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High phylogenetic diversity of *Flavobacterium* spp. isolated from a hardwater creek, Harz Mountains, Germany

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Abstract

Of 930 strains isolated on nutrient-poor media from water samples taken at four sites along a 370-m-long stretch of a hardwater creek, Westerhöfer Bach, Harz Mountains, Germany, more than half had swarming capacity, many of which with slimy appearance. MALDI-TOF spectroscopic analysis of 60 randomly picked isolates grouped about 45% of them in different orders of *Proteobacteria*, while about 55% clustered with a representative of the genus *Flavobacterium*. The latter affiliation was supported by positive flexirubin reaction and fatty acid profiles performed on a subset of these isolates. Partial 16S rRNA gene sequence analysis of more than 100 swarming strains also supported the protein-based analysis, and suggested high phylogenetic diversity of the flavobacterial isolates at the intrageneric level. Phylogenetic analysis of almost complete gene sequences obtained for 41 strains and type strains of *Flavobacterium* species refined the phylogeny, allowing the conclusions that only some isolates are members of described species, and that the phylogenetic depth of the other lineages is indicative of the presence of about 20 novel *Flavobacterium* species, providing support by the polyphasic approach to systematics. The phylogenetic breadth and depth of diversity of *Flavobacterium* spp. in this creek are very similar to those in epilithon samples from the nutrient-rich River Taff in Cardiff (O'Sullivan, L.A., Rinna, J., Humphreys, G., Weightman, A.J., Fry, J.C., 2006. Culturable phylogenetic diversity of the phylum 'Bacteroidetes' from river epilithon and coastal water and description of novel members of the family Flavobacteriaceae: *Epilithonimonas tenax* gen. nov., sp. nov. and *Persicivirga xylanidelens* gen. nov., sp. nov. Int. J. Syst. Evol. Microbiol. 56, 169–180.), prompting speculation about the rationale for this finding.

Keywords: *Flavobacterium*; Phylogeny; 16S rRNA gene sequence similarities; Freshwater microbial diversity

Introduction

Hardwater creeks like the Westerhöfer Bach in the Harz Mountains, Germany, are physicochemically characterized by fast carbonate precipitation driven by CO_2 degassing (Usdowski et al. 1979). This reaction leads to the formation of calcareous tufas, also referred to as tufa stromatolites (Riding 1991), i.e. porous, poorly stratified or laminated friable carbonate rocks. Usually, low-magnesium calcite is the main mineral component (Irion and Müller 1968). The rate of calcite deposition can be high in Westerhöfer Bach,

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e.g. 47 kg/year/m of creek length in 1979 (Usdowski et al. 1979), leading to rapid calcification of objects placed or fallen into the creek. Besides the inorganic formation of calcite (Herman and Lorah 1988), the role of plants and cyanobacteria in CO_2 degassing and calcite deposition has been discussed due to their acting as nucleation sites (Pentecost and Riding 1986) and trapping of detrital particles by their exopolysaccharides.

However, in contrast to some marine stromatolite-forming biofilms (Gerdes et al. 2000; Steppe et al. 2001; Kawaguchi and Decho 2002; Neilan et al. 2002; Kawaguchi et al. 2003; Burns et al. 2005; Papineau et al. 2005), the microbial species and exopolymer composition of cyanobacteria-rich biofilms involved in tufa stromatolite formation is still poorly known. While eukaryotic algae and cyanobacteria have been identified tentatively on the basis of their morphology and cytological characters (e.g. Fritsch 1950; Pentecost 1978; Freytet and Plet 1996; see also Arp et al. 2001 for additional references), information on the non-cyanobacterial prokaryotes is even more scarce. A rich diversity of phylogenetically novel flavobacteria has recently been reported in biofilms on stones collected from the river Taff in Cardiff, UK (O'Sullivan et al. 2006). The aerobic and anaerobic metabolism of these heterotrophs, including the formation and hydrolysis of exopolymers and its influence on the precipitation of carbonate within the top-layer biofilm, possibly extending into deeper layers of the porous tufa, should not be underestimated in discussions of the turnover of microbial mats and growth of stromatolites. In order to elucidate the microbial composition of water and biofilms in hardwater creeks, a combination of molecular and culture approaches is applied here within a project aiming at a better understanding of mineralisation processes. The present study analyses the dominant cultured aerobic heterotrophic bacteria in order to assess the origin of bacteria potentially found as components in biofilms of the same creek.

Material and methods

Sampling area

The Westerhöfer Bach is located on the western slopes of the Harz Mountains, close to Westerhof village, about 30 km north of Göttingen (Deutsche Grundkarte Kalefeld, Westerhof, coordinates 75.4/37.0). The spring discharges from a karst aquifer in mesozoic limestones; the creek then passes through dense forest for 370 m before entering an area of agricultural use. On the sampling date of 9 June 2005, the creek was up to 1 m wide and between 5 and 25 cm deep. Samples were taken at four sites (Fig. 1): the spring (station 1), and at downstream distances of 130 m (sta. 2), 280 m (sta. 3, cascade) and 320 m (sta. 4), respectively. Site 2 marked the downstream onset of distinct biofilm formation; site 3 was a small tufa cascade, about 2.5 m high and 5 m wide and rich in calcified plant material; site 4 was a narrow trench formed by calcite precipitation. Water flow was relatively slow at station 2, turbulent at station 3 (cascade), less turbulent again at station 4.

Isolation

Bacterial isolates were obtained by on-site streaking of 100 µl water samples directly onto agar plates of medium R2A (Difco 218263) and medium 51 (DSMZ Catalogue of Strains 2001), the latter containing per litre: beef extract (Lablemco, Oxoid) 5.0 g and agar 15 g,

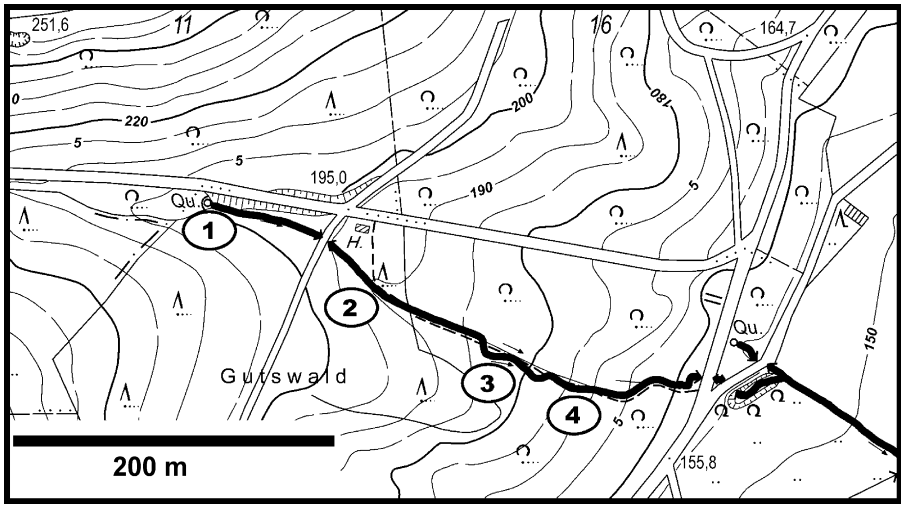

Fig. 1. Map of Westerhöfer Bach as it passes through dense forest for about 350 m, and locations of sampling sites.

pH 7.0. Plates were closed by means of parafilm, transported on ice to the laboratory within 2 h, and incubated in the dark at 10 °C and 20 °C. Purification of isolates followed standard microbiological practice. Purity was checked microscopically and was later confirmed by determination of partial 16S rRNA gene sequences. In the strain designations assigned, the first number (1–4) represents the sampling site, the second the medium and isolation temperature (1 = medium R2A, 10 °C; 2 = medium 51, 10 °C; 3 = medium R2A, 20 °C; 4 = medium 51, 20 °C), and the third represents the isolate number. The presence of flexirubin was tested according to Gosink et al. (1998).

MALDI-TOF analyses

Cells from a single colony were smeared onto a stainless-steel template, and immediately 1 µl of matrix (10 mg/ml 2,5-dihydroxybenzoic acid in water/acetonitrile (1:1) with 0.03% trifluoroacetic acid) was added. The cell/matrix mixture was air-dried at room temperature. Positive ion mass spectra were recorded from each colony, using a MALDI-TOF mass spectrometer (Ultraflex, Bruker, Bremen). Mass spectra were obtained from 2000 to 20,000 Da. All analyses were carried out in the linear and delayed extraction mode, yielding separation of protein peaks and a mass accuracy of at least 200 ppm. *Escherichia coli* strain DH5alpha with known mass values of ribosomal proteins was used for external calibration (Ryzhof and Fenselau 2001). For comparison of spectra, the peak lists of the strains were imported in AnagnosTec SARAMIS software, which includes a patented procedure for statistic evaluation and weighting of microorganism mass data.

16S rRNA gene sequence and fatty acid determination

Genomic DNA extraction, PCR-mediated amplification of the 16S ribosomal DNA, and purification of PCR products were carried out as described by Rainey et al. (1996). Purified PCR products were sequenced with a CEQ Dye terminator cycle sequencing Quick start kit (Beckman Coulter 608120) as described in the manufacturer's protocol. A Beckman Coulter CEQ 8000 Genetic Analysing system was used for electrophoresis of the sequence reaction products. The almost complete sequences (>1450 nt) of 41 isolates have been deposited at EMBL under accession numbers AM177392, AM167556–AM167566, and AM177612–AM166640. The ae2 editor (Maidak et al. 2001) was used to align the 16S rDNA sequences determined in this study against those of representatives of the main bacterial lineages available from public databases. A neighbour-joining dendrogram was constructed from a distance matrix, using the treeing algorithm of Felsenstein (1993). Bootstrap values were determined according to Felsenstein (1985).

Fatty acids where determined according to Miller and Berger (1984), using the MIDI System.

Results

As expected for karst springs, water chemistry in Westerhöfer Bach is characterised by high calcium and magnesium concentrations, and by high alkalinity, representing mainly hydrogen carbonate ions. Sulphate concentration is high, too, due to some dissolution of gypsum deposits embedded in the mesozoic limestones. The spring discharges at a pH of 7.3, a Ca^{2+} concentration of 140 mg L^{-1}, and a water temperature of 9.0 °C; the latter increases to 10.2 °C at 350 m downstream. CO_2 degassing can be observed by a drop in partial pressure (pCO_2) along the creek. Consequently, pH rises to rather constant values around 8.3 from 280 m downstream onward. A nearly ten-fold supersaturation with respect to calcite is reached between stations 2 and 3 where tufa deposits begin to spread. From here on, precipitation of $CaCO_3$ (calcite) is evidenced by a strong drop in alkalinity and calcium concentration. Additional chemical parameters are indicated in Table 1. Concentrations of silicate and nitrate decrease over the first 130 m; phosphate concentration is close to detection limits. Magnesium and sulphate contents remain constant, as no side springs enter the main creek.

A total of 960 bacterial isolates were obtained at the four sampling sites (214, 236, 280, and 230 isolates, respectively). A high proportion (41%) of cells were swarming, some of which were noticeably slimy (7.8% of total). All strains were isolated at 10 and 20 °C, but pure cultures grew faster at 20 °C. The vast majority of colonies were pigmented either whitish or yellowish. Twelve strains that formed purple colonies were later identified by 16S rRNA gene analysis as members of *Janthinobacter*. Pink and red pigmented colonies were virtually absent. Microscopic analysis of a small subset of 60 strains from differently pigmented colonies indicated the presence of a broad range of spherical to filamentous cells. Many of the swarming cells were filamentous, though short rods, often in chains, were also observed among the swarmers.

MALDI-TOF analysis

These 60 strains were analysed by MALDI-TOF in order to rapidly group isolates according to overall phenotypic resemblance. As the SARAMIS MALDI-TOF database focuses on strains from the

Table 1. Water chemistry data for spring and creek water in upstream region of Westerhöfer Bach

Sampling site	pH	Temp. (°C)	EC ($\mu S\,cm^{-1}$)	TA ($meq\,L^{-1}$)	pCO_2 (µatm)	$SI_{calcite}$ (log IAP/KT)	Ca^{2+} ($mg\,L^{-1}$)	NO_3^{2-} ($mg\,L^{-1}$)	Si^{4+} ($mg\,L^{-1}$)	Mg^{2+} ($mg\,L^{-1}$)	PO_4^{3-} ($\mu g\,L^{-1}$)	SO_4^{2-} ($mg\,L^{-1}$)
Spring, sta. 1	7.30	9.0	1088	4.76	10960	0.08	140	3.47	4.66	44.3	<1	305
Creek, sta. 2	8.03	9.2	1068	4.78	1995	0.78	132	2.75	4.40	44.3	<1	300
Tufa cascade, sta. 3	8.30	10.0	1023	3.90	871	0.92	120	2.61	3.99	43.3	3	302
Creek, sta. 4	8.32	10.2	1012	3.88	832	0.94	119	2.55	3.99	42.4	2	303

EC = electrical conductivity; pCO_2 = partial pressure of CO_2 (as compared to atmospheric pressure ~370 µatm); SI = saturation index with respect to carbonate mineral calcite (SI = 1 denotes ten-fold supersaturation); sta. = station; TA = total alkalinity (concentration of HCO_3^- and CO_3^{2-}).

clinical environment, the peak profile of only a single *Flavobacterium* species, *Flavobacterium hibernum,* was available for comparison. More than 30 strains clustered around this strain at similarity levels ranging from 50% to 30%. As judged from the peak dissimilarities and the resulting dendrogram (not figured), the isolates showed rich diversity (peak similarities from 80% to 30%). Other strains that did not group with the *Flavobacterium* isolates could not be affiliated to species by MALDI-TOF because of lack of reference organisms. These strains were identified by 16S rRNA gene sequence analysis (not shown) as members of *Alphaproteobacteria* (rhizobia), *Betaproteobacteria* (*Janthinobacterium*, *Zoogloea*), and *Gammaproteobacteria* (pseudomonads, aeromonads, enterobacteria). The number of Gram-positives isolates was small, with only two isolates assigned to *Arthrobacter ramosus* and *Rhodococcus erythropolis*, respectively.

Fatty acid analyses

In order to provide evidence that isolates identified as members by MALDI-TOF and 16S rRNA gene sequence similarity shared a chemotaxonomic signature of the genus *Flavobacterium*, a selection of strains were subjected to fatty acid analysis. Table 2 compiles the analyses of the type strains of *F. hibernum*, *Flavobacterium pectivorum* and *Flavobacterium frigidimaris*, and 10 isolates that were either closely related to the type strains or formed individual lineages according to sequence analysis (see below). All strains have large amounts of iso- and branched straight chain and branched fatty acids (<60%), and most of the individual fatty acids >than 7% of the total are present in each strain. In addition, several common fatty acids ranging between 2.0 and 8.0% are present. Differences in the composition of major fatty acids between type strains, as well as isolates falling into each of the species cluster, are mostly quantitative, not qualitative. Differences occur in minor compounds (<5%), even among strains of the same cluster. The only exception concerns isolates 3.3-35 and 1.1-21, representing individual lineages. Strain 3.3-35 is the only strain to show high amounts of C15:0 2OH and lower amounts of the summed feature i16:1w7c/i15:0 2OH. Strain 1.1-21 shows significantly higher proportions of 15:0 fatty acids.

Fatty acid similarities are visualised in Fig. 2. It is obvious that several isolates show lower similarity levels among each other and with type strains of *F. hibernum* and *F. frigidimaris* than the type strains show among each other.

Partial 16S rRNA gene sequence analysis

In order to obtain a more precise taxonomic assignment of swarming cells, more than 100 isolates

Table 2. Comparison of fatty acid profiles (>2%) of three type strains of *Flavobacterium* and some isolates from Westerhöfer Bach

Fatty acid	Taxon												
	F. pectinovorum DSM6368^T	*F. hibernum* DSM 2611^T	Isolate 3.4-10	Isolate 4.3-19	Isolate 1.1-23	*F. frigidimaris* DSM 15937^T	Isolate 4.4-16	Isolate 3.3-42	Isolate 4.4-14	Isolate 4.4-74	Isolate 2.4.2	Isolate 3.3-35	Isolate 1.1-21
	Cluster												
	F. pectinovorum/*F. hibernum*					*F. frigidimaris*					n.a.	n.a.	n.a.
i 15:1 G	5.64	3.56	4.73	5.02	5.77	3.02	3.22	5.54	7.90	7.07	5.17	6.30	4.87
i 15:0	25.06	17.38	24.73	23.95	13.11	14.76	20.83	15.65	23.97	22.82	24.53	25.32	18.27
ai 15:0	3.49	1.84	3.33	2.35	1.92	3.47	3.02	3.36	2.59	1.54	1.33	1.89	2.93
15:1 w6c		3.28		3.69	5.63	2.36		2.75	3.83	4.09	2.56	3.71	5.31
15:0	2.00	5.78	1.44	3.77	4.88	3.26	3.32	3.94	3.67	3.37	3.20	1.73	8.00
i 16:1 w7c/i 15:0 2OH	13.18	19.85	17.97	11.26	21.84	25.36	25.93	26.01	12.10	13.68	18.13	5.47	9.96
16:0	1.84	4.66	2.36			3.58	4.44	2.74					
i 15:0 3OH 15:0 2OH	11.45	8.44	10.39	10.26	8.46	7.35	7.96	7.69	9.53	9.25	9.75	10.94 18.34	8.46
i 17:1 w9c	8.95	4.53	6.18	10.17	9.52	4.02	2.44	3.23	8.94	10.57	7.13		8.28
i 17:1/ai 17:1 B	1.61			3.24	2.32				2.13	2.84	2.17	5.13	2.45
17:1 w6c		2.10		2.27	4.07	2.62		3.34	2.85	3.73	2.27	2.85	4.13
i 16:0 3OH	1.38	3.08			2.07	2.49		2.07					2.50
16:0 3OH	2.13	4.91	3.47			7.08	8.58	6.23			3.60		
i 17:0 3OH	14.94	10.37	12.94	13.92	11.93	9.01	6.44	7.24	12.40	12.75	11.66	12.09	12.70

n.a. = not applicable.

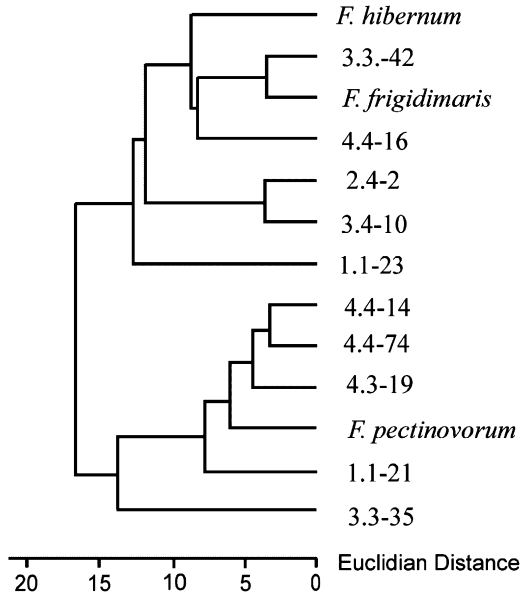

Fig. 2. Euclidian distance dendrogram of fatty acid relationships among strains indicated in Table 1.

originating from all four isolate sites were picked randomly and analysed by partial 16S rRNA gene sequences (around 500 bp). Some highly related isolate clusters emerged, showing >99.6% similarity among the isolates. Two of these comprised nine isolates each, one comprising isolates exclusively from the spring (sampling site 1), the second embracing nine isolates from three different sampling sites (2–4). Most isolates with 100% sequence identity were from the same sampling site. The data were then compared to the public database of sequences for type strains of 28 *Flavobacterium* species. Only three out of all isolates (all from sampling site 4) showed up to 99.3% similarity to the type strain of *F. pectinovorum*; the majority of isolates were less than 95% similar to any of the type strains. The phylogenetic diversity of *Flavobacterium* isolates was high, mirroring the impression obtained from MALDI-TOF analyses of whole cell proteins. The isolates clustered either apart from known species or were only distantly related to them. One cluster of 16 isolates, containing isolates from all four sampling sites, grouped with *F. pectinovorum* and *F. hibernum* (95.8–99.3% similarity). A second group of nine isolates from sampling sites 2–4 clustered with *Flavobacterium succinogenes* (93.3–95.5% similarity), while another group of eight isolates from sites 2–4 was related to *Flavobacterium saccharophilum* (95.2–97.1% similarity). Individual isolates showed a moderate degree of relatedness to certain other species (*Flavobacterium hydatis*, *Flavobacterium psychrophilum*, *Flavobacterium flevense*, and *F. frigidimaris*), but the majority of isolates constituted more than 10 individual clusters with no obvious relationship to described species. Inter-cluster sequence similarity ranged between 92 and 97%.

Full 16S rRNA gene sequence analysis

Almost complete 16S rRNA gene sequences (>1450 bp) were obtained from 41 isolates which represented the major isolate clusters emerging from partial gene sequence analysis. These sequences were compared to those of type strains of *Flavobacterium* species, and a neighbour joining (NJ) tree was generated. *Flavobacterium ferrugineum* and *Flavobacterium mizutaii* showed such low similarity to the other type isolates that their affiliation to *Flavobacterium* must be questioned; thus they were not included in the analysis. *Flavobacterium columnare* served as a root of the NJ tree. The type strains of *Flavobacterium* were phylogenetically well separated, the closest-related pair of type strains being *Flavobacterium frigoris* and *Flavobacterium gillisiae* (98.8% similarity). Several pairs were moderately related, sharing up to 98.6% similarity, but the majority shows only between 93.5 and 97% 16S rRNA gene sequence similarities. The majority of type strains are located within two major species clusters (I and II in Fig. 3).

The analysis of almost complete sequences from the hardwater creek isolates confirmed the notion of their high phylogenetic diversity. As judged from the lowest similarity values at which type strains are divided, isolates showing higher than 99.0% sequence similarity are affiliated with a single phylospecies (marked with an asterisk in Fig. 3). On the basis of this delineation some isolates can be assigned to described species, such as six isolates to *F. pectinatum* and 5 isolates to *F. saccharovorum*. The remaining phylogenetically isolated strains can be defined as twenty novel phylospecies which, if supported by taxonomic evidence, may represent unique centres of diversity and therefore potential new species. The situation is different with the isolates from the River Taff, where a significantly higher number of isolates were closely similar to type strains of described species (O'Sullivan et al. 2006).

Discussion

A previous study on a large number of environmental isolates (Tindall et al. 2000) has shown the power of Fourier-Transformed Infrared Spectroscopy (FTIR) to sort organisms according to similarities in patterns generated by infrared excitation. Recently, the MALDI-TOF spectroscopic approach has demonstrated superior potential by being faster than FTIR and capable of processing more samples per run. Because it also detects ribosomal proteins as well as other housekeeping proteins (Fenselau and Demirev 2001), it has provided

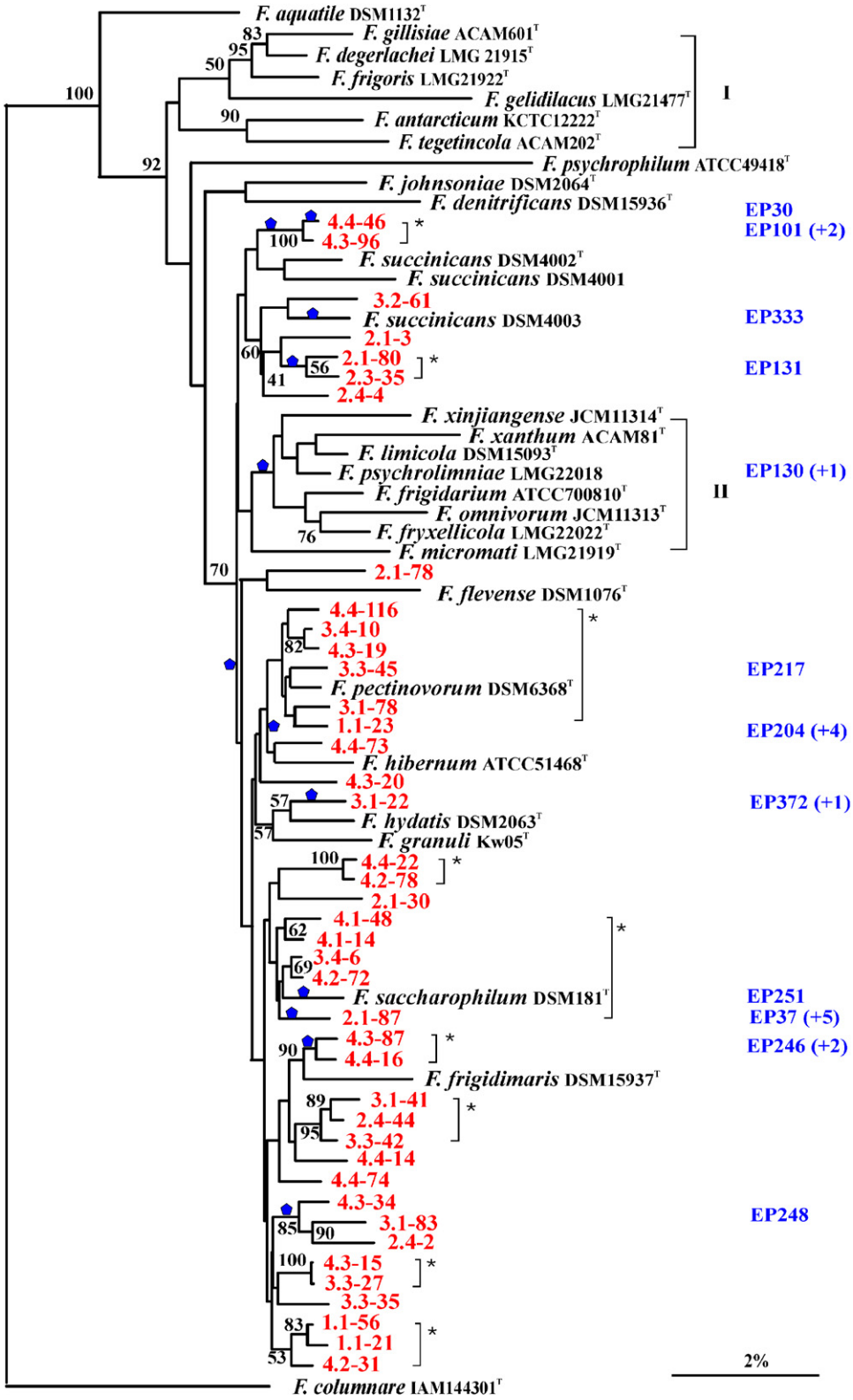

Fig. 3. Neighbour-joining dendrogram of 16S rRNA gene sequence similarities (Felsenstein 1993) among *Flavobacterium* species and isolates (in red) from Westerhöfer Bach. Other type strains of *Flavobacterium* species cluster outside this core group. Scale bar: 2% sequence divergence. Numbers at branching points refer to bootstrap values (500 resamplings; Felsenstein 1985). Clusters I and II refer to major groups of described species; strain clusters marked by * show >99% sequence similarity and are considered members of the same phylospecies; isolates without asterisks are considered phylogenetically distinct and represent individual phylospecies; blue pentagons indicate approximate phylogenetic positions of clusters of *Flavobacterium* isolates (in blue; numbers of phylogenetically close isolates in brackets) from River Taff, Cardiff, UK.

the basis for a phylogenetic assessment of protein pattern analysis. This approach, in combination with a chemotaxonomic and a phylogenetic method, was used to characterise the high number of isolates from the upstream region of Westerhöfer Bach. The creek shows extensive stromatolite formation starting about 130 m downstream at sampling site 2, where pH is 0.73 units higher than at the spring. In order to characterise the microflora, more than 900 isolates were obtained using two nutrient-poor media. Rapid screening by MALDI-TOF gave the first indication of a high diversity among members of a single genus (*Flavobacterium*, about 50%), while the remaining isolates predominantly were also Gram-negative, belonging to different orders and genera of the phylum *Proteobacteria*. Affiliation with the genus *Flavobacterium*, tentative on the basis of MALDI-TOF spectrometry because of lack of reference organisms, was attempted by flexirubin analysis, fatty acid analysis, and 16S rRNA gene sequence analyses. All three approaches confirmed the MALDI-TOF data. While the presence of flexirubin is a genus-specific trait, fatty acids profiles are conventionally generated for species characterisation. Data obtained for type strains and isolates indicated that all major fatty acids occurred in all but one isolate. To assess the taxonomic importance of qualitative and quantitative differences in the fatty acid composition, additional isolates besides the type strain would have to be investigated for a given species.

The study presented here was designed to examine the diversity of cultivable bacteria in order to obtain a glimpse of those aerobic prokaryotes that may be involved in the formation of stromatolite biofilms or negatively affect stability of the latter. It was not designed to quantitatively assess the diversity at each sampling site. We are well aware of the limitations of cultivation regimes as regards diversity assessment (Amann et al. 1995), especially when only two media and two temperatures are used for isolation and cultivation, and varying isolate numbers were selected for the assessment of phylogenetic diversity (10 at site 1; 17 at site 2; 27 at site 3; 52 at site 4). Isolates retrieved from sites 2, 3 or 4 are present in several of the multi-strain phylospecies, whereas new phylospecies composed only of isolates from a single sampling site are rare. The sequences of all but one isolate from the spring are identical; sequences of this phylospecies are not found at downstream sampling sites except in one isolate from site 4. The discharge of water varied between 3.5 and 6 l/s in 1979 (Usdowski et al. 1979), but is assumed to be stable now (G. Arp, pers. comm.). One should, therefore, assume rapid mixing of bacterial cells originating from upstream sites with those introduced downstream via organic matter and water received by the stream, as well as from biofilm deterioration, unless significant changes in physico-chemical parameters dramatically influence the viability of cells. Indeed, except for the absence of the spring phylotype at downstream sites, most phylogenetic clusters contain, to a varying extent, isolates from the three downstream sampling sites. Analysis of the same, and high numbers of, isolates from each sampling site are needed to assess the special distribution of isolates in detail.

Only a few of the 102 flavobacteria analysed by partial 16S rRNA gene sequence analysis were highly related to described species. Similar results were obtained when almost complete sequences were analysed. The only species found to be highly to moderately related to the isolates were *F. pectinovorum*, *F. hibernum*, *F. saccharophilum*, and *F. frigidimaris*. The respective affiliations of individual strains with described species have been reported, e.g. with *F. hibernum* (Brambilla et al. 2001; Lee et al. 2006); *Flavobacterium aquatile* (Crump et al. 1999), *Flavobacterium johnsonae* (Karpouzas et al. 2005), *F. frigidimaris* (Oikawa et al. 2005), to name a few. Flavobacteria are mainly isolated from freshwater fish, temperate freshwater and, though rarely, from soil, but increasingly also from cold environments, predominantly from the Antarctic (Bernadet and Bowman 2005). Most flavobacteria are psychrotolerant, which agrees with their isolation from the cold waters of Westerhöfer Bach (9–10 °C). Of the close phylogenetic neighbours of the isolates, *F. saccharophilum* and *F. flavense* have been isolated from groundwater, lakes and rivers, *F. hibernum* originates from Antarctic oligotrophic lakes, whereas *F. hydatis* and *F. succinicans* are the aetiological agents of fish disease.

The high diversity of cultured members of a single prokaryotic genus within the water column of a short region of the Westerhöfer Bach is paralleled only by isolates from epilithon samples scraped off the surfaces of stones from the River Taff at Cardiff, UK (O'Sullivan et al. 2006). In the latter study, phylogenetic analysis based on partial sequences of the 16S rRNA gene, showed 34 isolates clustering around *Flavobacterium* species, e.g. *F. johnsoniae*, *F. frigidarium*, *F. saccharophilum*, *F. hibernum*, and *F. hydatis*, some of which also have been identified as nearest neighbours in the present study. Merging the sequences from the two studies to a single tree reveals high sequence similarity between some clusters, and site-specific diversity in others (Fig. 3). However, hardly any of the phylospecies defined in either study contains representatives of the other. Not only is the high diversity of strains from the same genus within a given ecosystem surprising, but also the phylogenetic similarity of isolates in two apparently different water systems is puzzling: the nutrient-rich River Taff and the hardwater creek Westerhöfer Bach with isolates recovered within a few hundred metres from the spring.

How can the genomic closeness of strains from geographically separated habitats be explained? One option is the discharge of groundwater containing a

diverse population of flavobacterial organisms that have originated from a common set of ancestors present at an ancient geological time. This hypothesis requires the presence of a rich flavobacterial flora already at the discharge source, which is not observed in the Westerhöfer Bach example. The low total prokaryotic abundance at the spring ($<5\times10^2$ cells ml^{-1} of water on the media used) excludes the possibility that the discharged water is polluted by farming activities.

The second option is the continuous entry of flavobacterial phylotypes into the river/stream. This requires the constant leaking of a diverse population of these organisms from soil, roots, plant material and other sources into the flowing water, or in the case of the River Taff, also into side arms. The Westerhöfer Bach has multiple small areas of surface flooding into which material from the adjacent forest soils may seep. This alternative option may explain the limited diversity at the spring and increasing diversity at the downstream sampling sites. However, most *Flavobacterium* species have been isolated from freshwater habitats, whereas reports of occurrence in soil are much less frequent. A survey of public literature (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) and sequence (http://www.ebi.ac.uk) databases retrieved no records of any great variety of flavobacterial genotypes in culture-independent studies on soil habitats. However, flavobacteria have been reported to thrive in sediments and microbial mats of lakes, as well as at glacial run-off sites and in frozen, organic-rich soil (summarized in Bernadet and Bowman 2005). Moreover, due to slow growth under suboptimal enrichment conditions they may be overlooked on enrichment plates.

Though the second of the above hypotheses appears to be more reasonable, it does not explain the phylogenetic differences between the flavobacteria isolated at both sites. However, whether the diversity difference is authentic or due to sampling limitations remains an open question. It must be concluded that more extensive sampling and analysis, including non-culture techniques, will even increase the phylogenetic diversity of isolates identified at either site. Subsequent molecular and culture analyses of biofilm communities will determine whether or not flavobacteria are components of recent stromatolites, and the role they play in biofilm turnover. As judged from the origin of the *Flavobacterium* isolates from the River Taff, these organisms should be able to form biofilms on stones.

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References

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.

Arp, G., Wedemeyer, N., Reitner, J., 2001. Fluvial tufa formation in a hard-water creek, Deinschwanger Bach, Franconian Alb, Germany). Facies 44, 1–22.

Bernadet, J.-F., Bowman, J.P., 2005. The genus *Flavobacterium*. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community, 3rd ed., release 3.20. Springer, New York ⟨http://link.springer-ny.com/link/service/books/10125/⟩.

Brambilla, E., Hippe, H., Hagelstein, A., Tindall, B.J., Stackebrandt, E., 2001. 16S rDNA diversity of cultured and uncultured prokaryotes of a mat sample from Lake Fryxell, McMurdo Dry Valleys, Antartica. Extremophiles 5, 22–33.

Burns, B.P., Seifert, A., Goh, F., Pomati, F., Jungblut, A.-D., Serhat, A., Neilan, B.A., 2005. Genetic potential for secondary metabolite production in stromatolite communities. FEMS Microbiol. Lett. 243, 293–301.

Crump, B.C., Armbrust, E.V., Baross, J.A., 1999. Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary and the adjacent coastal ocean. Appl. Environ. Microbiol. 65, 3192–3204.

DSMZ Catalogue of Strains, 2001. Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. Braunschweig, Germany.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 30, 783–791.

Felsenstein, J., 1993. PHYLIP Phylogenetic Inference Package, version 3.5.1. Department of Genetics, University of Washington, Seattle, USA.

Fenselau, C., Demirev, P.A., 2001. Characterization of intact microorganisms by MALDI mass spectrometry. Mass Spectrom. Rev. 20, 157–171.

Freytet, P., Plet, A., 1996. Modern freshwater microbial carbonates: the *Porphyridium* stromatolites (tufa-travertine) of south-eastern Burgundy (Paris basin, France). Facies 34, 219–238.

Fritsch, F.E., 1950. *Porphyridium incrustatum* (Naeg.) Gom., an important member of the lime incrusted communities of flowing waters. Biol. Jahrb. 17, 27–39.

Gerdes, G., Klenke, T., Noffke, N., 2000. Microbial signatures in peritidal siliciclastic sediments: a catalogue. Sedimentology 47, 279–308.

Gosink, J.J., Woese, C.R., Staley, J.T., 1998. *Polaribacter* gen. nov., with three new species, *P. irgensii* sp. nov., *P. franzmannii* sp. nov. and *P. filamentus* sp. nov., gas vacuolate polar marine bacteria of the *Cytophaga–Flavobacterium–Bacteroides* group and reclassification of '*Flectobacillus glomeratus*' as *Polaribacter glomeratus* comb. nov. Int. J. Syst. Bacteriol. 48, 223–235.

Herman, J.S., Lorah, M.M., 1988. Calcite precipitation rates in the field: measurement and prediction for a travertine-depositing stream. Geochim. Cosmochim. Acta 52, 2347–2355.

Irion, G., Müller, G., 1968. Mineralogy, petrology and chemical composition of some calcareous tufa from the Schwäbische Alb, Germany. In: Müller, G., Friedman, G.M. (Eds.), Recent Developments in Carbon Sedimentology in Central Europe. Springer, Heidelberg, pp. 157–171.

Karpouzas, D.G., Fotopoulou, A., Menkissoglu-Spiroudi, U., Singh, B.K., 2005. Non-specific biodegradation of the organophosphorus pesticides, cadusafos and ethoprophos, by two bacterial isolates. FEMS Microbiol. Ecol. 53, 369–378.

Kawaguchi, T., Decho, A.W., 2002. Isolation and biochemical characterization of extracellular polymeric secretions (EPS) from modern soft marine stromatolites (Bahamas) and its inhibitory effect on $CaCO_3$ precipitation. Prep. Biochem. Biotechnol. 32, 51–63.

Kawaguchi, T., Sayegh, H.A., Decho, A.W., 2003. Development of an indirect competitive enzyme linked immunosorbent assay to detect extracellular polymeric substances (EPS) secreted by the marine stromatolite-forming cyanobacteria, *Schizothrix* sp. J. Immunoassay Immunochem. 24, 29–39.

Lee, C.C., Smith, M., Kibblewhite-Accinelli, R.E., Williams, T.G., Wagschal, K., Robertson, G.H., Wong, D.W.S., 2006. Isolation and characterization of a cold-active xylanase enzyme from *Flavobacterium* sp. Curr. Microbiol. 52, 112–116.

Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker Jr., C.T., Saxman, P.R., Farris, R.J., Garrity, G.M., Olsen, G.J., Schmidt, T.M., Tiedje, J.M., 2001. The RDP-II Ribosomal Database Project. Nucl. Acids Res. 29, 173–174.

Miller, L., Berger, T., 1984. Bacterial identification by gas chromatography and whole cell fatty acids. Gas Chromatography Application Note 228-41. Hewlett-Packard Co., Palo Alto, CA.

Neilan, B.A., Burns, B.P., Relman, D.A., Lowe, D.R., 2002. Molecular identification of cyanobacteria associated with stromatolites from distinct geographical locations. Astrobiology 2, 271–280.

O'Sullivan, L.A., Rinna, J., Humphreys, G., Weightman, A.J., Fry, J.C., 2006. Culturable phylogenetic diversity of the phylum 'Bacteroidetes' from river epilithon and coastal water and description of novel members of the family Flavobacteriaceae: *Epilithonimonas tenax* gen. nov., sp. nov. and *Persicivirga xylanidelens* gen. nov., sp. nov. Int. J. Syst. Evol. Microbiol. 56, 169–180.

Oikawa, T., Yamamoto, N., Shimoke, K., Uesato, S., Ikeuchi, T., Fujioka, T., 2005. Purification, characterization, and overexpression of psychrophilic and thermolabile malate dehydrogenase of a novel Antarctic psychrotolerant, *Flavobacterium frigidimaris* KUC-1. Biosci. Biotechnol. Biochem. 69, 2146–2154.

Papineau, D., Walker, J.J., Mojzsis, S.J., Pace, N.R., 2005. Composition and structure of microbial communities from stromatolites of Hamelin Pool in Shark Bay, Western Australia. Appl. Environ. Microbiol. 71, 4822–4832.

Pentecost, A., Riding, R., 1986. Calcification in cyanobacteria. In: Leadbeater, B.S.C., Riding, R. (Eds.), Biomineralization of Lower Plants and Animals. Clarendon, Oxford, pp. 73–90.

Pentecost, A., 1978. Blue-green algae and freshwater carbonate deposits. Proc. R. Soc. London B 200, 43–61.

Rainey, F.A., Ward-Rainey, N., Kroppenstedt, R.M., Stackebrandt, E., 1996. The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsaceae* fam nov. Int. J. Syst. Bacteriol. 46, 1088–1092.

Riding, R., 1991. Classification of microbial carbonates. In: Riding, R. (Ed.), Calcareous Algae and Stromatolites. Springer, Berlin, pp. 21–51.

Ryzhof, V., Fenselau, C., 2001. Characterization of the protein subset desorbed by MALDI from whole bacterial cells. Anal. Chem. 73, 746–750.

Steppe, T.F., Pinckney, J.L., Dyble, J., Paerl, H.W., 2001. Diazotrophy in modern marine *Bahamina stromatolites*. Microb. Ecol. 41, 36–44.

Tindall, B.J., Brambilla, E., Steffen, M., Neumann, R., Pukall, R., Kroppenstedt, R.M., Stackebrandt, E., 2000. Cultivatable microbial diversity: gnawing at the Gordian knot. Environ. Microbiol. 2, 310–318.

Usdowski, E., Hoefs, J., Menschel, G., 1979. Relationship between ^{13}C and ^{18}O fractionation and changes in major element composition in a recent calcite-depositing spring – a model of chemical variations with inorganic $CaCO_3$ precipitation. Earth Planet. Sci. Lett. 42, 267–276.