

Aquirufa antheringensis gen. nov., sp. nov. and *Aquirufa nivalisilvae* sp. nov., representing a new genus of widespread freshwater bacteria

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Abstract

Three bacterial strains, 30S-ANTBAC, 103A-SOEBACH and 59G-WUEMPEL, were isolated from two small freshwater creeks and an intermittent pond near Salzburg, Austria. Phylogenetic reconstructions with 16S rRNA gene sequences and, genome based, with amino acid sequences obtained from 119 single copy genes showed that the three strains represent a new genus of the family *Cytophagaceae* within a clade formed by the genera *Pseudarcicella*, *Arcicella* and *Flectobacillus*. BLAST searches suggested that the new genus comprises widespread freshwater bacteria. Phenotypic, chemotaxonomic and genomic traits were investigated. Cells were rod shaped and were able to glide on soft agar. All strains grew chemoorganotrophically and aerobically, were able to assimilate pectin and showed an intense red pigmentation putatively due to various carotenoids. Two strains possessed genes putatively encoding proteorhodopsin and retinal biosynthesis. Genome sequencing revealed genome sizes between 2.5 and 3.1 Mbp and G+C contents between 38.0 and 42.7 mol%. For the new genus we propose the name *Aquirufa* gen. nov. Pairwise-determined whole-genome average nucleotide identity values suggested that the three strains represent two new species within the new genus for which we propose the names *Aquirufa antheringensis* sp. nov. for strain 30S-ANTBAC^T (=JCM 32977^T=LMG 31079^T=DSM 108553^T) as type species of the genus, to which also belongs strain 103A-SOEBACH (=DSM 108555=LMG 31082) and *Aquirufa nivalisilvae* sp. nov. for strain 59G-WUEMPEL^T (=LMG 31081^T=DSM 108554^T).

The phylum *Bacteroidetes* contains Gram-negative bacteria, which occur in a broad variety of habitats all over the world and show different lifestyles and physiologies [1]. The *Cytophaga-Flavobacteria* cluster belongs to this phylum and is characterized by rod-shaped, non-spore-forming, frequently pigmented and gliding, chemoorganotrophic bacteria [2]. They are often capable of degrading biopolymers such as agar, cellulose, chitin, pectin and keratin [3]. Bacteria belonging to the *Cytophaga-Flavobacteria* cluster occur in high abundances in many freshwater and marine systems and play an important role in these ecosystems in respect to the uptake and degradation of dissolved organic material [2]. A large family within this cluster are the *Cytophagaceae*, which comprise 30 genera and about 150 species. Phylogenetic analyses based on 16S rRNA gene sequences suggested that this family encompass divergent clades which cannot be well distinguished from members of the families *Cyclobacteriaceae* and *Flammeovirgaceae* [4]. Genome-scale

phylogenies inferred from whole proteomes revealed that the *Cytophagaceae* as a family and some of their genera are not monophyletic [1].

Within a cooperation project between schools and science (Sparkling Science program), which pursues the aim to isolate and taxonomically describe new bacterial species, 125 teenagers from high schools took samples from various freshwater habitats around the city of Salzburg (Austria), measured basic water chemistry parameters and inoculated agar plates with the collected water samples. By screening these plates, we found several strains belonging to an obviously undescribed genus affiliated with the family *Cytophagaceae*. From nine pure cultures, three were selected for further analysis. Some students joined the lab and helped with the phenotypic characterizations of the strains. Furthermore, some students created, with the help of a nomenclature advisor, the proposed genus and species names.

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Keywords: *Aquirufa*; *Cytophagaceae*; freshwater; genome; whole genome average nucleotide identity (gANI).

Abbreviations: DTS, tryptone–soyotone; gANI, whole-genome average nucleotide identity; IMG/ER, Integrated Microbial Genomes/Expert Review; NSY, nutrient broth–soyotone–yeast extract; R2A, Reasoner's 2A.

The Whole Genome Shotgun project and 16S rRNA gene sequences have been deposited at DDBJ/ENA/GenBank. The accession numbers of strain 30S-ANTBAC^T are SEWZ000000000 (genome) and MK449343 (16S rRNA gene), strain 103A-SOEBACH SEWY000000000 (genome) and MK449347 (16S rRNA gene), strain 59G-WUEMPEL^T SEWX000000000 (genome) and MK449345 (16S rRNA gene).

Two supplementary figures and one supplementary table are available with the online version of this article.

So, here we describe a new genus belonging to the *Cytophagaceae* which seems to be common in various aquatic freshwater habitats. As type species for this new genus we propose *Aquirufa antheringensis* gen. nov., sp. nov. with its type strain 30S-ANTBAC^T. Furthermore, we propose to establish an additional species within this genus, *Aquirufa nivalisilvae* for strain 59G-WUEMPEL^T.

HOME HABITATS AND ISOLATION

Strain 30S-ANTBAC^T was isolated from Antheringer Bach, a small freshwater creek running through the town Anthering, Austria, the approximate geographic coordinates are 47.877 N 13.006 E. Water sampled in November 2017 had a pH of 6.9 and a conductivity of 435 $\mu\text{S cm}^{-1}$. Strain 103A-SOEBACH was isolated from Soellheimer Bach, a small freshwater creek located in Salzburg, Austria, the approximate geographic coordinates are 47.826 N 13.049 E. Water sampled in May 2018 had a pH of 6.9 and a conductivity of 515 $\mu\text{S cm}^{-1}$. Strain 59G-WUEMPEL^T was isolated from a small intermittent freshwater pond located in a forest in Schneegattern (Lengau), Austria, the approximate geographic coordinates are 48.029 N 13.299 E. Water sampled in April 2018 had a pH of 7.5 and a conductivity of 163.7 $\mu\text{S cm}^{-1}$. All three strains were isolated by filtrating the samples and subsequent plating on agar plates. For strains 30S-ANTBAC^T and 103A-SOEBACH filters with 0.45 μm pore size and for strain 59G-WUEMPEL^T filters with 0.65 μm pore size were used. Nutrient broth–soyotone–yeast extract (NSY) agar plates [5] were utilized for 103A-SOEBACH, while Reasoner's 2A (R2A) agar plates [6] were used for strain 30S-ANTBAC^T and tryptone–soyotone (DTS) plates [7] with very low nutrient concentrations were used for strain 59G-WUEMPEL^T for the first cultivation steps, respectively. Nevertheless, all strains grew on NSY plates and in liquid NSY medium at pH 7.2 and were purified using these media.

PHENOTYPIC AND CHEMOTAXONOMIC CHARACTERIZATION

The temperature range for growth was tested on NSY agar plates exposed to increasing temperatures in 1 °C steps starting at 5 °C until no growth was observed. NaCl tolerance was tested using agar plates with various NaCl concentrations in 0.1 % (w/v) steps. For testing anaerobic growth, an anaerobic chamber and standard NSY agar plates as well as NSY plates supplemented with 2 g l⁻¹ NaNO₃ were used. For determination of cell morphology and cell dimensions, well-growing liquid cultures were fixed with 2 % paraformaldehyde, stained with DAPI (4',6-diamidino-2-phenylindole) and investigated by using an epifluorescence microscope (UV filter). To test the ability of the strains to glide, soft agar plates (1 g l⁻¹ yeast extract, 0.1 g l⁻¹ K₂HPO₄, 2.0 g l⁻¹ agar) were used [8]. One drop of a well-growing culture was placed in the centre of these test plates, as well as on standard NSY plates, respectively, incubated at room temperature and observed for several days. Assimilation of various substrates was tested using GEN III MicroPlates

(Biolog), which detect utilization of substrates as electron donors by the subsequent reduction of a tetrazolium redox dye. Cells from well-growing liquid cultures were centrifuged and added to the inoculum medium so that the OD of the culture correspond to 0.07 at a wavelength of 590 nm. The absorption was measured with a Multiskan FC (Thermo Scientific) at a wavelength of 595 nm after 48 h incubation at 20 °C. After subtracting the value of the negative control (without substrate), obtained values from 0.016 to 0.05 were regarded as weak utilization and for >0.05 as positive. The chemotaxonomic characterization of the strains included analyses of the composition of whole-cell fatty acids, polar lipids and respiratory quinones. They were carried out by the Identification Service, Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. For all chemotaxonomic analyses cells were inoculated into liquid NSY medium and harvested after 3 days of growth by centrifugation. For the whole-cell fatty acid composition, an Agilent Technologies 6890 N instrument, the Microbial Identification System (MIDI) Sherlock version 6.1 and the TSBA 40 database were used as described by Sasser [9]. Polar lipids and respiratory quinones were extracted and analysed as described by Tindall [10, 11] based on the method by Bligh and Dyer [12]. To investigate if the pigmentation of the cultures was caused by flexirubin, tests with 20 % KOH and 12 M HCl were performed [13].

Cells of all investigated strains were slim rods. They formed bright red, in older stages dark-red coloured, circular, and convex colonies with smooth surface on agar plates and showed a strong orange-red colouring in liquid media. Strain 59G-WUEMPEL^T grew up to a temperature of 35 °C, the other two strains stopped growing at lower temperatures (Table 1). All strains only tolerated low salt concentrations, showed no anaerobic growth and were able to glide and showed spreading over the whole soft agar plates within periods of 7 days (Table 1). Despite various efforts according to Bernardet *et al.* [13] the flexirubin test was negative for all investigated strains, maybe caused by other overlaying pigments. All three investigated strains assimilated pectin and Tween 40 and showed weak assimilation of acetoacetic acid, glucuronamide, and D-fructose-6-PO₄ (Table 1). Strain 59G-WUEMPEL^T showed weak assimilation of additional substrates (Table 1). All three investigated strains contained nearly the same fatty acids but in various amounts, C_{15:0} was only found in strain 30S-ANTBAC^T (Table 2). The amounts of the major fatty acids differed remarkably between strain 30S-ANTBAC^T and strain 103A-SOEBACH, which should be classified as the same species (see below). For example, iso-C_{15:0} constituted 20.3 % and 39.5 % of fatty acids in strains 30S-ANTBAC^T and 103A-SOEBACH, respectively (Table 2). These finding suggested, that the relative fatty acid amounts can vary a lot among strains belonging to the same species grown under comparable conditions. A study describing a new *Polynucleobacter* species (Betaproteobacteria) within the cryptic species complex PncC based on six investigated isolates came to the

Table 1. Traits characterizing the three strains

All strains have the following substrate usage characteristics in common: assimilation of pectin and Tween 40; weak assimilation of acetoacetic acid, glucuronamide and D-fructose-6-PO₄; no assimilation of L-histidine, propionic acid, D-lactic acid methyl ester, L-alanine, L-glutamic acid, L-aspartic acid, D-glucose-6-PO₄, dextrin, D-glucuronic acid, D-fructose, D-arabitol, α-D-glucose, α-hydroxy-butyric-acid, D-galactose, methyl β-D-glucoside, D-galacturonic acid, 3-methyl glucose, L-rhamnose, L-galactonic acid lactone, D-mannitol, formic acid, L-malic acid, D-gluconic acid, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, mucic acid, myo-inositol, trehalose, inosine, maltose, gentiobiose, β-hydroxy-L-DL-butyric acid, cellobiose, glycyl-L-proline, L-pyroglutamic acid, N-acetyl-D-galactosamine, glycerol, L-fucose, α-keto-glutaric acid, melibiose, lactose, D-fucose, L-lactic acid, turanose, N-acetyl neuraminic acid, quinic acid, D-sorbitol, D-malic acid, p-hydroxy-phenylacetic acid, raffinose, γ-amino-butyric acid, L-arginine, starchyose, gelatin, D-serine, D-saccharic acid, methyl pyruvate, α-keto-butyric acid, bromo-succinic acid, L-serine, citric acid and acetic acid. +, Positive; w, weak; -, negative

Characteristic	Strain 30S-ANTBAC ^T	Strain 103A-SOEBACH	Strain 59G-WUEMPEL ^T
Cell morphology	Rods	Rods	Rods
Mean cell length (μm)	1.7	1.8	1.6
Mean cell width (μm)	0.6	0.4	0.5
Temperature range for growth (°C)	5–32 (w)	5–31 (w)	5–35 (w)
NaCl tolerance (% w/v)	0–0.3 (w)	0–0.2 (w)	0–0.4
Anaerobic growth on NSY plates	–	–	–
Anaerobic growth on NSY+nitrate	–	–	–
Gliding ability	+	+	+
Pigmentation	Red	Red	Red
Flexirubin test	–	–	–
Assimilation of:			
Sucrose	–	–	w
D-Mannose	–	–	w
D-Salicin	–	–	w
D-Aspartic acid	–	–	w

same conclusion [14]. Regarding the polar lipids, all three strains presented here contained phosphatidylethanolamine and various unidentified polar lipids like aminolipids, phospholipids and aminophospholipids (Table 2, Fig. S1, available in the online version of this article). While for strain 30S-ANTBAC^T seven polar lipids could be separated, strain 59G-WUEMPEL^T showed a more diverse pattern with three additional unidentified polar lipids. The main respiratory quinone for all three strains presented here was MK7. Additionally, strain 30S-ANTBAC^T and strain 103A-SOEBACH contained traces of MK6 (Table 2).

GENOMIC CHARACTERIZATION

DNA extraction and genome sequencing were performed as described previously [15]. A shotgun library was paired-end sequenced on an Illumina MiSeq instrument (2×300 bp). *De novo* assemblies were performed by using the software SPAdes version 3.13.0 [16], details are given in Table 3. The obtained genome sequences were annotated by the NCBI Prokaryotic Genome Annotation Pipeline and for further comparative analyses by the Integrated Microbial Genomes/Expert Review (IMG/ER) annotation pipeline and incorporated in the IMG database [17]. The IMG Genome ID and GenBank accession numbers of all three investigated strains and strain HME7025 (Kim et al., ‘*Pseudarcicella* sp.’ HME7025 genome sequencing and assembly, unpublished data), which is closely related to the three investigated strains, are listed in Table 3.

The size of the genomes of strain 30S-ANTBAC^T and strain 103A-SOEBACH was 2.5 Mbp, while strain 59G-WUEMPEL^T had a size of 3.1 Mbp (Table 3). All strains had relatively low G+C contents of less than 45 mol% (Table 3). Interestingly, members of the nearest relatives of the new genus had genome sizes twice as big (Table 4). Additionally, among 60 genomes of members of the family *Cytophagaceae* and related taxa, strain 30S-ANTBAC^T and strain 103A-SOEBACH not only possessed the smallest genome size but also the highest coding density. While coding densities of the other 58 genomes ranged from 85.6 % to 93.5 % coding bases (average 89.5 %, median 89.4 %), these two genomes were characterized by a value of 94.8 %. On the other hand, the G+C content of their genomes of 43 mol% was close to the average of the other *Cytophagaceae* genomes (range 35–56 mol%, average 45 mol%), suggesting that the genomes of these two strains underwent evolutionary genome streamlining, which was decoupled from changes in G+C content.

The gene content of the three investigated strains and strain HME7025, which is closely related to strain 59G-WUEMPEL^T (see above), showed interesting pattern, some genes had all strains in common and some differed between them (Table 5). All four strains had genes putatively encoding proteins associated with gliding motility, which confirmed the observed gliding on soft agar plates. Only strain 30S-ANTBAC^T and strain 103A-SOEBACH possessed genes putatively encoding a proteorhodopsin and a β-carotene

Table 2. Chemotaxonomy: polar lipids, respiratory quinones and fatty acid composition (%) of the three investigated strains

Fatty acids with values less than 0.5 percent for all strains are not listed. +, Detected; –, not detected, TR, trace.

Chemotaxonomic property	Strain 30S-ANTBAC ^T	Strain 103A-SOEBACH	Strain 59G-WUEMPEL ^T
Polar lipids:			
Phosphatidylethanolamine	+	+	+
Unidentified aminolipids	–	1	1
Unidentified amino-phospho-lipids	2	2	3
Unidentified polar lipids	4	4	5
Respiratory quinones:			
MK7	+	+	+
MK6	TR	TR	–
Fatty acids:			
C _{14:0}	2.9	1.0	0.4
C _{15:0}	2.2	–	–
C _{15:1} ω6c	1.5	2.0	1.1
C _{16:1} ω5c	11.9	6.8	3.0
C _{17:1} ω6c	1.1	1.3	1.4
iso-C _{11:0}	3.6	2.6	1.2
iso-C _{13:0}	0.8	0.8	1.4
iso-C _{15:0}	20.3	39.5	34.4
anteiso-C _{11:0}	0.5	0.3	0.2
anteiso-C _{13:0}	0.5	0.4	0.7
anteiso-C _{15:0}	5.6	8.3	10.7
C _{16:0} 3-OH	1.0	0.5	0.3
Iso-C _{15:0} 3-OH	13.4	11.7	10.5
Iso-C _{17:0} 3-OH	1.1	2.5	3.3
Iso-C _{17:1} ω9c	0.4	0.8	2.0
Summed feature 1*	0.3	1.0	1.0
Summed feature 2*	1.3	0.5	0.2
Summed feature 3*	24.7	9.2	16.8
Summed feature 4*	1.1	2.9	3.0
Unknown 14.959†	2.4	4.5	5.0

*Summed features represent groups of fatty acids which could not be separated by GLC and the MIDI system. Summed feature 1 contains iso-C_{15:1} h, C_{13:0} 3-OH, iso-C_{15:1} l l/H and iso-C_{15:1} l; summed feature 2 contains C_{12:0} ALDE, unknown 10.928, iso-C_{16:1} l, C_{14:0} 3-OH and iso-C_{16:1} l; summed feature 3 contains iso-C_{15:0} 2-OH and C_{16:1} ω7c; summed feature 4 contains iso-C_{17:1} l and anteiso-C_{17:1} B/l l.

†Unknown 14.959 is an unknown compound with an ECL of 14.959.

Table 3. Genome characteristics of the three investigated strains and strain HME7025

Characteristic	Strain 30S-ANTBAC ^T	Strain 103A-SOEBACH	Strain 59G-WUEMPEL ^T	Strain HME7025
Number of scaffolds	14	17	40	–
K-mer coverage	260	235	107	–
N50 value (Mbp)	0.64	0.58	0.78	–
Genome size (Mbp)	2.5	2.5	3.1	3.1
G+C content (mol%)	42.6	42.7	38.0	37.9
ANI with 30S-ANTBAC ^T	100.0	96.5	70.8	71.0
ANI with 103A-SOEBACH	96.5	100.0	71.0	71.1
ANI with 59G-WUEMPEL ^T	70.8	71.0	100.0	97.4
ANI with HME7025	71.0	71.1	97.4	100.0
Accession number GenBank	SEWZ000000000	SEWY000000000	SEWX000000000	CP029346
IMG/ER Genome ID	2816332120	2816332126	2816332125	2811994884

Table 4. Comparison of the proposed genus *Aquirufa* and the three most closely related genera

Features for all genera in common: identified polar lipid, phosphatidylethanolamine; major respiratory quinone, MK-7. ++, Genes putatively associated with gliding, ND, not determined.

Feature	<i>Aquirufa</i> gen. nov.	<i>Arcicella</i>	<i>Pseudarcicella</i>	<i>Flectobacillus</i>
Number of species	2	4	1	6
Cell morphology	Rods	Various*	Rods	Rods
Range of cell length (µm)	1.6–1.8	2.5–6.3	1.0–2.0	1.5–10.0
Range of cell width (µm)	0.4–0.6	0.5–0.75	0.9–1.1	0.3–1.0
Pigmentation	Red	Pink or orange	Pink	Pink or orange
Flexirubins	No	No	ND	No
Carotenoids	All strains†	One species	ND	Some species
Motility	Gliding /+++†	No/+++†	No/+++†	No/+++†
Temperature range of growth (°C)	5–34	4–40	10–36	4–40
NaCl tolerance (%NaCl, w/v)	0–0.4	0–3.0	ND	0–4.0
Predominant fatty acids	Iso-C _{15:0} 3-OH, iso-C _{15:0}	C _{16:1} ω5c, iso-C _{15:0}	C _{16:1} ω5c, C _{18:1} ω7c	C _{16:1} ω5c, iso-C _{15:0}
Summed feature	C _{16:1} ω7c, C _{16:1} ω7c, iso-C _{15:0} 2-OH	C _{16:1} ω7c, C _{16:1} ω6c	C _{16:1} ω7c, iso-C _{15:0} 2-OH	
G+C content (mol%)	38–43	34–44	38†	38–40
Genome size (Mbp)	2.5–3.1†	5.9†	6.2†	6.2†
Genome available (Table 3, S1)	All strains	<i>Arcicella aurantiaca</i>	<i>Pseudarcicella hirudinis</i>	<i>Flectobacillus major</i>
References	This study	[40]	[35]	[41–44]

*Rods, vibrioid, curved or spiral-shaped.

†Based on genome data.

Table 5. Comparison of the presence and absence of selected genes of the three investigated strains and strain HME7025

+, Present; –, absent.

Genes putatively encoding	Strain 30S-ANTBAC ^T	Strain 103A-SOEBACH	Strain 59G-WUEMPEL ^T	Strain HME7025
Motility:				
Proteins associated with gliding	+	+	+	+
Utilization of light:				
Proteorhodopsin	+	+	–	–
Biosynthesis of 7,8-dihydro-β-carotene	+	+	+	+
β-Carotene 1, 15'-monooxygenase	+	+	–	–
Pigments:				
Biosynthesis of β, γ and ζ-carotene	+	+	+	+
Biosynthesis of β-cryptoxanthin, canthaxanthin, phoenicoxanthin and astaxanthin	+	+	+	+
Transport systems:				
ABC-type: phospholipid, lipoprotein	+	+	+	+
myo-Inositol, lipopolysaccharide	–	–	+	+
MFS-transporter: nitrate/nitrite	+	–	+	+
Inorganic nutrients:				
Nitrate reductase (assimilatory)	+	–	+	+
Nitrite reductase (assimilatory)	+	–	+	+
Anaerobiosis:				
Nitrous oxide reductase	–	+	+	+
Oxidative stress:				
Catalase-peroxidase (EC:1.11.1.21)	+	+	–	–
Cytochrome c peroxidase (EC:1.11.1.5)	+	+	–	–

15,15'-monooxygenase. But all four strains had genes putatively encoding enzymes for the complete biosynthesis of 7,8-dihydro- β -carotene. The latter one serves as substrate for the monooxygenase, which produces retinal, the cofactor for the light driven proton pump proteorhodopsin [18]. Furthermore, only strain 30S-ANTBAC^T and strain 103A-SOEBACH possessed genes putatively encoding a catalase-peroxidase and a cytochrome c peroxidase, which could protect bacteria from oxidative stress.

All three investigated strains and strain HME7025 encoded genes annotated for the biosynthesis of various carotenoids (Table 5). Concerning the ABC-type transport systems the four strains showed different patterns (Table 5). Regarding nitrogen metabolism the four strains showed some shared genes with an interesting distribution pattern among them. All strains except 103A-SOEBACH possessed a gene cluster, which encompassed genes putatively necessary for the uptake and assimilation of nitrate and nitrite (Table 5). All strains except 30S-ANTBAC^T possessed a gene cluster with genes putatively encoding enzymes responsible for the reduction of nitrous oxide to nitrogen, which is the last step of the dissimilatory denitrification (see below).

PHYLOGENY

Phylogenetic and phylogenomic reconstructions were performed by using almost full length sequences of the 16S rRNA gene and an aligned concatenated set of 119 single copy marker proteins [19], respectively.

For the phylogenetic tree in Fig. 1, 16S rRNA gene sequences from the type species of all genera currently classified as *Cytophagaceae* [1, 20], of further species closely related to the investigated strains, of *Prevotella melaninogenica* serving as outgroup, and of the investigated strains were used. The sequence set was aligned by using the software MEGA X [21]. The trimmed alignment consisted of 1336 alignment positions. With the same software the data set was tested for the best fitting model and thus a maximum-likelihood phylogenetic tree was calculated using Kimura's two-parameter model [22] as follows: discrete gamma distribution (G=5), 500 replications, gaps were partial deleted, which resulted in 1306 used positions. In addition, neighbour-joining and maximum-parsimony trees were calculated by using the corresponding algorithms.

Although the 16S rRNA gene tree showed that the major branching close to the root of the family *Cytophagaceae* were only supported by low bootstrap values, the clade comprising species of the genera *Flectobacillus*, *Arcicella*, *Pseudarcicella* and the new taxon represented by the investigated strains was very well supported in all calculated trees. Within this clade, the phylogenetic tree showed a clear structure supported by high bootstrap values. The new taxon appeared to be closest related to *Pseudarcicella hirudinis*. The 16S rRNA gene sequence of the type strain of this species had a similarity of 93 % to that of strain 30S-ANTBAC^T. Nevertheless, it was not clear if the 16S rRNA gene tree could fully resolve the positions of the strains belonging

to the new taxon. The fact, that 16S rRNA gene sequences of different species within a genus are quite similar was reported from other taxonomic groups [23].

To improve the phylogenetic placement of the members of the new genus, genome data sets were used to calculate a RAxML tree [24]. Of the 120 protein-encoding genes recommended by Parks *et al.* [19], one gene (protein family TIGR00095) was not present in any of the available genomes of members of the *Cytophagaceae*. Sequences of the remaining 119 protein families were concatenated and aligned by using MAFFT [25]. This resulted in a sequence alignment of 50 907 amino acid positions. This alignment was filtered by GBLOCKS (version 0.91b) [26] for highly variable positions. The chosen settings resulted in reduction of the alignment to 37 796 positions (74 % of positions) in 556 selected blocks. A maximum-likelihood tree (Fig. 2) was reconstructed using RAxML 8.2.10 [24], as implemented on the CIPRES Science Gateway version 3.3 [27], under the Protein CAT model with auto selection of a protein substitution matrix (model settings PROTCATAUTO), and 100 bootstrapping iterations.

The phylogenetic reconstruction revised the placements of the members of the *Cytophagaceae* and confirmed the clade comprising members of the genus *Flectobacillus*, *Arcicella*, *Pseudarcicella* and the new taxon. The latter was now clearly separated from the other three genera. (Fig. 2).

ECOLOGY

The three strains presented in this study were isolated from either standing or running freshwater systems. All home habitats had in common that they were small ones. Thus, all habitats were in close contact to terrestrial systems. This corresponded with the fact that all strains are obviously able to degrade the polymer pectin, which is a component of the cell wall of terrestrial plants. The ability of all investigated strains to glide could also be a hint to their lifestyle in small and not permanent waters, which potentially allow them to move on moist surfaces. Strains 30S-ANTBAC^T and 103A-SOEBACH could have, via proteorhodopsin, the opportunity to use sunlight as a supplementary energy source, which indicates that the strains live in at least temporarily light exposed habitats. All investigated members of the new genus-like taxon possessed a gene cluster putatively encoding for enzymes responsible for the reduction of nitrous oxide to nitrogen. This last step of the dissimilatory nitrate reduction is used by some bacteria to transfer protons across the membrane for energy conservation and was considered as the least oxygen tolerant step of nitrogen respiration [28]. However, a recent study [29] from a marine system revealed that N₂O-consuming bacteria were also present and active in oxygenated surface water, this might be attributed to anoxic micro-environments created by particles. Even though the members of the new taxon grew aerobically, the potential ability to use N₂O could also be an advantage at the water sediment interface of small or ephemeral water bodies, where anaerobic conditions may occur.

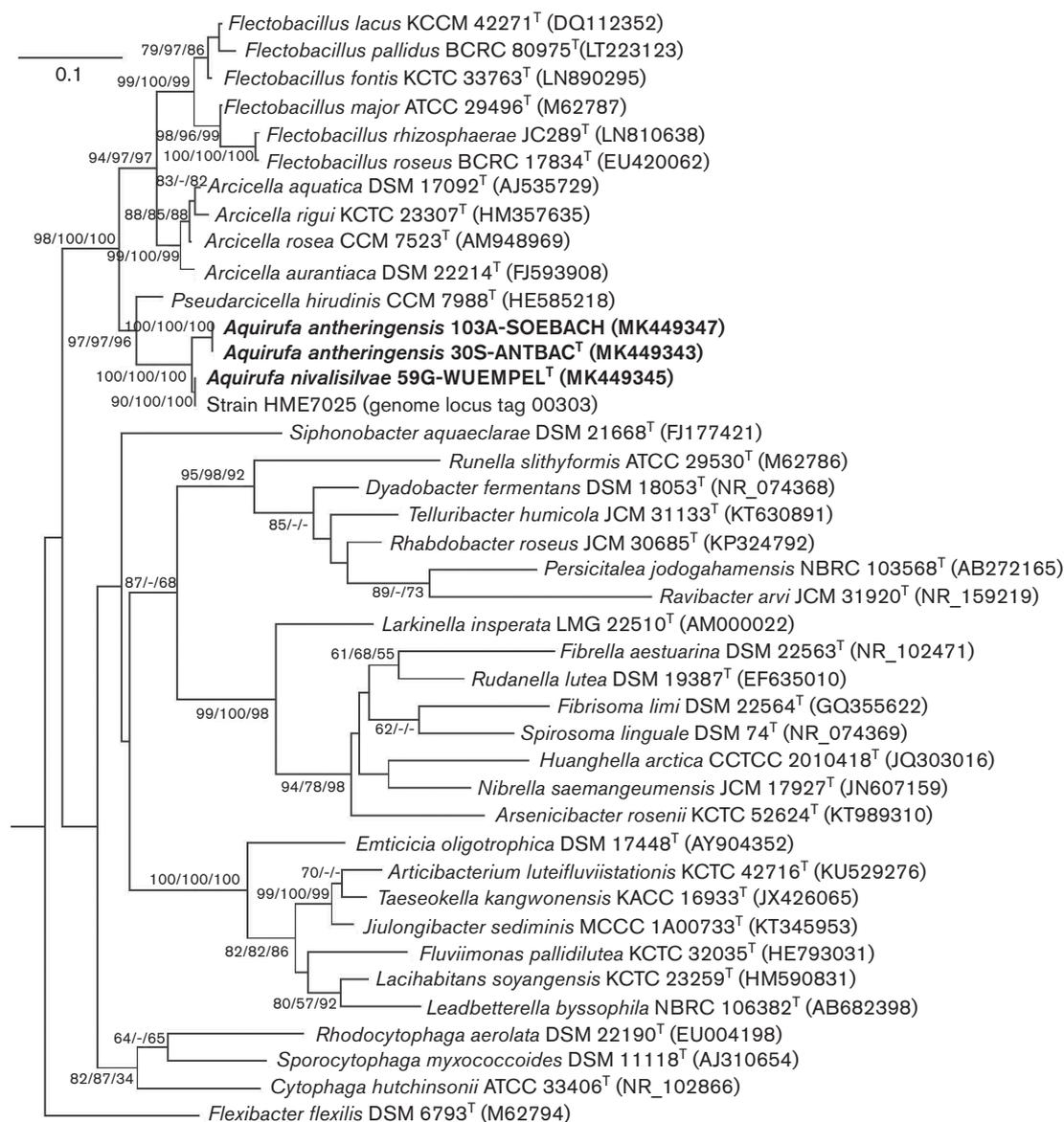


Fig. 1. Reconstruction of the phylogenetic position of the investigated strains based on almost full length 16S rRNA gene sequences (1336 alignment positions). Sequences from all genus type species of the family *Cytophagaceae*, and further species closely related to the investigated strains were used. Shown is the maximum-likelihood tree. Bootstrap values are shown from left to right for maximum-likelihood, neighbour-joining, and maximum-parsimony trees calculated with the same sequence set. Bar, 0.01 substitutions per nucleotide position. The tree was rooted with *Prevotella melaninogenica* DSM 7089^T (not shown, AY323525).

BLAST searches with the 16S rRNA gene sequences of 30S-ANTBAC^T and 59G-WUEMPEL^T revealed that the new genus-like taxon represents a common and widespread group of freshwater bacteria. Even when taking only the 100% and 99% hits into account, more than 100 sequences from uncultured and cultured bacteria originating from all over the world were reported (Fig. S2). This included detections from various inland water systems like rivers, lakes, ponds, groundwater and lake sediments in the USA, Canada, the Arctic, China, Japan,

Africa and Europe. For instance, sequences from environmental samples putatively belonging to the new genus-like taxon were found in high-altitude lakes of the eastern Tibetan plateau [30], the temperate Ipswich and Parker River in Massachusetts, USA [31], the humic lake Grosse Fuchskuhle, Germany [32], and in sediments of the freshwater Lake Kasumigaura, Japan [33]. Nevertheless, only a very few isolates were found in databases corresponding to the 16S rRNA genes from strains 59G-WUEMPEL^T and 30S-ANTBAC^T with identities of more

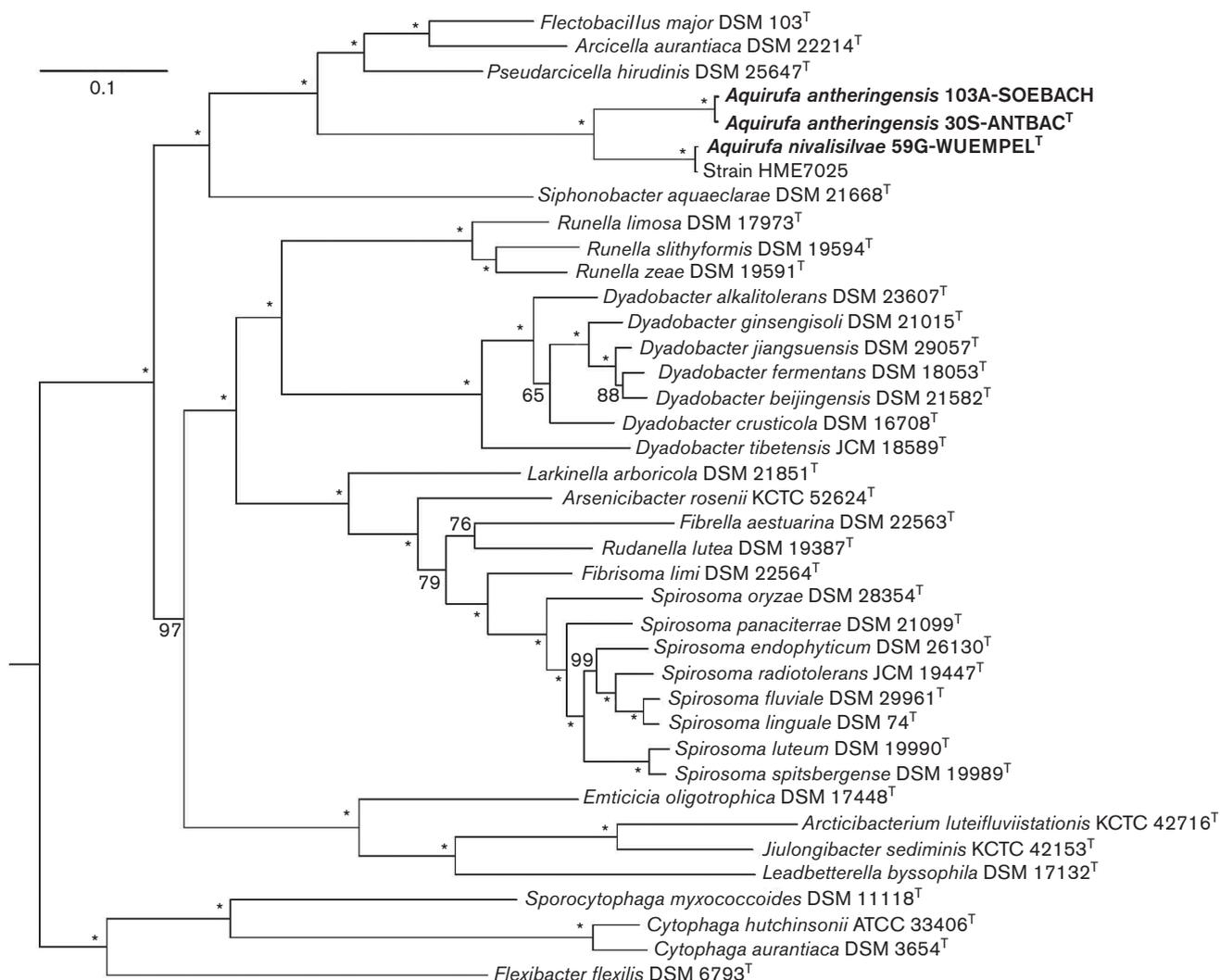


Fig. 2. Phylogenomic RAxML tree calculated with amino acid sequences obtained from 119 single copy genes from genomes of species belonging to the family *Cytophagaceae*. Asterisk, bootstrap value 100%. Bar, 0.2 substitutions per nucleotide position. Accession numbers of the genome sequences are listed in Tables S1 and 3. The tree was rooted with *Prevotella melaninogenica* DSM 7089^T (not shown).

than 98.7%, which is according to Chun *et al.* [34] a similarity value potentially separating different species. In the case of 59G-WUEMPEL^T six strains sharing 16S rRNA gene similarities of >99.7% were isolated from a mesotrophic lake in the Republic of Korea (strains HME7025, IMCC25912 and HMD1017), from the sediment of a freshwater lake in Japan [33], from an unknown source in China (strain D11), and from freshwater in Taiwan (strain CAR-16). In case of strain 30S-ANTBAC^T, only two isolates sharing 16S rRNA gene similarities of >99.3%, one from an artificial mesotrophic lake in the Republic of Korea (strain HME7208) and one from Lake Maarsseveen, The Netherlands (strain MI3-53), could be found.

PROPOSAL OF THE NEW GENUS *AQUIRUF* GEN NOV. AND THE TWO SPECIES *AQUIRUF* *ANTHERINGENSIS* SP. NOV. AND *AQUIRUF* *IVALISILVAE* SP. NOV.

In the 16S rRNA gene tree, strains 30S-ANTBAC^T, 103A-SOEBACH and 59G-WUEMPEL^T formed their own clade, well separated from the closest related genera. With their nearest relative *Pseudarcicella hirudinis* they showed low 16S rRNA gene sequence similarities of less than 94.5%. The separation of the clade formed by the new strains was confirmed and more pronounced by the phylogenomic tree (Fig. 2). Pairwise whole genome average nucleotide identity (gANI) values

calculated with the type strains of *Pseudarcicella hirudinis* and *Arcicella aurantiaca* were only slightly higher (71.8%) than pairwise values calculated (i) for any of the three new strains and the *P. hirudinis* type strain (in all three cases about 69%), and (ii) for any of the three new strains and the *A. aurantiaca* type strain (in all three cases 69–70%). A comparison of the new taxon with the closest related genera *Pseudarcicella*, *Arcicella* and *Flectobacillus* is given in Table 4. The new taxon differed in pigmentation, in predominant fatty acid composition and in the combination of some other features from all other genera (Table 4). The patterns of the polar lipids of the three investigated strains (S1) and *Pseudarcicella hirudinis* [35] differed strongly. The most striking point is the genome size of the new taxon, which is approximately half of that of these nearest related genera. These points and the phylogenetic reconstructions (Fig. 1, Fig. 2), as well as 16S rRNA gene similarities and gANI values suggested that these three strains represent a new genus, for which we propose the name *Aquirufa* gen. nov.

To test which of the three investigated strains belong to the same species, pairwise gANI values were calculated (Table 3). While strains 30S-ANTBAC^T and 103A-SOEBACH had a gANI value of 96.5% which is slightly higher than the proposed cut-off of 95–96% separating two species [34, 36–39], indicated the remaining gANI values of rounded 71% (Table 3) that the three investigated strains represented two different species [34, 36–39]. These findings were confirmed by the phylogenomic reconstructions with multiple amino acid sequences (Fig. 2), which separated the proposed novel species on different branches with branch length, i.e. evolutionary distances, similar to those between other type strains belonging to the same genus within the family *Cytophagaceae* (Fig. 2). The pairwise gANI value between strains 59G-WUEMPEL^T and HME7025 of 97.4% suggested, that these strains need to be considered as members of the same species. At first glance, the results of the gene content concerning the nitrogen metabolism disagree with the classification of the investigated strains into two species, since strains 30S-ANTBAC^T and 103A-SOEBACH classified as one species differed in possessing gene clusters putatively encoding for nitrate/nitrite assimilation and nitrous oxide reduction, respectively (see above). According to the pangenome concept, these two gene clusters could be regarded as auxiliary genes representing parts of the flexible genome of the strains. Such genes could be exchanged by intra- or even interspecific horizontal gene transfer [15], which might explain, why the clusters are present or absent in strains belonging to the same species. In the case of the predicted assimilatory nitrate reductase, the gene sequence similarity between strains HME7025 and 59G-WUEMPEL^T, probably belonging to a single species, was relatively high (97%), while the latter and strain 30S-ANTBAC^T showed 70%. In contrast, for the predicted nitrous oxide reductase and flanking genes, the sequence similarity between strains HME7025 and 59G-

WUEMPEL^T was the same (97%), while the latter and strain 103A-SOEBACH showed 88% similarity of the genes. This value is much higher than the whole genome sequence similarity of 71% between these two strains and could imply that for this gene cluster horizontal gene transfer across species boundaries might be possible.

Some features distinguished the three strains from each other. Only strain 59G-WUEMPEL^T weakly assimilated sucrose, D-mannose, D-salicin and D-aspartic acid (Table 1). Furthermore, while strain 30S-ANTBAC^T had only four unidentified polar lipids, strain 59G-WUEMPEL^T had five. The two strains 30S-ANTBAC^T and 103A-SOEBACH considered as belonging to the same species, shared highly similar G+C values but differed in this characteristic from the third strain.

DESCRIPTION OF *AQUIRUF* GEN. NOV.

Aquirufa gen. nov. [A.qui.ru'fa. L. n. *aqua*, water; L. adj. *rufus*, red; N.L. fem. n. *Aquirufa*, a red (bacterium) isolated from water].

Cells form rods and grow chemoorganotrophically and aerobically. Colonies grown on NSY or R2A agar are bright red, in older stages dark red pigmented, circular and convex with smooth surface. Liquid cultures in NSY or R2A medium have an intense red-orange colouring. Cells are able to glide on soft agar. Major respiratory quinone is MK-7, predominant fatty acid is iso-C_{15:0}, identified polar lipid is phosphatidylethanolamine. Based on phylogenetic reconstructions with 16S rRNA gene sequences and amino acid sequences obtained from 119 single copy genes, respectively, the genus belongs to the family *Cytophagaceae*. G+C content is in the range of 38–43 mol%. The type species of the new genus is *Aquirufa antheringensis*.

DESCRIPTION OF *AQUIRUF* *ANTHERINGENSIS* SP. NOV.

Aquirufa antheringensis (an.the.rin.gen'sis. N.L. fem. adj. *antheringensis*, isolated from Antheringer Creek).

Cells form rods, about 1.7 μm long and 0.6 μm wide. Colonies grown on NSY or R2A agar are bright red, in older stages dark red, pigmented, circular and convex with smooth surface. Liquid cultures in NSY or R2A medium have an intense red-orange colouring. Cells are able to glide on soft agar. Growth occurs at 5–32 °C and in 0–0.3% (w) NaCl. Cells assimilate pectin and Tween 40, weakly assimilate acetoacetic acid, glucuronamide and D-fructose-6-PO₄, and do not assimilate L-histidine, propionic acid, D-lactic acid methyl ester, L-alanine, L-glutamic acid, L-aspartic acid, D-glucose-6-PO₄, dextrin, D-glucuronic acid, D-fructose, D-arabitol, α-D-glucose, α-hydroxy-butyric-acid, D-galactose, methyl β-D-glucoside, D-galacturonic acid, 3-methyl glucose, L-rhamnose, L-galactonic acid lactone, D-mannitol, formic acid, L-malic acid, D-gluconic acid, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, mucic acid, myo-inositol, trehalose, inosine, maltose, gentiobiose, β-

hydroxyl-D,L-butyric acid, cellobiose, glycyl-L-proline, L-pyroglutamic acid, N-acetyl-D-galactosamine, glycerol, L-fucose, α -keto-glutaric acid, melibiose, lactose, D-fucose, L-lactic acid, turanose, N-acetyl neuraminic acid, quinic acid, D-sorbitol, D-malic acid, p-hydroxy-phenylacetic acid, raffinose, γ -amino-butyric acid, L-arginine, stachyose, gelatin, D-serine, D-saccharic acid, methyl pyruvate, α -keto-butyric acid, bromo-succinic acid, L-serine, citric acid, sucrose, D-mannose, D-salicin, D-aspartic acid and acetic acid. Major fatty acids are C_{16:1} ω 5c, iso-C_{15:0}, iso-C_{15:0} 3-OH and summed feature 3 (iso-C_{15:0} 2-OH and C_{16:1} ω 7c). Polar lipids are phosphatidylethanolamine, unidentified aminolipids, unidentified amino-phospho-lipids and unidentified polar lipids. Respiratory quinones are MK7 and traces of MK6.

The type strain is 30S-ANTBAC^T (=JCM 32977^T =LMG 31079^T=DSM 108553^T), which was isolated from a small creek with medium conductivity and nearly neutral pH located in Anthering, Austria. The genome of the type strain is characterized by a size of 2.5 Mbp and a G+C content of 42.6 mol%.

DESCRIPTION OF *AQUIRUFALIVALISILVAE* SP. NOV.

Aquirufa nivalisilvae [ni.va.li.si.l'vae. L. adj. *nivalis*, snow covered; L. n. *silva*, forest; N.L. gen. n. *nivalisilvae*, from the forest of Schnee (snow)gattern].

Cells form rods, about 1.6 μ m long and 0.5 μ m wide. Colonies grown on NSY or R2A agar are bright red, in older stages dark red, pigmented, circular and convex with smooth surface. Liquid cultures with NSY or R2A medium have an intense red-orange colouring. Cells are able to glide on soft agar. Growth occurs at 5–35 °C and in 0–0.4 % (w) NaCl. Cells assimilate pectin and Tween 40, weakly assimilate acetoacetic acid, glucuronamide, D-fructose-6-PO₄, sucrose, D-mannose, D-salicin, and D-aspartic acid, but do not assimilate L-histidine, propionic acid, D-lactic acid methyl ester, L-alanine, L-glutamic acid, L-aspartic acid, D-glucose-6-PO₄, dextrin, D-glucuronic acid, D-fructose, D-arabitol, α -D-glucose, α -hydroxy-butyric-acid, D-galactose, methyl β -D-glucoside, D-galacturonic acid, 3-methyl glucose, L-rhamnose, L-galactonic acid lactone, D-mannitol, formic acid, L-malic acid, D-gluconic acid, N-acetyl-D-glucosamine, N-acetyl- β -D-mannosamine, mucic acid, myo-inositol, trehalose, inosine, maltose, gentiobiose, β -hydroxyl-D,L-butyric acid, cellobiose, glycyl-L-proline, L-pyroglutamic acid, N-acetyl-D-galactosamine, glycerol, L-fucose, α -keto-glutaric acid, melibiose, lactose, D-fucose, L-lactic acid, turanose, N-acetyl neuraminic acid, quinic acid, D-sorbitol, D-malic acid, p-hydroxy-phenylacetic acid, raffinose, γ -amino-butyric acid, L-arginine, stachyose, gelatin, D-serine, D-saccharic acid, methyl pyruvate, α -keto-butyric acid, bromo-succinic acid, L-serine, citric acid and acetic acid. Major fatty acids are iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{15:0} 3-OH and summed feature 3 (iso-C_{15:0} 2-OH and C_{16:1} ω 7c). Polar lipids are phosphatidylethanolamine, one unidentified

aminolipid, three unidentified amino-phospho-lipids and five unidentified polar lipids. The respiratory quinone is MK7.

The type strain is 59G-WUEMPEL^T (=LMG 31081^T =DSM 108554^T), which was isolated from a small intermittent freshwater pond with low conductivity and nearly neutral pH located near Lengau, Austria. The genome of the type strain is characterized by a size of 3.1 Mbp and a G+C content of 38.0 mol%.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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