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Culturable aerobic bacteria from the upstream region of a karst water rivulet

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Summary. The composition of 681 aerobic and heterotrophic strains that were isolated on two different media was assessed at four sampling points along a ~300 m stretch of a karst water rivulet. Based on partial sequence analysis of 16S rRNA genes, members of 35 genera were identified; however, only a few species dominated as their representatives were repeatedly isolated at different sampling sites. Determination of the phylum affiliation showed that the isolates included members of Bacteriodetes (especially the genus *Flavobacterium*) and *Proteobacteria* (mainly *Pseudomonas* and *Stenotrophomonas*). MALDI-TOF analysis and/or similarities of partial sequences of flavobacterial strains resulted in the generation of almost complete 16S rRNA gene sequences for 100 isolates, about 60 of which may represent novel phylospecies. The number as well as the intra-phylum distribution of the isolates changed with distance from the discharge site. While phylogenetically restricted at the spring, diversity increased at downstream sampling sites. [**Int Microbiol** 2008; 11(2):91-100]

Key words: Bacteriodetes · *Proteobacteria* · MALDI-TOF analysis · freshwater · karst

Introduction

Within the framework of a broad study on the coupling of the geosphere and the biosphere by microbial processes, the geobiology of organo- and biofilms in a northern-Germany karst hardwater creek, the Westerhöfer Bach, was investigated. Water with a calcium content exceeding 120 mg Ca^{2+}/l is considered very hard, and this rivulet has a water hardness of 140 mg Ca^{2+}/l. The partial pressure of CO_2 drops along the rivulet, and the pH increases from 7.3 to 8.3 because of CO_2 degassing. Water chemistry data from the Westerhöfer Bach were recently reported [12]. While the role of cyanobacteria in calcite nucleation, fabric formation, and early diagenesis within tufa-forming biofilms has been long emphasized [23,27,28,31], the contribution of aerobic and heterotrophic bacteria has only recently been explored in detail [13,37,38]. Most of those studies concentrated on marine stromatolites and tufa deposits whereas reports on non-marine environments are much less frequent [5,21,28,39]. In general, the bacterial diversity of karst water has not been thoroughly assessed. A preliminary survey on the phylogenetic identity of 100 isolates revealed the dominance of flavobacteria [12,15,34]. In order to investigate in greater detail the taxonomic diversity of aerobic bacteria, bacterial strains present in samples obtained at the discharge site of Westerhöfer Bach as well as three downstream sampling sites of the rivulet were isolated on R2A medium. This study serves as the starting point for a thorough assessment of bacteria involved in biofilm formation, which itself may contribute to the formation of tufa sediments.

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Materials and methods

Sampling and isolation of strains. Sampling locations were site 1 (the discharge site), site 2 (110 m downstream of the spring), site 3 (262 m downstream of the spring), and site 4 (295 m downstream of the spring, on top of an extensive tufa formation). The bottled water samples were directly placed in ice, brought back to the facility within 2 h, and plated the same day. The isolation of strains from the Westerhöfer Bach (named WB in the following text) on low nutritional content media (R2A [Difco, Detroit, MI] and medium 51) in June 2005 has been previously described [12]. Plates were incubated at 8 and 21ºC and colonies were picked randomly

over a period of 3 weeks whenever new colonies emerged. Following their isolation, the strains were transferred onto the same medium. Cultures were maintained in glycerol at 8°C in R2A.

Molecular assessment of isolates and determination of diversity. DNA extraction and PCR amplification of the 16S rRNA genes were carried out as described [41]. Partial gene sequences were obtained using the 530 reverse primer (5′-GKATTACCGCGGCKGCTG-3′). PCR amplificons were purified by using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Almost complete sequences were generated by Seqlab (Göttingen, Germany) using DNA released from boiled cells. The sequences were assembled with the Sequencher software, version 4.1 for Macintosh (Genes Codes, Ann Arbor, MI). The sequences of all partial sequences were first analyzed by BLAST [3] and partial as well as almost complete sequences were automatically aligned according to the SILVA database release 92 (Ribocon, Bremen, Germany) using the SINA aligner (Ribocon) [40] and the ARB program [32]. Similarity values but not dendrograms of relationships were generated for partial 16S rRNA gene sequences. Phylogenetic relationships of almost complete sequences were determined using neighbor-joining [20] and maximum-likelihood analyses [19,36]. As the vast majority of sequences were highly related (>96% similarity) among each other and to those of type strains, no additional treeing algorithms were applied. EMBL accession numbers of the almost complete 16S rRNA genes (AM934630-AM934798) were obtained for single representatives of strain clusters that showed <98.5% similarity with phylogenetically nearest neighbors. Partial sequences were deposited under FM161352–FM161880.

Pairwise distance matrices served as input to DOTUR [44] for clustering the sequences into operational taxonomic units (OTUs) at 99 and 98% sequence similarity, thus generating rarefaction values, the Simpson and Shannon diversity indices, and the estimated ACE and Chao1 values.

MALDI-TOF analysis. Cellular extracts were analyzed according to the ethanol/formic acid extraction protocol recommended by Bruker Daltonics. Isolated colonies (ca. 10 mg) from R2A agar were washed with 300 µl MilliQ Water and 900 µl ethanol. The biomass was pelleted by centrifugation at 13,000 ×*g* twice for 3 min after which the pellet was resuspended in 50 µl acetonitrile and the suspension mixed with 50 µl 70% formic acid. The cells were collected at 13,000 ×*g* for 3 min and the supernatant fluid retained for MALDI analysis. Samples of 1.5 µl were spotted on the MALDI target plate and allowed to air-dry; immediately afterwards, the sample was overlaid with 1.5 µl of matrix solution (α-cyano-hydroxy-cinnaminic acid in 50% aqueous acetonitrile containing 2.5% trifluoroacetic acid) and air-dried.

Samples were analyzed by MALDI using a Microflex L20 mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a N_2 laser. All spectra were recorded in linear, positive-ion mode. The acceleration voltage was 20 kV. Spectra were collected as a sum of 500 shots across a spot. Data were acquired between 2000 and 20,000 Da for analysis. The MALDI BioTyper software, version 1.0 (Bruker Daltonics) was used to process the raw spectra and to compare the spectra in order to classify the strains. Spectra recorded in 16 replicates per strain were smoothed, their baselines were corrected, and peak lists generated automatically according to the parameter settings. Main spectra were created by extraction of the specific peak information from the individual spectra of a particular strain. Based on similarity scores of the main spectra, dendrograms were constructed that showed the clustering of strains by their mass spectra.

Results

Affiliation of isolates to phyla and estimation of diversity. The 9–10°C cold water carried a rather low bacterial load, about 3×10^2 (site 1), 6×10^3 (site 2), and 2×10^4 (sites 3 and 4) colony-forming units (CFU)/ml on R2A medium. Cyanobacterial and diatom diversity, analyzed in parallel by Thomas Friedl (personal communication), was evident in the water and, as determined by a non-culture approach, in biofilm samples (Cousin and Stackebrandt, unpublished). Isolation of heterotrophic strains was hampered by dominating swarming flavobacteria and by colonies with a slimy appearance, both of which impaired the growth of slower developing colonies. A total of 681 isolates were recovered from the rivulet sampling sites: 34, 172, 205, and 270, respectively, from sites 1–4.

Initial BLAST hit analyses of the 5′-500 nucleotides of the 681 16S rRNA gene sequences indicated that for the majority of WB isolate sequences highest scores were obtained with clone sequences either from uncultured bacteria or from unclassified strains belonging to the phyla Bacteriodetes, *Firmicutes, Proteobacteria*, and *Actinobacteria* (not shown). Subsequent comparison of the BLAST hit results with alignment-based similarity values between sequences of isolates and type strains demonstrated the excellent agreement with the BLAST results at the genus level. Members of the Bacteriodetes phylum almost exclusively belonged to the genus *Flavobacterium*, while members of *Pseudomonas* (*Gammaproteobacteria*) dominated in the phylum *Proteobacteria*. ARB analysis affiliated 656 isolates to 38 genera; 25 strains clustered with two undescribed taxa of *Betaproteobacteria* and *Gammaproteobacteria* (Table 1).

The distribution of organisms along the rivulet changed within the first upstream 110 m. While mainly flavobacteria (68% of total) were isolated from the discharge site, flavobacteria (47–59%), pseudomonads (15–20%), and stenotrophomonads (6–9%) were recovered most frequently from downstream sampling sites. Rarely, firmicutes (1.2%) and, except for site 2, actinobacteria (2.2%) were isolated (Table 1). The percentage distribution of the isolates relative to the various phyla and classes is indicated in Table 1, while phylum distribution per site is indicated graphically in Fig. 1, alongside the rivulet profile.

To calculate operational taxonomic units (OTUs) and diversity indices, information obtained from the four sampling sites was pooled. Individual data of the three downstream sampling sites were similar but different from data obtained from the discharge site (not shown). The number of OTUs calculated from rarefaction curves (not shown) increased from 129 to 182 when shifting from a 98 to a 99% similarity threshold (Table 2). The estimated OTUs determined by ACE and Chao1 were similar but higher (53%) than those determined by rarefaction curves. Shannon's index of sequence diversity at 99 and 98% similarity for sites 2, 3, and 4 were similar, i.e., 3.6–4.31 and 3.21–3.88, respectively. Compared to the corresponding values of 1.54 and

Table 1. Affiliation of isolates to phyla/class and genera based upon the analysis of partial 16S rRNA gene sequences, using the ARB treeing program [32]. Major taxa (>5% of total) are shown in bold

		Sites				Total	
Phylum/class	Genus	1	2	3	4	Number	%
Alphaproteobacteria	*Rhizobium*	1				1	0.1
	Bradyrhizobium			1	1	2	0.3
	Sphingomonas		1			1	0.1
Betaproteobacteria	*Acidovorax*	1		1	1	3	0.4
	Deefgea		3	2		5	0.7
	Duganella	1		2		3	0.4
	Iodobacter		1	4	4	9	1.3
	Janthinobacterium	1	1	3	2	7	1.0
	Massilia	1	2	3	1	7	1.0
	Pigmentiphaga				1	1	0.1
	Polaromonas		1			1	0.1
	Rhodoferax		1			1	0.1
	Undescribed	1	1	2	3	7	1.0
Gammaproteobacteria	*Acinetobacter*		1	1		2	0.3
	Aeromonas		6	2	7	15	2.2
	Buttiauxella			1	1	2	0.3
	Erwinia		3	6		9	1.3
	Escherichia			1		1	0.1
	"Mariprofundis"		3	2	1	6	0.9
	Pantoea			1		1	0.1
	Pectobacterium			1		1	0.1
	Pseudomonas	1	25	30	53	109	16.0
	Rahnella		2	1	5	8	1.2
	Rheinheimera			5		5	0.7
	Serratia		2	1	1	4	0.6
	Shewanella		4			4	0.6
	Stenotrophomonas		10	19	20	49	7.2
	Yersinia		1			1	0.1
	Undescribed		3	9	6	18	2.6
Bacteroidetes	*Chryseobacterium*	1		1	1	3	0.4
	Flavobacterium	23	80	101	158	362	53.2
	Hymenobacter		1			1	0.1
	Pedobacter	1	4	3		8	1.2
	Sphingobacterium				1	1	0.1
Firmicutes	*Bacillus*		2	1		3	0.4
	Sporosarcina		4		1	5	0.7
Actinobacteria	*Arthrobacter*	1	8	1	2	12	1.8
	Leifsonia	1				1	0.1
	Nocardia		1			1	0.1
	Rhodococcus		1			1	0.1
Total of 40 genera		34	172	205	270	681	100

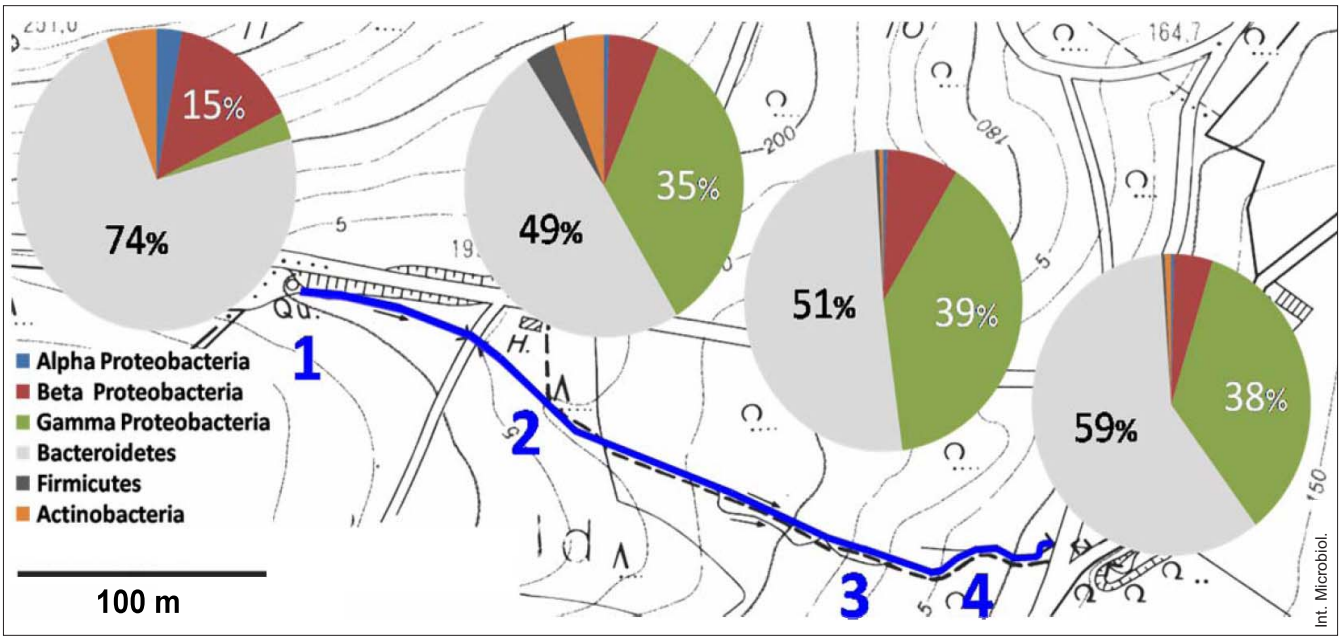

Fig. 1. Taxonomic distribution of partial 16S rRNA gene sequences from bacteria isolated from four sites (1–4) along the Westerhöfer Bach (flow direction from west to east; site 1 is the discharge site).

1.49, determined for sequence similarities between strains isolated at the discharge site, diversity was significantly increased at the downstream sampling sites. Similarly, as indicated by Simpson's index of diversity, which ranged from 0.01 to 0.04 at 99% similarity at the three downstream sites, diversity was higher than that at the discharge site (0.39). The values for the combined four sampling sites indicated a moderately higher diversity (Table 2).

Affiliation of isolates to species. Since information other than the 16S rRNA gene sequences is lacking for those organisms entered into public nucleic acid sequence databases as being "uncultured" and "unclassified", the SILVA database [40] was restricted to type strains of described species of those genera embracing the majority of isolates from WB. A few strains fell outside the described genera; two of these have been described as new genera [46,49]. The decision whether isolates represent strains of a known species or constitute a putative new phylospecies was made based on a similarity threshold value of 98.5% of the partial 16S rRNA gene sequences. This conclusion derived from the finding that similarity values >98% have been determined for several highly related pairs of type strains of species belonging to the genera *Pseudomonas* and *Flavobacterium* for the same sequence stretch (5′-terminal 450 nucleotides) as used for isolates of this study. For some species within *Pseudomonas* (Table 3) and *Flavobacterium* (see below), similarities were even higher than 98.5%. Decreasing the value to 97.5% resulted in a non-discriminatory clustering of several type strains into the same phylogenetic group.

A second method, MALDI-TOF analysis of whole cells, was used to evaluate the phenetic coherence within two

Table 2. Estimated numbers of operational taxonomic units (OTUs) for the combined four samples using parametric (rarefaction) and non-parametric estimators (Chao1 and ACE), and diversity indexes

		Number of OTUs			
Similarity (%)	Rarefaction	ACE	Chao 1	Shannon	Simpson's (D)
99	182	275.92 (240.80–332.02)[a]	280.18 (237.30–356.32)	4.54 (4.45–4.64)	0.019
98	129	170.43 (151.78–204.38)	165.12 (145.93–206.08)	4.16 (4.07–4.26)	0.026

[a]In parentheses, 95% confidence intervals.

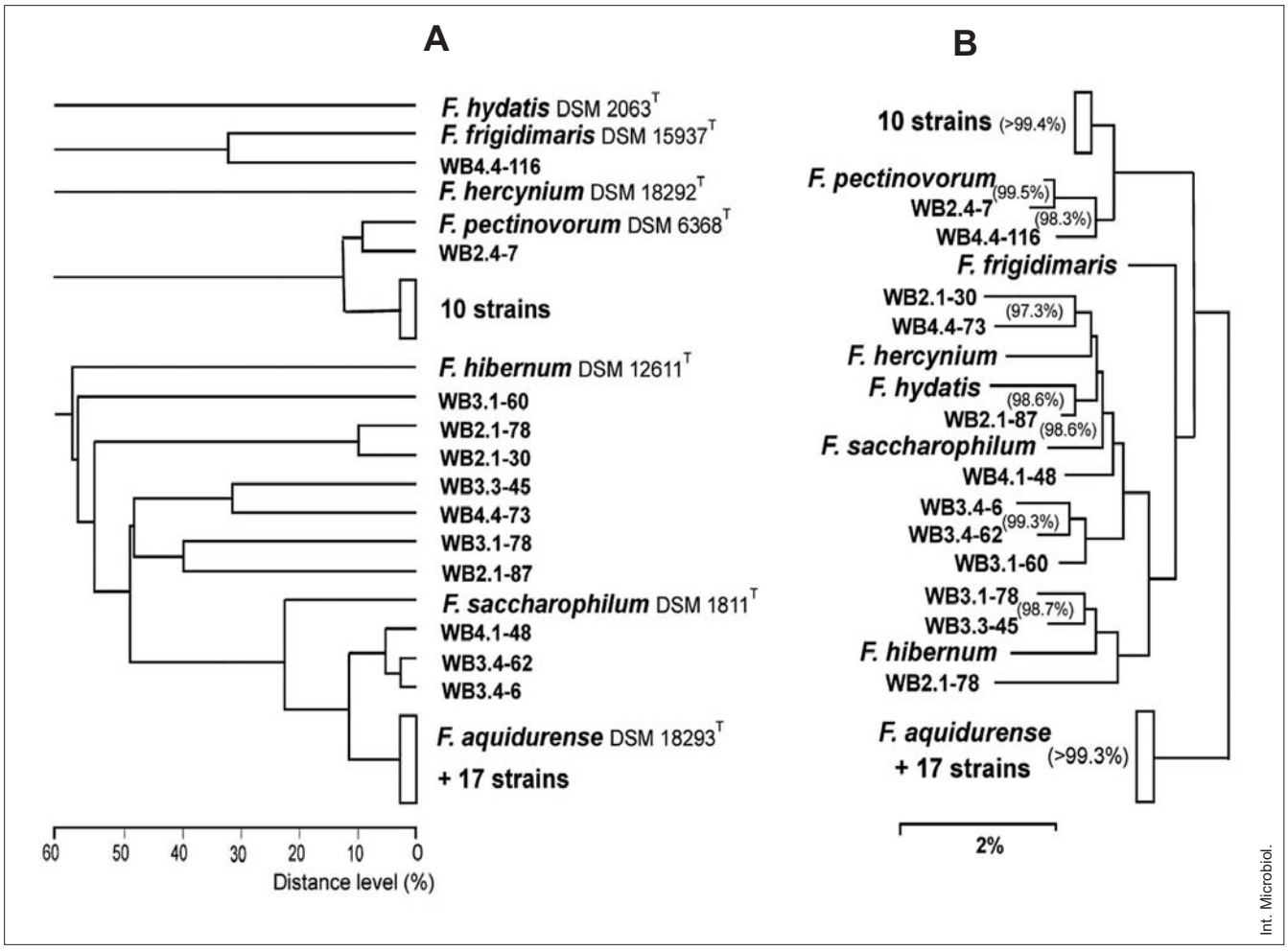

Fig. 2. Comparison of distance level dendrogram of (**A**) MALDI-TOF analysis of whole cells, with (**B**) a neighbor-joining dendrogram of partial 16S rRNA sequences (similarity values in parentheses). Strain numbers as well as the strain composition of the two highly related strain clusters are identical in the two dendrograms. Bar, 0.02 substitutions per nucleotide position.

genomic clusters of flavobacterial strains exhibiting >99% sequence similarity. This was done by comparing their mass spectra of abundantly occurring, ionized biomolecules, including ribosomal proteins. Figure 2 compares the dendrogram of protein similarities (Fig. 2A) with a neighbor-joining dendrogram of sequence relatedness (Fig. 2B). It is obvious that, except for strains WB3.1-78 and WB3.3-45, flavobacteria that showed <2% partial 16S rRNA gene sequence dissimilarities showed less than 20% divergence in their MALDI-TOF spectra. The two methods are therefore in accordance with respect to the clear separation of type strains from each other, the tight clustering of genomically and phenetically highly similar strains, and the separate position of those strains considered by 16S rRNA analyses to be phylospecies.

The intrageneric diversity of the isolates from WB is too rich and the unidentified species too numerous to be discussed in detail; a more detailed analysis is therefore restricted to two genera, *Pseudomonas* and *Flavobacterium*. After a preview of relatedness, as determined on the basis of partial 16S rRNA gene sequences, almost complete sequences were generated for 97 strains (*Flavobacterium*, 94; *Pseudomonas*, 3), which according to the partial gene sequences potentially represent novel phylospecies. Restricting a highly related strain cluster to a single representative by no means allows the conclusion that the other strains may not represent novel phylospecies. Strains sharing between 98.8 and 100% 16S rRNA gene sequence similarity may nonetheless represent novel species but in these cases more elaborate methods need to be applied to confirm the status of species. According to present day taxonomic practice, species with a 16S rRNA gene sequence level of >98.8% are delineated from each other mainly on the basis of DNA–DNA similarity values in combination with differentiating phenotypic properties [45].

Table 3. Affiliation of isolates to named *Pseudomonas* species or species groups on the basis of >98.5% 16S rRNA partial gene sequence similarity values

Species/species group[a]	Number of isolates
P. grimontii[b], *P. trivialis, P. simiae*	34
P. avellanae, P. meliae, P. fredericksbergensis	14
P. mediterraneae	3
P. salmonii, P. psychrophila, P. fragi	16
P. graminis	7
P. reinekei, P. koreensis	2
P. jessenii	12
P. putida	1
P. peli	1
P. panacis , P. brenneri	1
P. gessardii, P. libanense	8
P. migulae	12
P. tolaasii, P. lurida, P. costantinii	29

[a]Only up to 3 representatives are indicated.
[b][Refs. 8,9,48].

Gammaproteobacteria. Many of the isolates (109, 45% of total) of this class clustered with species of the genus *Pseudomonas*. Phylogenetically, this genus contains highly related species clusters, as 16S rRNA gene sequence similarities of partial and almost complete sequences are >98.5%, as evidenced by the clustering of *P. grimontii, P. rhodesiae, P. extremorientalis, P. simiae, P. trivialis,* and *P. orientalis* [6,25]. At a threshold value for the partial sequences of >98.5%, 84% of pseudomonas isolates were assigned to species or species groups but without precise affiliation to a defined species (Table 3).

Based upon nearly complete 16S rRNA gene sequences, three pseudomonad strains were found to comprise three novel phylospecies: WB3.2-34 [nearest neighbor is *P. lutea,* 98.4% (AM934696)], WB4.4-63 [*P. asplenii,* 98.4% (AM934697)], and WB4.4-99 [*P. viridiflava,* 98% AM934698)].

Flavobacterium. The high number of 362 *Flavobacterium* isolates is remarkable but enrichment bias due to the cultivation medium cannot be excluded. Members of this genus, which comprises 45 species [http://www.bacterio.cict.fr], are common inhabitants of a wide range of temperate and cold soil and water habitats. At the discharge site of the rivulet, 78% of isolates belonged to the genus *Flavobacterium* and 75% of these to *F. aquidurense* [15]. Representatives of this species were never found at any of the downstream sampling sites. While the phylogenetic diversity of flavobacteria was poor in the spring water (4 phylospecies), it increased significantly over the following 110 m (39 phylospecies) as well as further downstream (34–37 phylospecies). In contrast to the situation in the genus *Pseudomonas*, however, only about half of the flavobacterial isolates could be affiliated to named species. The remaining isolates formed 90 individual lineages or strain clusters that shared between 92 and 98.5% sequence similarity with *Flavobacterium* type strains. Only two major novel strain clusters (35 and 55 strains) included isolates from all sampling sites, except the discharge site (Fig. 3).

Nearly complete 16S rRNA gene sequences were obtained for isolates representing individual lineages or strain clusters on the basis of partial sequences. At the 98.5% similarity threshold established for the recognition of novel phylospecies, some strains were affiliated to described species (*F. frigidimaris, F. hibernum, F. hercynium*) but 40 new lineages, putative new species, were identified that showed <98.5% sequence similarity among themselves and with the type strains of described species (Fig. 3).

Discussion

It was the goal of this study to identify the major cultivable, aerobic non-phototrophic components of bacterial diversity in a karst water rivulet. The authors are aware of the limitation of this approach for the assessment of a more global view of bacterial diversity, which requires the inclusion of culture-independent techniques [4,24]. The rationale of working with organisms rather than with bio-information was laid down in the multi-partner project proposal to provide cultured organisms for subsequent studies on the establishment of calcium-carbonate-precipitating biofilms from phototrophic and non-phototrophic components.

The number of CFU/ml isolated on R2A medium was low in the 9–10°C cold Westerhöfer rivulet, although within 320 m it increased from 3×10^2 to 2×10^4 CFU/ml. The low percentage (1.5%) of cell numbers at the discharge site confirmed the 4% reported by Beier et al. [10] for other German running waters. Superimposing the number of bacteria reported to thrive in cold Alpine and Antarctic freshwater lakes [7,16,43], other German rivers [10,22], and natural plankton samples [18] (between 10^6 and 5×10^6) on the 2×10^4 CFU/ml obtained in the Westerhöfer rivulet, then the latter would account for 0.4–0.7% of the total cell numbers in this habitat. This narrow percentage range is in the same order of magnitude as indicated for cultured bacteria in freshwater, mesophilic lakes, and unpolluted estuarines [4,26].

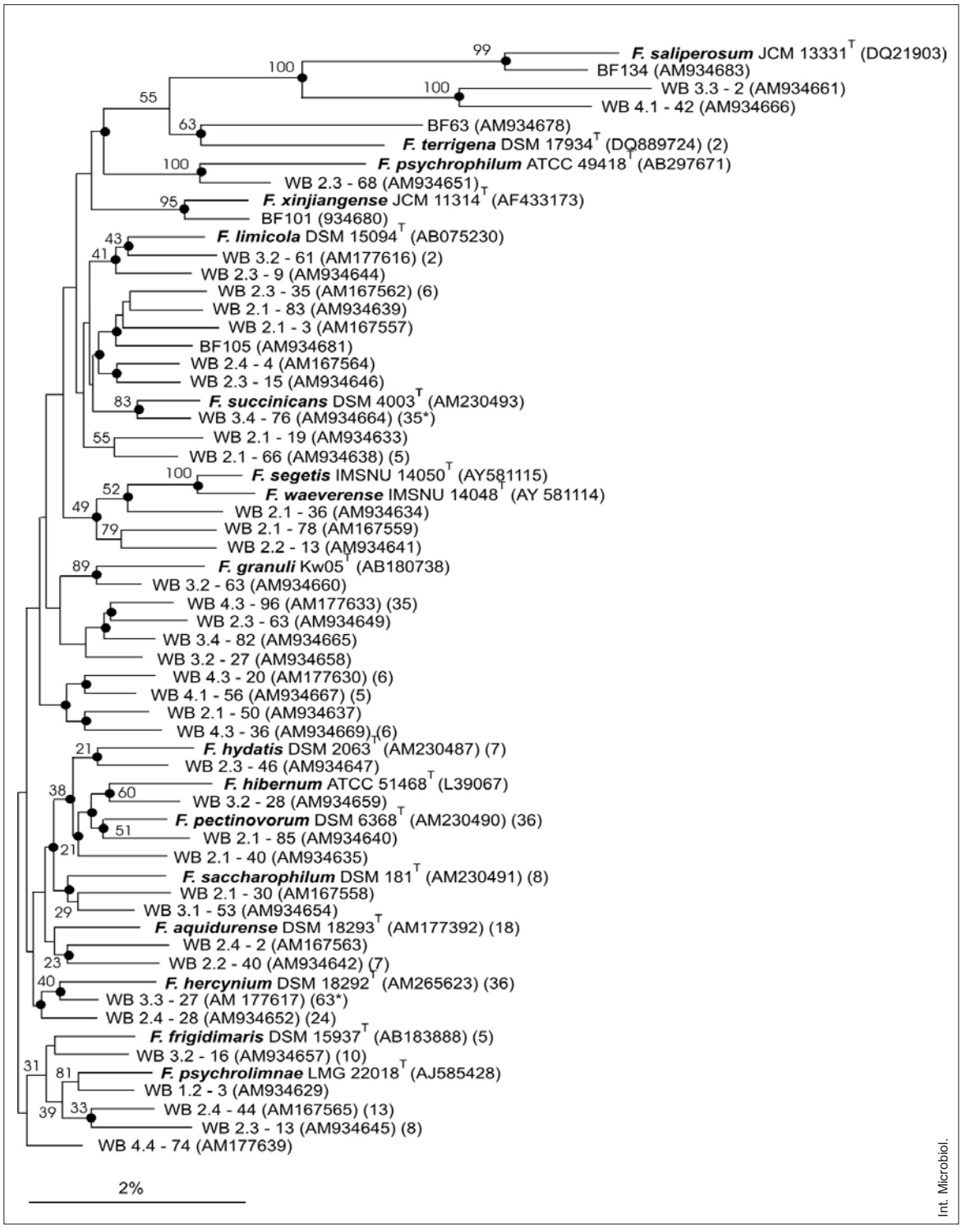

Fig. 3. Neighbor-joining tree showing the phylogenetic relationship of isolates of the Westerhöfer Bach to a selection of type strains of the genus *Flavobacterium* based on sequence similarity of nearly complete 16S rRNA genes. Type strains that appeared to be only distantly related were omitted for reasons of readability. Filled circles indicate nodes also recovered reproducibly with maximum likelihood calculation. Numbers at the nodes are bootstrap values (only values >30% are shown) from 1000 replicates. Numbers in parentheses at the end of the line indicate the number of isolates affiliated to that species. Tree calculation was done with the ARB software package (http://www.arb-home.de [32]). The sequences of *F. ceti* CCUG 52969^T (AM292800) and *F. gelidilacus* LMG 21477^T (AJ440996) were used as outgroup (not shown) to define the root of the tree. EMBL accession numbers are given in parentheses. Bar, 0.02 substitutions per nucleotide position.

The 40 bacterial genera to which the isolates could be affiliated, based on a comparison of partial 16S rRNA gene sequences with sequences in the SILVA database, were restricted to four bacterial phyla, i.e., Bacteriodetes, *Proteobacteria* (especially *Gammaproteobacteria*), and some *Firmicutes* and *Actinobacteria*. The members of these genera often dominate in broad assessments of diversity, not only in culture but also in non-culture studies [e.g., refs. 29,33,47]. The abundant occurrence of *Flavobacterium* spp. has been reported in studies on other, diverse environments [17,30,50,51], where these bacteria may account for almost 100% of the total bacterioplankton community [17]. None of these studies, however, consisted of in-depth analyses of the phylogenetic structure of this genus, although several studies used quantitative PCR [1,17] FISH [1], and CARD-FISH [2] hybridization techniques to evaluate the abundance of members of Bacteriodetes. An accompanying high proportion of *Gammaproteobacteria*, especially *Pseudomonas*, was also reported. The lack of abundance of *Firmicutes* (gram-positive, low-G+C bacteria) in bacterioplankton communities of lakes and rivers was in accordance with the data of Beier et al. [10] and Zwart et al. [52] but the presence of *Firmicutes* was reported in other studies [14,35]. Hence, with respect to the presence of major taxa of culturable aerobic bacteria, karst hard water does not represent a unique, highly selective environment. However, a significant difference regarding the isolates assigned to major phylogenetic groups was the detection of novel diversity, as judged by partial and almost complete 16S rRNA gene sequences.

Although 362 flavobacterial strains were isolated, the majority of which (339) came from the three downstream sampling sites, not more than 80–158 isolates were recovered from any of these sites. Compared to reports on estimates of cell numbers per ml of freshwater samples, this was a minute fraction of the potential abundance and diversity of any taxon thriving at any of these sites. Despite the low numbers of isolates, the diversity of the 16S rRNA gene sequences was surprising. While only five flavobacterial phylospecies were detected at the discharge site, 39 phylospecies, 22 of which may represent novel species, were found as near as 110 m downstream (Fig. 2) and new phylospecies emerged at sites further downstream. The same was true for members of the genus *Pseudomonas*, which were almost absent in the discharge water but present in considerable numbers at sampling site 2. The absence in downstream sites of strains most abundant at the discharge site and the rapid increase in phylogenetic diversity and strains at downstream sampling sites could be due to changes in biotic and abiotic water variables or to the entry of new microbiota introduced by soil runoff water. The first option is not convincing as members of *Flavobacterium* are metabolically very versatile and similar [11,15]. Also, the water residence time is short (about 25–32 min over 320 m, 15–20 cm/s; G. Arp, personal communication) for the length of the rivulet analyzed, making it unlikely that competition for nutrients is a controlling factor. The second option is more likely, as the rivulet bed is deeper than the surrounding forest soil, with water continuously seeping into the rivulet. This is the case not only after rainfall but was also observed during all sampling visits. Many of the genera listed in Table 1 are known to thrive in terrestrial habitats [42] and their dispersion by means of water may explain why many of the isolates likely belong to cosmopolitan taxa.

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