LN998996	PNUC_RS00010	Pnuc_0002	640470144
trpE	Anthranilate synthase, aminase component (EC 4.1.3.27)	LN998997	PNUC_RS00805	Pnuc_0148	640470301

[§]GenBank locus tag

[§§]old GenBank locus tag, = IMG locus tag

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Table 6

Whole genome Average Nucleotide Identity (gANI) values of strain MWH-K35W1^T with the genomes of the endosymbiont *P. necessarius* STIR1 and six free-living type strains of species affiliated with subcluster PnecC, respectively. Analyses were performed by using the IMG/ER system [31]. Exchanging of subject and query genome resulted in all pairwise calculations in almost identical gANI values and only slightly different Alignment Fractions (AF) values.

Strain	gANI (%)[a]	AF (%)[a]
P. sphagniphilus MWH-Weng1-1^T	77.01	0.62
P. wuianus QLW-P1FAT50C-4^T	77.72	0.68
P. yangtzensis MWH-JaK3^T	78.15	0.69
P. sinensis MWH-HuW1^T	79.91	0.72
P. duraquae MWH-MoK4^T	81.97	0.73
P. asymbioticus QLW-P1DMWA-1^T	77.63	0.65
P. necessarius STIR1 (Endosymbiont)	78.06	0.55

[a]Genome of MWH-K35W1^T served as query genome