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Rufibacter quisquiliarum sp. nov., a new member of the phylum Bacteroidetes isolated from a bioreactor treating landfill leachate

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Abstract:	A new bacterium, CAI-18bT, was isolated from a bioreactor that treated landfill leachate using an oligotrophic growth medium. Phylogenetic analysis based on the 16S rRNA gene revealed that strain CAI-18bT is a member of the genus Rufibacter, showing 97.1% pairwise similarity value to Rufibacter roseus H359T, 96.4% to Rufibacter tibetensis 1351T, 96.4% to Rufibacter glacialis MDT1-10-3T and 96.0% to Rufibacter immobilis MCC P1T. Strain CAI-18bT was rod-shaped, motile, oxidase and catalase positive. The predominant fatty acids were iso-C15:0 (24.1%) and iso-C17:1 I (22.3%), the major respiratory quinone was MK-7, and the predominant polar lipids were phosphatidylethanolamine and an unknown aminophospholipid. The G + C content of the genomic DNA of strain CAI-18bT was 50.7 mol%. The new bacterium can be distinguished from the related type strains based on its capability for the assimilation of N-acetylglucosamine and gentiobiose. On the basis of the phenotypic, chemotaxonomic and molecular data, strain CAI-18bT is considered to represent a new species, for which the name Rufibacter quisquiliarum sp. nov. is proposed. The type strain is CAI-18bT (=DSM 29854T=NCAIM B.02614T).

Rufibacter quisquiliarum sp. nov., a new member of the phylum *Bacteroidetes* isolated from a bioreactor treating landfill leachate

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAI-18b^T is KM083132.

A new bacterium, CAI-18b^T, was isolated from a bioreactor that treated landfill leachate using an oligotrophic growth medium. Phylogenetic analysis based on the 16S rRNA gene revealed that strain CAI-18b^T is a member of the genus *Rufibacter*, showing 97.1% pairwise similarity value to *Rufibacter roseus* H359^T, 96.4% to *Rufibacter tibetensis* 1351^T, 96.4% to *Rufibacter glacialis* MDT1-10-3^T and 96.0% to *Rufibacter immobilis* MCC P1^T. Strain CAI-18b^T was rod-shaped, motile, oxidase and catalase positive. The predominant fatty acids were iso-$C_{15:0}$ (24.1%) and iso-$C_{17:1}$ I (22.3%), the major respiratory quinone was MK-7, and the predominant polar lipids were phosphatidylethanolamine and an unknown aminophospholipid. The G + C content of the genomic DNA of strain CAI-18b^T was 50.7 mol%. The new bacterium can be distinguished from the related type strains based on its capability for the assimilation of N-acetylglucosamine and gentiobiose. On the basis of the phenotypic, chemotaxonomic and molecular data, strain CAI-18b^T is considered to represent a new species, for which the name *Rufibacter quisquiliarum* sp. nov. is proposed. The type strain is CAI-18b^T (=DSM 29854^T=NCAIM B.02614^T).

Members of the phylum *Bacteroidetes* colonize various types of habitats, including saline and freshwater, soil, compost, activated sludge, dairy products and gastrointestinal tract of animals (Kirchman, 2002; McBride *et al.*, 2014; Thomas *et al.*, 2011). These diverse, Gram-negative, rod-shaped bacteria are also known as degraders of high molecular weight organic matter, such as proteins and polysaccharides (Kirchman, 2002). The number of species within this phylum has been doubled between 2009 and 2014 (Munoz *et al.*, 2016), while the increase in the prokaryotic species names validly published was only 30% in the same period (Parte, 2014), which indicates that strains belonging to *Bacteroidetes* gained relatively increased interest in the last few years.

A recent cultivation-based analysis of various aquatic habitats in Romania using oligotrophic media resulted in the isolation of bacterial strains representing potentially new species (Felföldi *et al.*, 2015). One of the new isolates, strain CAI-18b^T, showed low pairwise similarity values of partial 16S rRNA gene sequences to members of the genera *Nibribacter* and *Rufibacter*, which represent a new branch within the family *Cytophagaceae* (order *Cytophagales*, phylum *Bacteroidetes*). Both genera were described in the last few years and contained only a single species. *Nibribacter koreensis* was isolated from estuarine water (Kang *et al.*, 2013), while *Rufibacter tibetensis* was isolated from soil (Abaydulla *et al.*, 2012). However, very recently, three additional *Rufibacter* species have been described, *Rufibacter immobilis* from a saline lake (Polkade *et al.*, 2015), *Rufibacter roseus* from radiation-polluted soil (Zhang *et al.*, 2015) and *Rufibacter glacialis* from glacier soil (Liu *et al.*, 2016). This study is aimed the polyphasic characterization of the *Rufibacter*-related bacterial strain CAI-18b^T. Based on the obtained results, this strain is supposed to represent a novel species of the genus *Rufibacter* for which the name *Rufibacter quisquiliarum* sp. nov. is proposed.

Strain CAI-18b^T was isolated from a bioreactor, which treated the leachate of a landfill site located in Odorheiu Secuiesc (Harghita County, Transylvania, Romania). For isolation, a diluted R2A-

based medium was used, which contained 360 mL R2A medium (DSMZ medium 830, www.dsmz.de), 1.33 g $CaCl_2$ and 1.81 g NH_4Cl in 1 l final volume (pH 8.0) and was solidified with 20 g l^{-1} agar. The standard dilution plating technique was applied to obtain isolates from the samples with incubation at room temperature (20-22 °C). Subsequently, strain CAI-18b^T was maintained on normal R2 agar medium (pH 8.5-8.8) at 28 °C. The type strains for side-by-side analyses, *Rufibacter tibetensis* CCTCC AB 208084^T and *Nibribacter koreensis* JCM 17917^T, were maintained on the same medium and at the same temperature. Temperature, pH and salt concentration optima were determined at 4, 10, 20, 25, 30, 37 and 45 °C, at pH from 4 to 11 (with intervals of 1) and with NaCl concentration from 0 to 5% (w/v, with intervals of 1%), respectively, as described previously by Felföldi *et al.* (2014).

Motility of strain CAI-18b^T was studied by native preparation, cell morphology was observed after Gram staining according to Claus (1992). Oxidase activity was examined as given by Tarrand & Gröschel (1982), while catalase reaction was checked as described by Barrow & Feltham (2004). Caseinase and phosphatase activities were determined as described by Smibert & Krieg (1994). Acid production from D-glucose was checked by the oxidative and fermentative test according to Hugh & Leifson (1953). Additional metabolic tests were performed with API 50 CH, API 20 NE and API ZYM (bioMérieux) systems following the instructions given by the manufacturer. Susceptibility of the strains to antibiotics was studied on R2A plates using antibiotic-containing discs (Bio-Rad) after 3 days of incubation at 28 °C. Growth under anaerobic condition was examined using agar slant cultures on R2A medium incubated for one week in an anaerobic chamber (Forma Scientific) at room temperature.

Analyses of isoprenoid quinones, cellular fatty acids, polar lipids and the determination of DNA base composition were performed as given in Felföldi *et al.* (2011).

The 16S rRNA gene sequence of strain CAI-18b^T was amplified as described previously (Máthé *et al.*, 2014). Purification and sequencing of PCR products were carried out by the LGC Genomics Ltd (Berlin, Germany). Sequence alignment with the closest related type strains and clones was conducted with SINA (Pruesse *et al.*, 2012). Phylogenetic analysis (which included the search for the best-fit models) was performed with the MEGA 6.0 software (Tamura *et al.*, 2013).

Sequencing the 16S rRNA gene of strain CAI-18b^T resulted in 1434 nucleotides. Based on this data, the most closely related species (represented by type strains) were identified by the EzTaxon-e server (Kim *et al.*, 2012). *Rufibacter roseus* H359^T (=CPCC 100615^T=KCTC 42217^T) shared 97.1%, *Rufibacter tibetensis* 1351^T (=CCTCC AB 208084^T=NRRL B-51285^T, type species of the genus) 96.4%, *Rufibacter glacialis* MDT1-10-3^T (=CGMCC 1.9789^T=NBRC 109705^T) 96.4% (since the 16S rRNA gene sequence of the type strain is currently not available in EzTaxon-e, comparison was performed with Blast, Zhang *et al.*, 2000), *Rufibacter immobilis* MCC P1^T (=MCC 2268^T=CCTCC AB 2013351^T) 96.0% and *Nibribacter koreensis* GSR3061^T (=KACC 16450^T=JCM 17917^T) 94.4% pairwise similarity value based on the 16S rRNA gene, while members of all other genera showed lower than 92.2% similarities to strain CAI-18b^T. The phylogenetic analysis of the 16S rRNA gene (Fig. 1) also confirmed the distinct position of strain CAI-18b^T within the order *Cytophagales* (phylum *Bacteroidetes*). Incorporating environmental clones in the 16S rRNA gene similarity analysis (Supplementary Fig. S1, available in IJSEM Online), it has been shown that this group of bacteria (i.e. members of the genera *Rufibacter* and *Nibribacter*) is rather versatile, since they were detected from many different habitats, including various aquatic and soil types, air samples, plant-, animal- and human-associated environments.

Cells of strain CAI-18b^T were Gram-negative, rod-shaped (Supplementary Fig. S2, available in IJSEM Online), motile, aerobic and mesophilic with a characteristic heterotrophic metabolism (Table 1). Based on the results of the phenotypic and biochemical investigations, strain CAI-18b^T

could be distinguished from the studied type strains, since the new strain was capable for the assimilation of N-acetylglucosamine, D-lactose and gentiobiose in contrast to the negative results observed with related type strains. Moreover, according to antibiotic sensitivity tests (Supplementary Table S1, available in IJSEM Online; Kang *et al.*, 2013; Polkade *et al.*, 2015; Zhang *et al.*, 2015), all studied related strains were susceptible to ampicillin, while strain CAI-18b^T showed resistance to this antibiotic.

The major respiratory quinone of CAI-18b^T was menaquinone MK-7, which is the characteristic respiratory quinone of the family *Cytophagaceae* (McBride *et al.*, 2014). The fatty acid pattern of strain CAI-18b^T was dominated by iso-$C_{15:0}$ (24.1%) and iso-$C_{17:1}$ I (22.3%), and in lower amounts $C_{17:1}\omega6c$ (6.9%), $C_{16:1}\omega7c$ (5.7%), anteiso-$C_{15:0}$ (5.6%) and several other components have been detected (Table 2). Comparing these data with related strains analyzed in this study (Table 2) and with those obtained by others (Polkade *et al.*, 2015; Zhang *et al.*, 2015; Liu *et al.*, 2016), fatty acids contributing >10% could be very variable within the genus *Rufibacter* even if the same or similar cultivation conditions were applied. A major fatty acid of strain CAI-18b^T, iso-$C_{15:0}$, was present in less than half of the amount found in *R. tibetensis* CCTCC AB 208084^T and *R. glacialis* MDT1-10-3^T (9.8% and 8.9%, respectively), while some important fatty acids detected in the new strain were completely missing in other *Rufibacter* species: e.g. $C_{17:1}\omega6c$ (6.9% in CAI-18b^T) was not present in strain *R. roseus* H359^T (strain showing the highest 16S rRNA gene similarity to our novel bacterium), and the fatty acid iso-$C_{15:0}$ 2-OH (3.3% in CAI-18b^T) was not detected in *R. roseus* H359^T, *R. tibetensis* CCTCC AB 208084^T and *R. glacialis* MDT1-10-3^T, while $C_{15:0}$ (3.4% in CAI-18b^T) was not detected in *R. roseus* H359^T, *R. glacialis* MDT1-10-3^T and *R. immobilis* MCC P1^T.

The polar lipid pattern of strain CAI-18b^T was complex with the dominance of phosphatidylethanolamine and an unknown aminophospholipid, and additionally two other unknown aminophospholipids, three unknown phospholipids and two unknown lipids were detected

as minor components (Supplementary Fig. S3, available in IJSEM Online). Similar polar lipid compositions have been reported for other *Rufibacter* species (Kang *et al.*, 2013; Polkade *et al.*, 2015; Zhang *et al.*, 2015; Liu *et al.*, 2016).

The genomic G+C content value of strain CAI-18b^T is 50.7 mol% (HPLC), which falls within the range reported for the type species and other members of the genus *Rufibacter* (43.9-52.6 mol%; Abaydulla *et al.*, 2012; Polkade *et al.*, 2015; Zhang *et al.*, 2015; Liu *et al.*, 2016), but is higher by 7 mol% than this of *R. roseus* H359^T (43.9 mol%), the strain having the highest 16S rRNA gene similarity to CAI-18b^T. According to Mesbah *et al.* (2011), the mol% G+C range within a species is less than 3% and is not higher than 10% within a genus, therefore the genomic G+C content of strain CAI-18b^T supported the view that the new strain belongs to the genus *Rufibacter*.

Based on the comparative data presented in this study, strain CAI-18b^T is considered to represent a novel species within the genus *Rufibacter*, for which the name *Rufibacter quisquiliarum* sp. nov. is proposed.

Description of *Rufibacter quisquiliarum* sp. nov.

Rufibacter quisquiliarum (quis.qui.li.a'rum. L. gen. fem. pl. n. *quisquiliarum* of waste, of rubbish).

Cells are short, rod-shaped (0.3-0.5 x 0.7-1.6 μm) and motile. Colonies on R2A agar medium (pH 8.8) are pinkish-red-colored, circular and raised with an average diameter of 2 mm. Growth occurs at 4-45 °C (optimum, 20-37 °C), at pH 7-11 (optimum, pH 8-10) and 0-2% (w/v) NaCl concentration. Positive for oxidase, catalase and caseinase activities. Negative for oxidative and fermentative acid production from D-glucose, nitrate reduction, indole production, urease and

phosphatase enzyme activities. D-Galactose, D-glucose, D-mannose, N-acetylglucosamine, esculine, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-trehalose, starch (amidon), glycogen and gentiobiose are assimilated, while all other carbon sources in the API 50 CH and API 20NE tests are not assimilated. According to API 20NE and API ZYM tests, positive for gelatinase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase (weak), trypsine, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase; and negative for arginine dihydrolase, lipase (C14), α-chymotrypsine, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase enzyme activities. The major fatty acids are iso-C15:0 (24.1%) and iso-C17:1 I (22.3%), the predominant polar lipids are phosphatidylethanolamine and an unknown aminophospholipid.

The G + C content of the genomic DNA is 50.7 mol%.

The type strain is CAI-18b^{T} (=DSM 29854^{T}=NCAIM B.02614^{T}) which was isolated from a bioreactor treating landfill leachate.

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FIGURE LEGENDS

Fig. 1. Phylogenetic position of CAI-18b^T and related type strains based on the 16S rRNA gene. Phylogenetic tree has been constructed based on 1340 nucleotide positions using the maximum likelihood (ML) method with Kimura 2-parameter nucleotide substitution model. Bootstrap values >50% for the ML (left) and neighbor-joining (right) methods are shown. Branches recovered with both treeing methods are marked with black dots. GenBank accession numbers are given in parentheses. Bar, 0.05 substitutions per nucleotide.

TABLES

Table 1. Phenotypic and biochemical characteristics of CAI-18b^T and related type strains.

Strains: 1, CAI-18b^T (=DSM 29854^T); 2, *Rufibacter roseus* H359^T; 3, *R. tibetensis* CCTCC AB 208084^T; 4, *Nibribacter koreensis* JCM 17917^T. All strains had rod-shaped cells, formed pinkish-red colonies. All strains were positive for oxidase, catalase, caseinase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsine, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase activities; and positive for the assimilation of D-glucose, D-maltose, D-trehalose and amidon (starch). All strains were negative for nitrate reduction (both to nitrite and nitrogen gas), indole production, arginine dihydrolase and urease activities, for the assimilation of D-mannitol, capric acid, citrate, phenylacetic acid, glycerol, erythritol, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, β-methyl-D-xylopyranoside, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, α-methyl-D-mannopyranoside, α-methyl-D-glucopyranoside, amygdalin, arbutin, salicin, D-melezitose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate, 5-ketogluconate; and for β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase enzyme activities. Symbols: +, present; -, absent; w, weak reaction. NT, not tested. Data are from the present study (unless otherwise indicated). API ZYM enzyme activity data is not available for strain *R. roseus* H359^T.

Characteristic	***1***	***2****	***3***	***4***
Motility	+	+	+	-
Temperature range (optimum) (°C)	4-45 (20-37)	4-37 (30)	20-37 (25-30)	10-45 (20-25)
pH range (optimum)	7-11 (8-10)	6-9 (7)	7-11 (8-9)	6-11 (9-10)
NaCl concentration for growth (%)	0-2	0-4	0-2	0-2
Assimilation of (API 50 CH)				
D-Arabinose	-	+	-	-
D-Galactose	+	+	-	-
D-Fructose	-	+	-	-
D-Mannose	+	+	-	-
N-Acetylglucosamine	+	-	-	-

Characteristic	*1*	*2**	*3*	*4*
Esculin	+	+	+	-
D-Cellobiose	+	+	+	-
D-Lactose	+	-	-	-
D-Melibiose	+	+	-	-
D-saccharose (sucrose)	-	+	-	-
Inulin	-	+	-	-
D-Raffinose	-	+	-	-
Glycogen	+	+	-	-
Gentiobiose	+	-	-	-
Glucose fermentation (API 20 NE)	-	+	-	-
Gelatine hydrolysis (API 20 NE)	+	-	+	+
Assimilation of (API 20 NE)				
L-Arabinose	-	-	+	-
Adipic acid	-	-	-	+
Malic acid	-	-	-	+
Enzyme activity (API ZYM)				
Lipase (C14)	-	NT	-	+
Cystine arylamidase	w	NT	+	+
α-Chymotrypsine	-	NT	+	-
α-Galactosidase	+	NT	+	-
β-Galactosidase	-	NT	+	-
β-Glucosidase	+	NT	+	-
DNA G + C content (mol%)	50.7	43.9	46.8†	44.9‡

*All data from Zhang *et al.* (2015).

†Data from Abaydulla *et al.* (2012).

‡Data from Kang *et al.* (2013).

Table 2. Major fatty acids of CAI-18b^T and related type strains.

Strains: 1, CAI-18b^T (=DSM 29854^T); 2, *Rufibacter roseus* H359^T; 3, *R. tibetensis* CCTCC AB 208084^T; 4, *Nibribacter koreensis* JCM 17917^T; -, not detected; TR, <1.0%. Data are from the present study, except fatty acid data of *R. roseus* H359^T. All strains were grown on R2A medium (pH 8.0) for 3 days at 25 °C, except *R. roseus* H359^T, which was grown on TSA medium for 3 days.

Fatty acid	***1***	***2****	***3***	***4***
iso-$C_{15:0}$	24.1	30.5	9.8	22.9
iso-$C_{17:1}$ I	22.3	32.9‡	16.1	24.8
$C_{17:1}\omega6c$	6.9	-	13.9	6.1
$C_{16:1}\omega7c$	5.7	6.3‡	8.3§	1.7
anteiso-$C_{15:0}$	5.6	4.5	6.2	4.0
$C_{15:0}$	3.4	-	9.8	2.1
iso-$C_{16:1}$ H	3.4	-	1.8	3.8
iso-$C_{15:0}$ 2-OH	3.3	-	-§	1.1
$C_{15:1}\omega6c$	3.2	-	6.1	2.1
iso-$C_{16:0}$	3.1	-	3.0	4.6
iso-$C_{17:0}$ 3-OH	3.1	3.8	2.4	3.8
$C_{16:1}\omega5c$	2.9	5.8	4.3	3.1
iso-$C_{15:0}$ 3-OH	2.5	2.3	1.8	2.5
Summed feature 1†	1.7	2.4	1.3	2.6
iso-$C_{14:0}$	1.7	-	TR	1.7
iso-$C_{16:0}$ 3-OH	1.5	-	1.7	1.4
iso-$C_{15:1}$ G	0.6	-	1.9	5.4
$C_{16:0}$	TR	2.1	1.1	TR
$C_{17:1}\omega8c$	TR	-	2.6	TR

*Data from Zhang *et al.* (2015).

†Summed features represent two or three fatty acids that cannot be differentiated using the MIDI system. Summed feature 1: iso-$C_{15:1}$ I/H and/or $C_{13:0}$ 3-OH.

‡These two fatty acids were detected as summed feature components (Summed feature 3, $C_{16:1}\omega7c/C_{16:1}\omega6c$ and Summed feature 4, iso-$C_{17:1}$ I/anteiso-$C_{17:1}$ B, respectively) in the case of this strain.

§These two components were detected as Summed feature 3 in the case of this strain.

Figure 1

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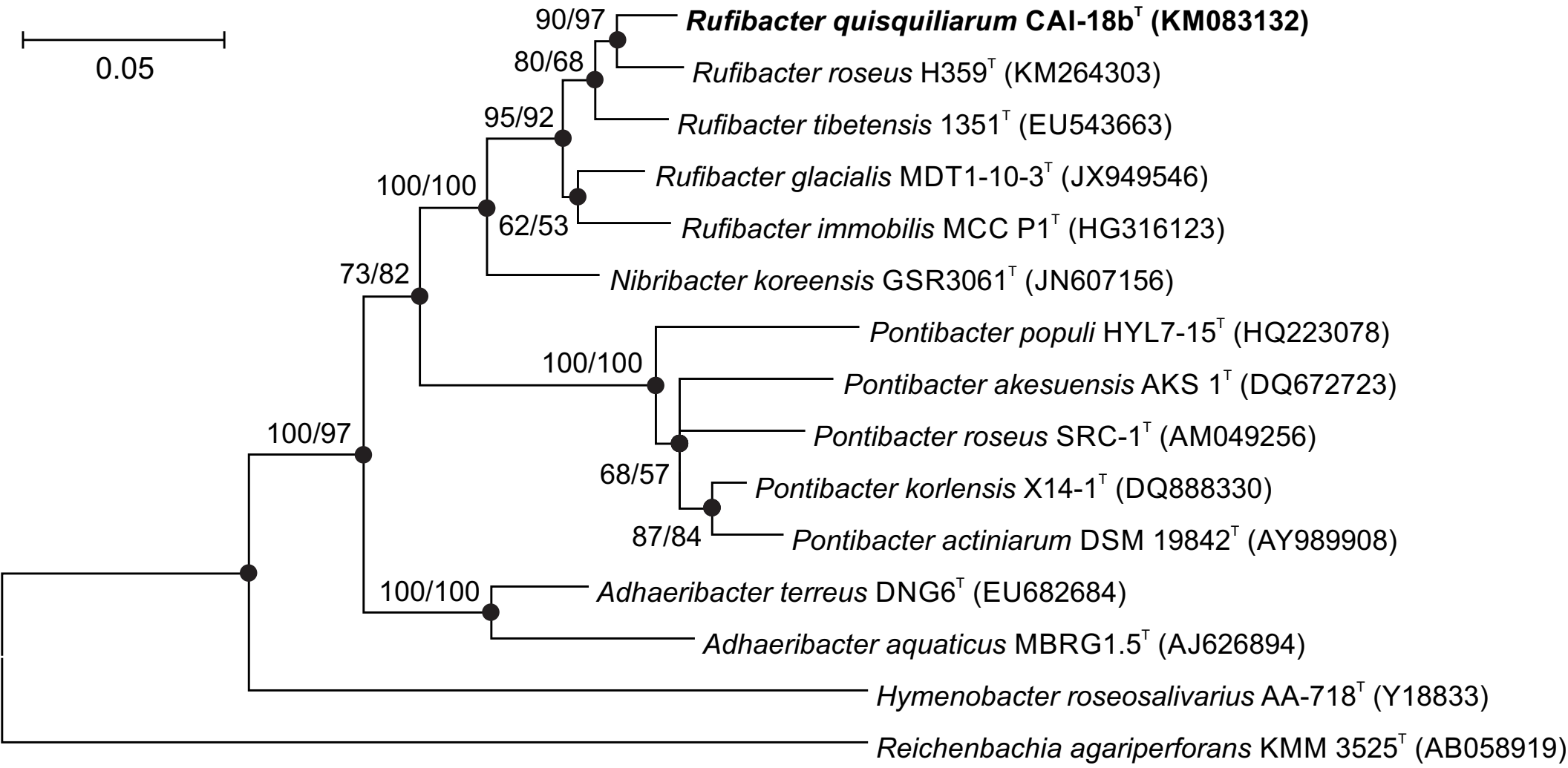

Supplementary material for

Rufibacter quisquiliarum sp. nov., a new member of the phylum Bacteroidetes isolated from a bioreactor treating landfill leachate

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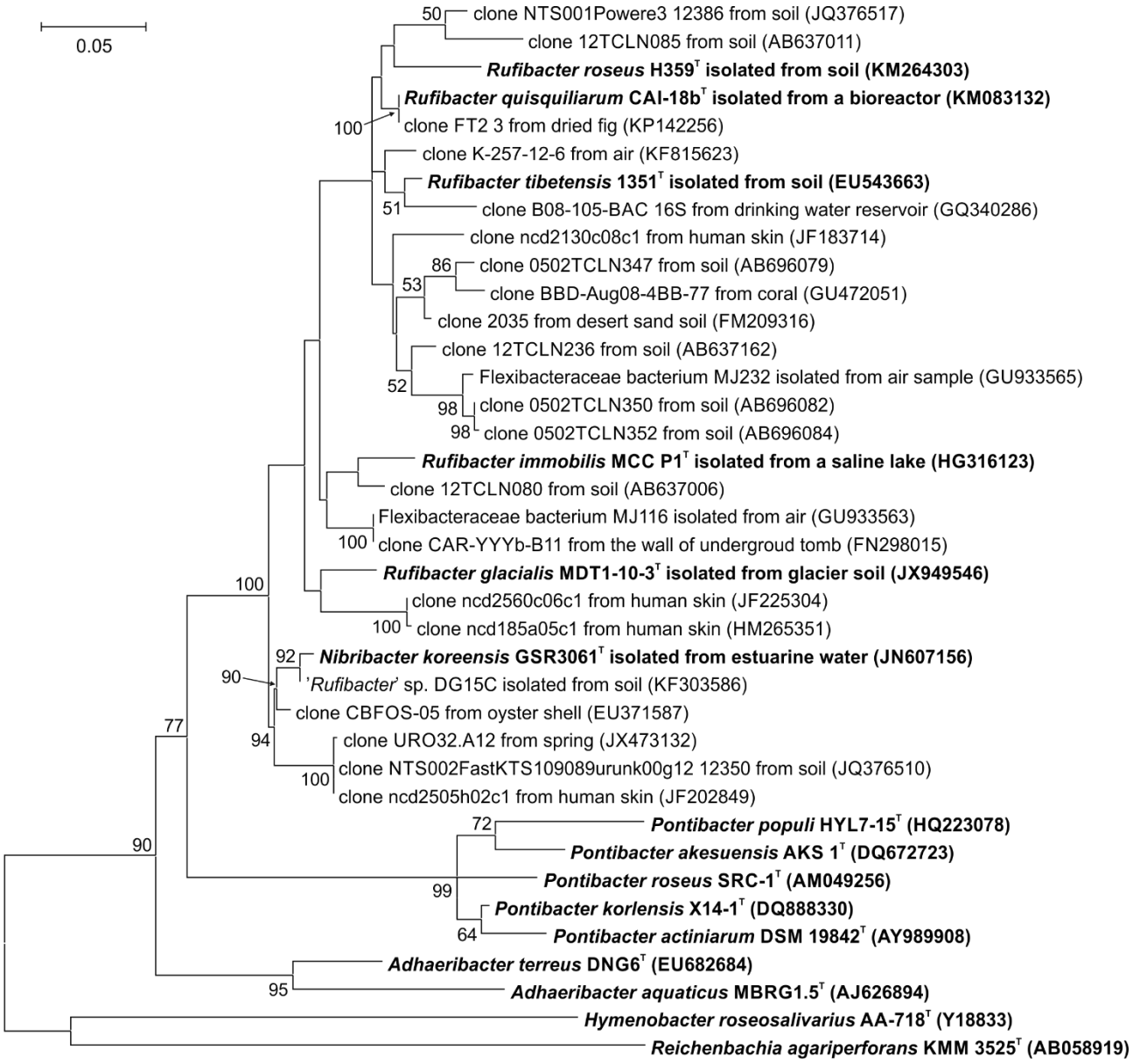
Supplementary Table S1. Antibiotic resistance of CAI-18b^T and related type strains.

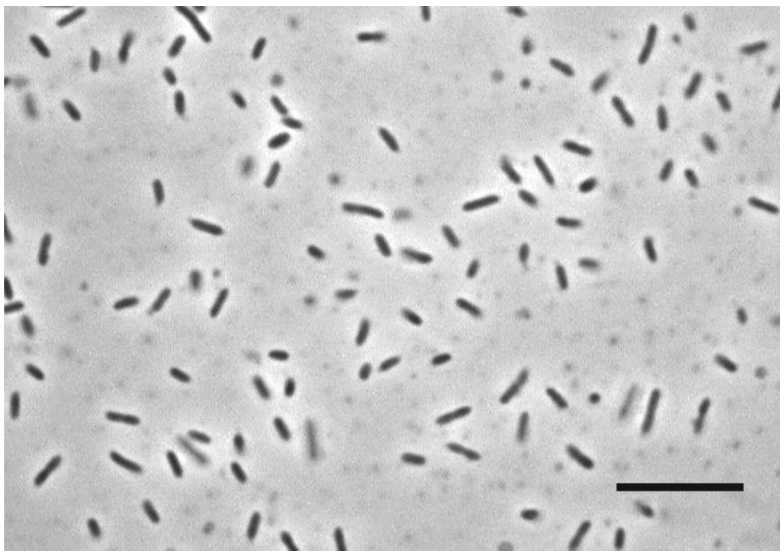
Strains: 1, CAI-18b^T (=DSM 29854^T); 2, *Rufibacter tibetensis* CCTCC AB 208084^T; 3, *Nibribacter koreensis* JCM 17917^T. Size of inhibition zone is given in cm. Data are from the present study. All strains were grown on R2A medium (pH 8.8) for 3 days at 28 °C.

Antibiotic	***Disk Content***	***1***	***2***	***3***
Amoxicillin + Clavulanic acid	20 + 10 µg	0.1	1.6	0.7
Ampicillin	10 µg	0.3	1.7	1.7
Cefoxitin	30 µg	1	2.6	1.8
Cefuroxime	30 µg	0.2	0.4	0.8
Clindamycin	2 µg	1.3	2.2	1.6
Erythromycin	15 µg	0.8	1.8	1.5
Gentamycin	120 µg	0.4	0.6	0.7
Gentamycin	10 µg	0	0.2	0.2
Imipenem	10 µg	3.5	3.8	3
Meropenem	10 µg	1.5	2	2
Neomycin	30 UI	0.1	0.2	0.2
Netilmicin	30 µg	0.2	0.3	0.3
Piperacillin + Tazobactam	100 + 10 µg	1.2	2.8	1.8
Polymyxin B	300 UI	0	0.1	0.1
Vancomycin	30 µg	1.3	1.2	0.5



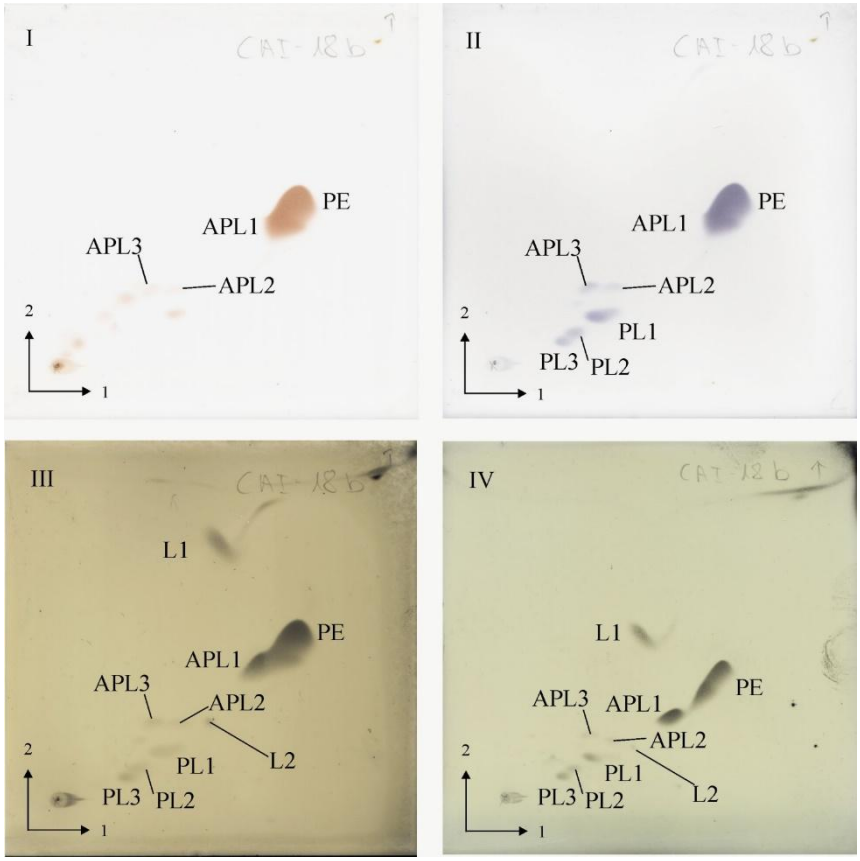
Supplementary Fig. S1. Phylogenetic distribution of *Rufibacter*- and *Nibribacter*-related environmental 16S rRNA gene clone sequences.

Phylogenetic tree has been constructed based on 600 nucleotide positions using the maximum likelihood (ML) method with Kimura 2-parameter nucleotide substitution model. Bootstrap values >50% are shown. Type strains (according to Fig. 1.) are highlighted with bold letters. GenBank accession numbers are given in parentheses. Bar, 0.05 substitutions per nucleotide.



Supplementary Fig. S2. Phase-contrast micrograph from cells of strain CAI-18b^{T}.

Native preparation, after 3 days of incubation on R2A agar. Bar, 5 μm.



Supplementary Fig. S3. Polar lipid profile of strain CAI-18b^{T}.

Two-dimensional TLC of polar lipids after spraying with ninhydrin and heating at 100 °C for 10 minutes (I, aminolipids), after spraying with molybdenum blue (II, phospholipids), after spraying with molybdenum blue (Sigma) and subsequent heating at 200 °C for 15 min (III, total lipids) and after spraying with 20% (w/v) ethanolic phosphomolybdic acid (Sigma) and subsequent heating at 200 °C for 15 min (IV, total lipids). Chloroform/methanol/water (65:25:4, by vol.) was used in the first direction (1), followed by chloroform/acetic acid/methanol/water (80:15:12:4, by vol.) in the second direction (2). Abbreviations: PE, phosphatidylethanolamine; APL1-3, unknown aminophospholipids; PL1-3, unknown phospholipids; L1-2, unknown lipids.