Published in final edited form as:
Int J Syst Evol Microbiol. 2018 August ; 68(8): 2593–2601. doi:10.1099/ijsem.0.002880.

Polynucleobacter hirudinilacicola sp. nov. and *Polynucleobacter campilacus* sp. nov. both isolated from freshwater systems

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Abstract

Strains MWH-EgelM1-30-B4^T and MWH-Feld-100^T were isolated from the water columns of two freshwater systems. Both strains represent delicate bacteria not easy to work with in laboratory experiments. Phylogenetic analyses of the 16S rRNA genes suggested that both strains were affiliated with the genus *Polynucleobacter*. Both strains share 16S rRNA sequence similarities of > 99% with eight free-living *Polynucleobacter* type strains, all affiliated with the cryptic species complex PnecC. The full length 16S rRNA gene sequences of the two strains differ only in two and three positions, respectively, from the sequence of the closest related *Polynucleobacter* type strain. Genome sequencing of both strains revealed relatively small genome sizes of 2.0 Mbp and G+C contents of 45 mol%. Phylogenetic analyses based on nucleotide sequences of 319 shared protein-encoding genes consistently placed the two strains in taxon PnecC but did not suggest an affiliation with one of the previously described species. Pairwise analyses of whole genome average nucleotide identities (gANI) with representatives of all previously described *Polynucleobacter* species resulted in both cases throughout in values < 80%. Pairwise comparison of the genomes of the two new strains resulted in gANI values of 83.3%. All gANI analyses clearly suggested that strains MWH-EgelM1-30-B4^T and MWH-Feld-100^T represent two novel *Polynucleobacter* species. We propose for these novel species the names *P. hirudinilacicola* sp. nov. and *P. campilacus* sp. nov. and strain MWH-EgelM1-30-B4^T (=DSM 23911^T =LMG 30144^T) and MWH-Feld-100^T (=DSM 24007^T =LMG 29705^T) as the type strains, respectively.

The genus *Polynucleobacter* (family *Burkholderiaceae*, class *Betaproteobacteria*) and the species *P. necessarius* were described by Klaus Heckmann and Helmut J. Schmidt as obligate endosymbionts dwelling in ciliates affiliated with the genus *Euplotes* [1]. The

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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.

DDBJ/EMBL/GenBank accession numbers
Polynucleobacter hirudinilacicola sp. nov. MWH-EgelM1-30-B4^T: NAIA00000000 (genome) and FN429662 (16S rRNA gene); *Polynucleobacter campilacus* sp. nov. MWH-Feld-100^T: NGUP00000000 (genome) and MG952228 (16S rRNA gene)

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symbiosis between the ciliates and the bacteria is mutually obligate, that is both partners rely on each other and cannot survive if separated [1, 2]. The obligate endosymbiotic lifestyle of these bacteria prevented their cultivation when separated from their hosts. Therefore, the type species of the genus, *P. necessarius*, is not represented by a type strain but by type material consisting of endosymbionts contained in a culture of *E. aediculatus* [1]. Unfortunately, this type material is not anymore available from the American Type Culture Collection (ATCC) or from any other culture collection [3].

More than ten years after description of the genus, it was recognized that a large group of free-living bacteria also belongs to that genus [4–7]. These free-living *Polynucleobacter* bacteria are ubiquitously present in the water column of standing freshwater systems [8–14]. Strains affiliated with this genus could be isolated from freshwater systems across all climatic zones and continents [7, 15]. Unusual traits of *Polynucleobacter* bacteria are their ability to penetrate 0.2 μm filters [16] and only weak growth in standard media as compared to their closest relatives [17]. Furthermore, some *Polynucleobacter* strains possess genes putatively encoding an apparatus for anoxygenic photosynthesis [18–20] or proteorhodopsins [21, 22]. Recent investigations revealed that a part of the genus *Polynucleobacter* named subcluster PnecC [23] represents a large cryptic species complex not resolvable by analyses of 16S rRNA gene sequences [3, 19]. At the time of writing, fourteen *Polynucleobacter* species were described, which is including only one endosymbiotic species.

Here we describe two new members of subcluster PnecC and propose to establish for these strains the new species *P. hirudinilacicola* sp. nov. and *P. campilacus* sp. nov.

Home habitat and isolation

Strains MWH-EgelM1-30-B4^T and MWH-Feld-100^T were both isolated from the water columns of freshwater systems by using the filtration acclimation method (FAM) and NSY medium [24]. Strain MWH-EgelM1-30-B4^T was isolated from the smallest (0.4 hectare surface area) of four ponds and lakes called Egelseen forming a chain connected by running waters. These systems are located (47.966°N, 13.125°E) at an altitude of about 590 m near the town Mattsee in Austria. This shallow pond was sampled from the shore line on 21. April 2006. The water sample from which the strain was isolated was characterized by a pH of 8.0, a conductivity of 353 μS cm^{-1}, and a water temperature of 11.4 °C. The water was stained by humic substances. Repeated sampling of the pond confirmed the alkaline pH (range 7.6 – 8.0) and the brownish water colour, which is an unusual combination in lakes and ponds of this area [8].

Strain MWH-Feld-100^T was isolated from Lake Feldsee (47.871°N, 8.033°E) located in the Black Forest, Germany. This oligotrophic cirque lake is located at an altitude of 1109 meters, has a surface area of about 10 hectare and a maximum depth of 32 meter. This habitat is characterized by a circum-neutral pH (6.4 - 7.5), low conductivity (18 - 24 μS cm^{-1}), very high transparency (clear water lake) and low concentrations of humic substances. The lake was sampled from the shore line on 30. December 2006.

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Genomic characterization

DNA extraction, genome sequencing, assembly and annotation were performed for both strains as described previously for other *Polynucleobacter* type strains [18, 21, 25]. The genomes of strains MWH-EgelM1-30-B4^T and MWH-Feld-100^T are in their general characteristics very similar to previously characterized genomes of *Polynucleobacter* type strains affiliated with subcluster PnecC (Table 1). Nevertheless, they differed in their gene content from all other type strains affiliated with subcluster PnecC, even from the closest related type strain *P. wuianus* QLW-P1FAT50C-4^T (Fig. 1, Table 2) [3, 18, 21, 22, 25]. Genome sizes of strains MWH-EgelM1-30-B4^T and MWH-Feld-100^T were 2.01 and 1.98 Mbp, respectively, and G+C contents were 45.3 and 45.2 mol%, respectively. The genomes of the two new type strains shared the presence of a gene cluster putatively encoding the apparatus of anoxygenic photosynthesis with the type strains of *P. wuianus* (Table 2, [18]), *P. duraquae* [3], *P. difficilis* [22], *P. acidiphobus* [22], and *P. rarus* [22]. Furthermore, the two new type strains shared with those strains the lack of genes encoding efficient pathways for assimilation of inorganic carbon. In addition, the genomes of the two new strains lacked any genes putatively encoding proteorhodopsins, which were previously discovered in the genomes of *P. meluiroseus* [22], *P. aenigmaticus* [21], and *P. cosmopolitanus* type strains [22]. Both strains MWH-EgelM1-30-B4^T and MWH-Feld-100^T shared with *P. duraquae* [3] and other *Polynucleobacter* strains [19] the presence of an Fe^{3+} ABC-type transporter and the lack of genes encoding an Fe^{2+} FeoAB transporter. This gene content signature linked to iron acquisition suggested that both strains are adapted to circum-neutral or alkaline waters but not to acidic habitats [19], which fits quite well to the environmental conditions characterizing the respective home habitat of the two new type strains. Interestingly, both strains differed in their iron acquisition genes, as well as in the lack of several inorganic nitrogen assimilation genes from the type strain of the closest related species *P. wuianus* (Table 2). Strains MWH-EgelM1-30-B4^T and MWH-Feld-100^T differed from each other in the presence of genes encoding for flagella. While strain MWH-Feld-100^T shared, for instance, with the type strain of *P. duraquae* [3], the presence of such genes, accompanied by a motile phenotype, strain EgelM1-30-B4^T seemed to completely lack the potential for motility.

Phylogeny

Reconstruction of the phylogenetic positions of strains MWH-EgelM1-30-B4^T and MWH-Feld-100^T with a suitable resolution was not possible by using 16S rRNA gene sequences. Tree construction based on this marker placed both strains in the cryptic species complex PnecC [3] within the genus *Polynucleobacter* (Supplementary Materials Fig. S1). As expected for members of this subcluster [3], both strains shared 16S rRNA gene sequence similarities of > 99% with the other type strains affiliated to this taxon.

The phylogenetic positions of the two new strains were reconstructed based on an alignment of 319 shared genes as described previously [22]. Briefly, nucleotide sequences of 319 genes shared by all *Polynucleobacter* type strains and *Cupriavidus metallidurans* CH34^T were extracted from genome sequences (Table 1) and aligned by using the software MAFFT [26]. This resulted in a total alignment length of 344,717 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 331,885 positions (96 %) in 253 selected blocks. The CIPRES Science Gateway V. 3.3 [28] was used to calculate a bootstrapped (100 iterations) RAxML tree [29] (Fig. 1). In accordance to the 16S rRNA gene phylogeny, this tree based on a large multi-gene alignment also placed strains MWH-EgelM1-30-B4^T and MWH-Feld-100^T in subcluster PnecC but did not suggest an affiliation with any previously described *Polynucleobacter* species. The multi-gene tree differed from a 16S rRNA tree calculated for the same taxa in the branching order of taxa not belonging to subcluster PnecC (Fig. 1, Suppl. Materials Fig. S1). In the multi-gene tree *P. cosmopolitanus*, *P. victoriensis* and *P. rarus* formed a joint branch but *P. rarus* was separated from the other two species in the 16S rRNA tree. By contrast, *P. difficilis* and *P. acidiphobus* formed a joint branch in the 16S rRNA tree (previously designated subcluster PnecB) but not in the multi-gene tree. The multi-gene tree suggested that strains MWH-EgelM1-30-B4^T and MWH-Feld-100^T were more closely related to each other than to any previously described type strain. The closest related *Polynucleobacter* species to both new strains was *P. wuianus* (Fig. 1). Whole genome Average Nucleotide Identity (gANI) analyses performed by using the IMG/M ER system [30] revealed that strains MWH-EgelM1-30-B4^T and MWH-Feld-100^T shared a value of 83.3% gANI. Both did not share ANI values > 80% with any previously described *Polynucleobacter* species (Fig. 1).

Phenotypic and chemotaxonomic characterization

The phenotypic and chemotaxonomic characterization was performed as described previously [17, 22]. The obtained results are presented in Tables 3 and 4. As many other *Polynucleobacter* strains, strains MWH-EgelM1-30-B4^T and MWH-Feld-100^T formed small circular, convex, colourless colonies with shiny surface on NSY agar plates [24]. Strain MWH-Feld-100^T differed from many PnecC strains in its growth performance on liquid NSY medium. While most *Polynucleobacter* strains established maximum optical densities (OD_{575nm}) of 0.1 or slightly higher, which is only about 10% of the maximum OD reached by members of the closest related genera *Cupriavidus* and *Ralstonia* [17], strain MWH-Feld-100^T reached even only OD values of 0.03-0.04 in this medium. The weak and slow growth performance of this strain made the comparative phenotypic characterization of the strain even more tedious than usual for *Polynucleobacter* strains. We cannot exclude that we overlooked weak growth of strain MWH-Feld-100^T on some substances when assessing the substrate spectra, due to measurements near the detection limit. The fact that only a relatively low number of four substrates resulted in good growth compared to eight to eleven compounds utilized as substrates by the related type strains [22] may also result from the comparatively weak growth of the type strain. On the other hand, a narrower substrate spectrum could explain the low maximum OD reached by the strain.

The analysis of the whole-cell fatty acid composition (Table 4) was carried out as described previously [31]. The cell masses were cultivated on R2A [32] 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 well visible at the lowest point of the slope the cell mass was harvested. The incubation periods for strains MWH-EgelM1-30-B4^T, MWH-Feld-100^T and QLW-P1FAT50C-4^T were 4, 13 and 7 days, respectively. The fatty acids of strain

MWH-EgelM1-30-B4^T had a composition similar to that of *P. wuianus* which is roughly representative for strains of the PnecC subcluster [18, 21, 22]. The fatty acid pattern of strain MWH-Feld-100^T, however, deviated from all these patterns by showing relatively high parts of $C_{16:0}$ and extraordinarily low percentages of $C_{18:1}$ ω7c (Table 4). These characteristics persisted when the temperature was changed to 22°C or incubation time was varied between 10 and 14 days.

Proposal of the new species *Polynucleobacter hirudinilacicola* sp. nov. and *Polynucleobacter campilacus* sp. nov.

The performed phylogenetic and gANI analyses clearly suggested that strains MWH-EgelM1-30-B4^T and MWH-Feld-100^T each represent new *Polynucleobacter* species. Both strains did not share gANI values of > 95% with any other type strains and also not with each other. This value is commonly accepted for species demarcation in prokaryotes [33], therefore it is clearly suggested that two new species should be established for the two investigated strains.

Strain MWH-EgelM1-30-B4^T can be discriminated from the two closest related type strains, i.e. *P. campilacus* sp. nov. MWH-Feld-100^T and *P. wuianus* QLW-P1FAT50C-4^T, by the lack of growth at NaCl concentrations above 0.1% (Table 3). Strain MWH-Feld-100^T can be discriminated phenotypically from strains *P. hirudinilacicola* sp. nov. MWH-EgelM1-30-B4^T and *P. wuianus* QLW-P1FAT50C-4^T by its ability to assimilate glycolate and D-sorbitole (Table 3).

The most efficient way to identify and discriminate *Polynucleobacter* type strains is by comparative analyses of partial sequences of the glutamine synthetase gene [34]. Such sequences can be obtained from *Polynucleobacter* strains by using the primers glnA1212F 5'-AGTWGCWCCWGTAGATACATTCC-3' and glnA1895R 5'-GTTGGGATCTTTGCATCTTCTTC -3'. All *Polynucleobacter* type strains possess similarity values < 91% for these sequences (Table 5), while several strains affiliated with the same species, i.e. *P. asymbioticus*, share similarities of > 99% for this marker sequence [35].

We propose to establish the new species *Polynucleobacter hirudinilacicola* sp. nov. and *Polynucleobacter campilacus* sp. nov. and to designate strains MWH-EgelM1-30-B4^T (=DSM 23911 ^{T} =LMG 30144^T) and MWH-Feld-100^T (=DSM 24007^T = LMG 29705^T) as the type strains, respectively.

Description of *Polynucleobacter hirudinilacicola* sp. nov.

Polynucleobacter hirudinilacicola (hi.ru.di.ni.la.ci'co.la. L. fem. n. *hirudo –inis* a leech; L. masc. n. *lacus* lake; L. suff. *–cola* (from L. masc. or fem. n. *incola*), inhabitant, dweller; N.L. masc. n. *hirudinilacicola*, inhabitant of leech lake (German Egelsee)).

Contains free-living *Polynucleobacter* strains dwelling in the water body of alkaline and probably also circum-neutral freshwater systems. Cells are short sometimes slightly curved

rods, 0.5-1.2 μm in length and 0.3–0.5 μm in width, depending on cultivation conditions. Aerobic and chemoorganoheterotrophic, anaerobic growth was not observed. Colonies grown on NSY agar are non-pigmented, circular and convex with smooth surface. Growth occurs up to 31 °C. Growth occurs in 0–0.1% (w/v) NaCl but not in 0.2 to 0.5% or higher. Assimilates acetate, pyruvate, oxaloacetate, fumarate, succinate, L-cysteine, and L-alanine. Does not assimilate glycolate, glyoxylate, propionate, oxalate, malate, citrate, levulinic acid, D-galacturonic acid, D-glucose, D-galactose, D- lyxose, D-fructose, D-sorbitole, L-histidine, L-aspartate, L-asparagine, L-serine, L-leucine, or betaine. Major fatty acids are $C_{16:1}$ ω7c, $C_{16:0}$, $C_{18:1}$ ω7c and feature 2 containing $C_{16:1}$ isoI and $C_{14:0}$-3OH. Encodes an ABC-type Fe^{3+} transport system but no FeoAB Fe^{2+} transporter. The type strain is MWH-EgelM1-30-B4^T (=DSM 23911 ^{T} =LMG 30144^T), isolated from Lake Egelsee in Austria. The type strain is characterized by a genome size of 2.01 Mbp and a G+C content of 45.3 mol%. The accession numbers of the whole genome and the 16S rRNA gene sequence are NAIA00000000 and FN429662, respectively.

Description of *Polynucleobacter campilacus* sp. nov.

Polynucleobacter campilacus (cam.pi.la'cus. L. masc. n. *campus*, field; L. gen. n. *lacus* of a lake, N.L. gen. n. *campilacus*, of field lake, referring to Lake Feldsee ('field lake') in Germany, indicating the site from which the type strain was isolated).

Contains free-living *Polynucleobacter* strains dwelling in the water body of circum-neutral and probably also alkaline freshwater systems. Cells are motile slightly curved rods, 0.5 – 2.4 μm in length and 0.3 – 0.7 μm in width, depending on cultivation conditions. Aerobic and chemoorganoheterotrophic, anaerobic growth was not observed. Colonies grown on NSY agar are non-pigmented, circular and convex with smooth surface. Growth occurs up to 28 °C and in 0 – 0.4% (w/v) NaCl but not at 0.5 to 0.7 % NaCl or higher. Assimilates glycolate, acetate, oxaloacetate, and D-sorbitole. Weakly assimilates pyruvate, malonate, D-mannose, L-leucine, and betaine. Does not assimilate glyoxylate, propionate, malate, fumarate, succinate, citrate, levulinate, oxalate, D-galacturonic acid, D-glucose, D-galactose, D-lyxose, D-fructose, L-fucose, L-glutamate, L-cysteine, L-alanine, L-histidine, L-aspartate, and L-asparagine. Major fatty acids are $C_{16:1}$ ω7c, $C_{16:0}$ and feature 2 containing $C_{16:1}$ isoI and $C_{14:0}$-3OH, content of $C_{18:1}$ ω7c. Encodes an ABC-type Fe^{3+} transport system but no FeoAB Fe^{2+} transporter. The type strain is MWH-Feld-100^T (=DSM 24007^T = LMG 29705^T), isolated from Lake Feldsee, Germany. The type strain is characterized by a genome size of 1.98 Mbp and a G+C content of 45.2 mol%. The accession numbers of the whole genome and the 16S rRNA gene sequence are NGUP00000000 and MG952228, respectively.

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 Aharon Oren for nomenclatural advice.

Funding information

This study was supported by the Austrian Science Fund (FWF) project 27160-B22.

Abbreviations

gANI	whole genome average nucleotide identity
IMG/M ER	Integrated Microbial Genomes with Microbiome Samples Expert Review (ER) companion system
ML	maximum-likelihood
NSY medium	nutrient broth soytone yeast extract medium
R2A medium	Reasoner's 2A medium
PnecC	cryptic species complex PnecC
FAM	filtration acclimation method
FL	free-living
E	endosymbiotic
OD	optical density
Mbp	mega base pairs

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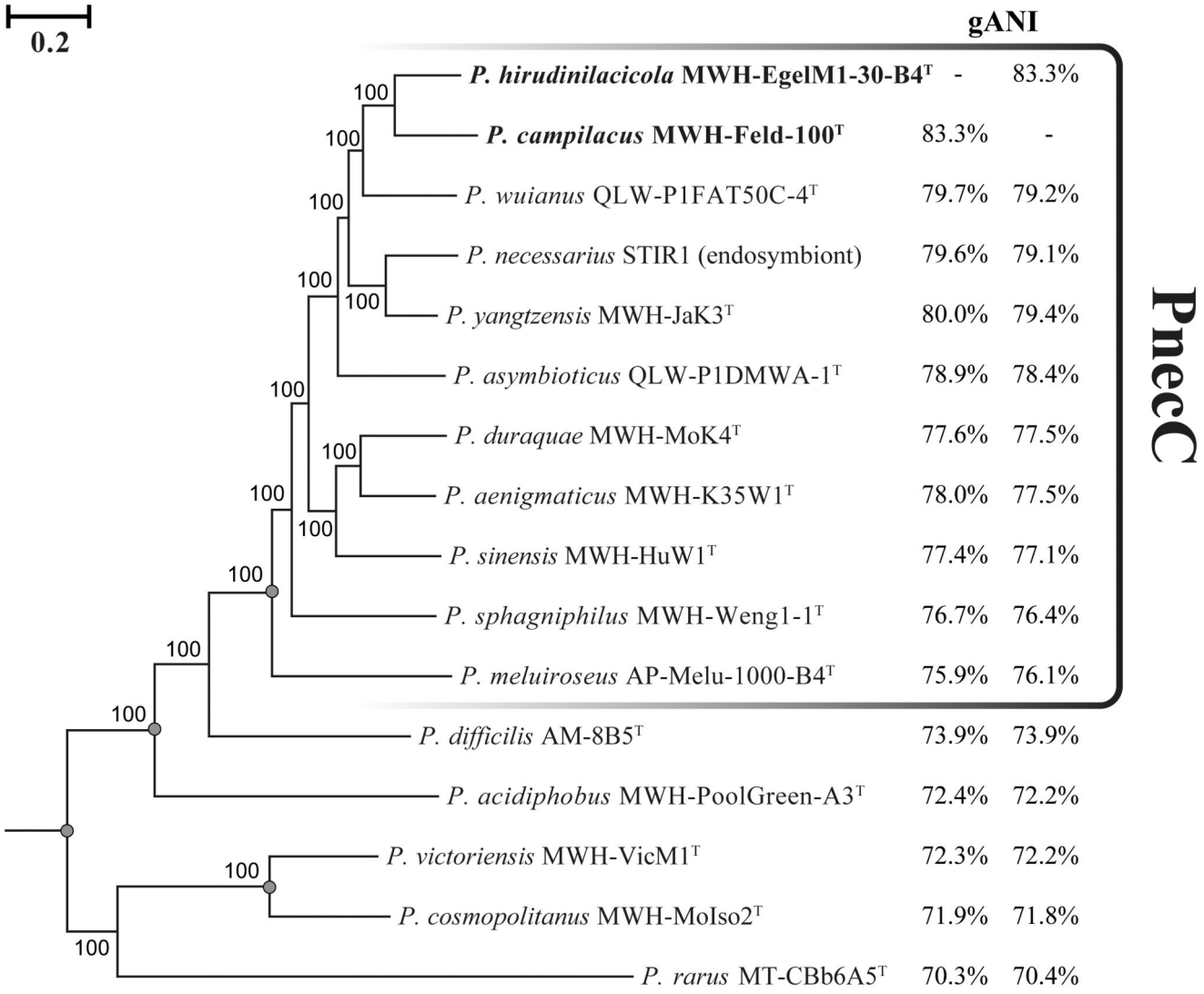

Fig. 1.

Reconstruction of the phylogenetic position of strains MWH-EgelM1-30-B4^T and MWH-Feld-100^T. Bootstrapped RAxML tree calculated with nucleotide sequences of 319 shared genes. Percentage values behind the strain names indicate gANI values obtained in pairwise comparisons of whole genome sequences with strains MWH-EgelM1-30-B4^T (first column) and MWH-Feld-100^T (second column), respectively. The tree was rooted with sequences of *Cupriavidus metallidurans* CH34^T (not shown, accession number: CP000352-CP000355 [39]). Accession numbers for the genomes of the shown taxa can be found in Table 1. Nodes present in a RAxML tree calculated with 16S rRNA gene sequences of the same taxa are labelled with filled circles (if bootstrap values were > 70%). Bar, 0.2 substitutions per nucleotide position.

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Int J Syst Evol Microbiol. Author manuscript; available in PMC 2018 September 21.

Table 1

Genome characteristics of *Polynucleobacter* type strains and an endosymbiotic strain affiliated with the species *P. necessarius*.

Species	Strain	*Polynucleo-bacter* subcluster	Life-style	Genome size (Mbp)	Scaf-folds	G+C content (mol%)	DDBJ/EMBL/ GenBank accession number	IMG Genome ID	Reference
P. hirudinilacicola sp. nov.	MWH-EgelM1-30-B4^T (=DSM 23911^T)	PnecC	FL	2.01	5	45.3	NAIA00000000	2590828818	This study
P. campilacus sp. nov.	MWH-Feld-100^T (= DSM 24007^T)	PnecC	FL	1.98	9	45.2	NGUP00000000	2590828843	This study
P. wuianus	QLW-P1FAT50C-4^T (= DSM 24008^T)	PnecC	FL	2.23	1	44.9	CP015922	2687453598	[18]
P. necessarius	STIR1 [host, *Euplotes aediculatus*]	PnecC	E	1.56	1	45.6	CP001010	2503982034	[36]
P. yangtzensis	MWH-JaK3^T (= DSM 21493^T)	PnecC	FL	2.05	42	45.4	LOJI01000000	2608642177	[3]
P. asymbioticus	QLW-P1DMWA-1^T (= DSM 18221^T)	PnecC	FL	2.16	1	44.8	CP000655	640427129	[37]
P. duraquae	MWH-MoK4^T (= DSM 21495^T)	PnecC	FL	2.03	1	45.2	CP007501	2634166443	[3]
P. aenigmaticus	MWH-K35K1^T (= DSM 24006^T)	PnecC	FL	2.14	37	46.0	NGUO00000000	2675903126	[21]
P. sinensis	MWH-HuW1^T (= DSM 21492^T)	PnecC	FL	2.32	19	45.5	LOJJ01000000	2630969031	[3]
P. sphagniphilus	MWH-Weng1-1^T (= DSM 24018^T)	PnecC	FL	2.04	17	45.6	MPIY01000000	2574179738	[25]
P. meluiroseus	AP-Melu-1000-B4^T (= DSM 103591^T)	PnecC	FL	1.89	11	46.6	OANS00000000	2710724120	[22]
P. difficilis	AM-8B5^T (= DSM 22349^T)	PnecB	FL	2.00	1	49.5	CP023276	2642422589	[22]
P. acidiphobus	MWH-PoolGreenA3^T (= DSM 21994^T)	PnecB	FL	1.85	1	48.2	CP023277	2642422590	[22]
P. victoriensis	MWH-VicM1^T (= DSM 21486^T)	PnecD	FL	1.63	3	43.1	FYEX00000000	2710264786	[38]
P. cosmopolitanus	MWH-MoIso2^T (= DSM 21490^T)	PnecD	FL	1.77	6	44.1	NJGG00000000	2642422582	[38]
P. rarus	MT-CBb6A5^T (= DSM 21648^T)	PnecA	FL	3.16	2	39.9	NTGB00000000	2645728114	[22]

FL, free-living; E, endosymbiotic

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Table 2

Comparison of the presence and absence of selected genes in strains *P. hirudinilacicola* sp. nov. MWH-EgelM1-30-B4^T, *P. campilacus* sp. nov. MWH-Feld-100^T, and the type strain representing the closest related species.

Genes putatively encoding	*P. hirudinilacicola* sp. nov. MWH-EgelM1-30-B4^T	*P. campilacus* sp. nov. MWH-Feld-100^T	*P. wuianus* QLW-P1FAT50C-4^T
Inorganic nutrients			
ABC-type Fe^{3+} transport system	+	+	-
feoAB genes (uptake of Fe^{2+})	-	-	+
ABC-type sulfate transport system	-	-	+
ABC-type Nitrate/Nitrite/Cyanate transporter	-	-	+
Nitrate reductase (assimilatory)	-	-	+
Nitrite reductase (assimilatory)	-	-	+
Cyanate lyase (releases NH_3 and 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	-	-	+
Anoxygenic photosynthesis			
Photosynthesis gene cluster	+	+	+
Motility			
Flagella genes	-	+	-
Oxidative stress			
Catalase	-	-	-

Table 3

Phenotypic characteristics of strains MW-Feld-100^T, MWH-EgelM1-30-B4^T and the type strain of the closest related species. All three strains have the following characteristics in common: Assimilation of acetate and oxaloacetate; no assimilation of oxalate, citrate, D-glucose, D-galactose, D-lyxose, and L-serine. +, increase in optical density (OD); w, weak increase in OD; -, no significant increase in OD. All presented data were obtained in the same lab under standardized conditions.

	***P. hirudinilacicola* sp. nov. MWH-EgelM1-30-B4^T (DSM 23911^T)**	***P. campilacus* sp. nov. MWH-Feld-100^T (DSM 24007^T)**	***P. wuianus* QLW-P1FAT50C-4^T (DSM 24008^T)**
Cell morphology	short rods	slightly curved rods	short rods
Cell length (μm)	0.5-1.2	0.5-2.4	0.6-1.7
Cell width (μm)	0.3-0.5	0.3-0.7	0.3-0.6
Temperature range of growth (°C)	5 - 31	5 - 28	5 - 34
NaCl tolerance (%NaCl, w/v)	0 - 0.1	0 - 0.4(w)	0 - 0.5
Anaerobic growth	-	-	-
Assimilation of:			
Glyoxylate	-	-	w
Glycolate	-	+	-
Propionate	-	-	+
Pyruvate	+	w	+
Malonate	-	w	w
Malate	w	-	+
Fumarate	+	-	+
Succinate	+	-	+
Levulinate	-	-	w
D-Galacturonate	-	-	w
D-Mannose	w	w	-
D-Fructose	-	-	w
L-Fucose	w	-	-
D-Sorbitole	-	+	-
L-Glutamate	w	-	+
L-Histidine	-	-	+
L-Aspartate	-	-	+
L-Cysteine	+	-	w
L-Alanine	+	-	+
L-Asparagine	-	-	w
L-Leucine	-	w	-
Betaine	-	w	-

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Table 4

Major fatty acid compositions of *P. hirudinilacicola* sp. nov. MWH-EgelM1-30-B4^T, *P. campilacus* sp. nov. MWH-Feld-100^T, and the type strain representing the closest related species. Compounds occurring at percentages of 0.2 or higher are given. Data for *P. wuianus* QLW-P1FAT50C-4^T were taken from Hahn *et al.* (2017) [18]. All presented data were obtained in the same lab under standardized conditions.

Fatty acid	***P. hirudinilacicola* sp. nov. MWH-EgelM1-30-B4^T DSM 23911^T**	***P. campilacus* sp. nov. MWH-Feld-100^T DSM 24007^T**	***P. wuianus* QLW-P1FAT50C-4^T DSM 24008^T**
$C_{10:0}$	-	3.1	-
$C_{12:0}$	6.8	3.6	4.0
$C_{14:0}$	0.6	0.8	0.2
$C_{15:0}$	-	0.3	-
$C_{16:0}$	26.3	38.9	18.5
$C_{17:0}$	-	0.4	-
$C_{18:0}$	0.7	1.0	0.9
$C_{14:1}$ ω5c	-	0.3	-
$C_{16:1}$ ω7c	35.7	40.1	39.1
$C_{18:1}$ ω9c	-	-	-
$C_{18:1}$ ω7c	15.7	0.5	27.8
11-methyl $C_{18:1}$ ω7c	3.3	-	2.7
$C_{12:0}$ 2-OH	0.3	-	1.0
$C_{16:1}$ 2-OH	-	-	0.5
Feature 2	10.2	11.0	4.7
Feature 7	0.5	-	0.4

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 5

Sequence similarity of partial (603 bp) glutamine synthetase gene (glnA) sequences. Accession numbers for the compared sequences are provided in Supplementary Materials Table S1. The sequences of *P. necessarius* STIR1 and *C. metallidurans* CH34^T were extracted from their genome sequences, all other sequences were obtained by using primers glnA1212F and glnA1895R [34].

Taxon	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)
(1) *P. hirudinilacicola* sp. nov. MWH-EgelM1-30-B4^T	90.9	90.9	89.9	92.5	89.7	90.0	90.2	87.1	84.4	83.1	84.2	82.1	87.4	85.2	82.8	77.6
(2)*P. campilacus* sp. nov. MWH-Feld-100^T		90.4	89.1	89.1	89.1	88.4	88.4	87.2	82.6	82.1	84.1	82.4	86.1	83.1	81.9	76.9
(3)*P. wuianus* QLW-P1FAT50C-4^T			91.0	91.4	92.9	89.7	88.1	87.7	84.4	84.9	84.2	82.8	87.2	84.6	81.9	76.9
(4)*P. necessarius* STIR1 (endosymbiont)				93.2	90.0	90.0	89.2	86.2	83.3	82.8	83.4	80.9	87.9	85.9	82.4	77.4
(5)*P. yangtzensis* MWH-JaK3^T					89.7	91.2	89.6	87.2	82.9	82.4	84.1	80.6	89.6	86.9	82.4	78.3
(6)*P. asymbioticus* QLW-P1DMWA-1^T						88.7	88.7	87.9	82.8	83.9	82.4	83.1	85.9	82.4	80.8	76.5
(7)*P. duraquae* MWH-MoK4^T							90.4	89.7	83.7	82.3	84.9	81.3	87.2	86.2	81.3	78.4
(8)*P. aenigmaticus* MWH-K35W1^T								89.6	84.1	81.9	85.1	81.9	85.7	84.1	81.8	76.3
(9)*P. sinensis* MWH-HuW1^T									83.1	82.9	84.4	80.8	84.4	82.4	79.9	76.6
(10)*P. sphagniphilus* MWH-Weng1-1^T										80.6	83.6	81.6	81.9	81.6	79.1	76.5
(11)*P. meluiroseus* AP-Melu-1000-B4^T											82.4	81.4	79.8	78.3	79.4	73.8
(12)*P. difficilis* AM-8B5^T												83.1	82.1	80.1	79.6	79.3
(13)*P. acidiphobus* MWH-PoolGreenA3^T													80.9	79.6	79.9	76.3
(14)*P. victoriensis* MWH-VicM1^T														92.9	83.4	78.3
(15)*P. cosmopolitanus* MWH-MoIso2^T															82.6	78.9
(16)*P. rarus* MT-CBb6A5^T																74.5
(17)*Cupriavidus metallidurans* CH34^T																