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.

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Members of the phylum 'Bacteroidetes' are important heterotrophs involved in cycling organic carbon in aquatic habitats. Their diversity has been studied by molecular methods in both freshwater and marine habitats and many novel genera and species within this phylum have been characterized in recent years. In this study, we examined the diversity of members of the 'Bacteroidetes' that could be readily isolated on solid media from river epilithon and coastal sea water. Most (93 %) of the 55 isolates confirmed as members of the 'Bacteroidetes' and examined by phylogenetic analysis of 16S rRNA gene sequences belonged to the Flavobacteriaceae. Furthermore, most (62 %) of these were almost certainly members of the genus *Flavobacterium* and all but one were from river epilithon. Conversely, the sea-water isolates were more widely distributed in clades containing other genera. Some of the isolates were deep-branching within phylogenetic trees and so could not be assigned to putative genera. Two of these deep-branching isolates were characterized by polyphasic taxonomy and are proposed as novel species within two new genera of the family Flavobacteriaceae. These are *Epilithonimonas tenax* gen. nov., sp. nov. (type strain EP105^T = NCIMB 14026^T = DSM 16811^T) and *Persicivirga xylanidelens* gen. nov., sp. nov. (type strain SW256^T = NCIMB 14027^T = DSM 16809^T).

INTRODUCTION

Members of the phylum 'Bacteroidetes' (previously known as *Cytophaga-Flavobacterium-Bacteroides*) are seemingly omnipresent in aquatic environments (Reichenbach, 1992). Members have been identified in a diverse range of freshwater and marine habitats, including temperate rivers (Böckelmann *et al.*, 2000), freshwater lakes (McCammon *et al.*, 1998), marine sediments (Humphry *et al.*, 2001), sea ice (Brown & Bowman, 2001), hypersaline lakes (Dobson *et al.*, 1993) and saline hot springs (Tenreiro *et al.*, 1997). Fluorescent *in situ* hybridization studies indicate that members of the 'Bacteroidetes' are second only to the Proteobacteria in terms of aquatic abundance (Glöckner *et al.*, 1999; Simon *et al.*, 1999; Cottrell & Kirchman, 2000b).

Bacteroidetes have been implicated as major utilizers of high-molecular-mass dissolved organic matter in marine ecosystems (Cottrell & Kirchman, 2000a) and are often abundant in nutrient-rich waters where biomacromolecules accumulate (Reichenbach, 1989). Despite their abundance and apparent ecological significance, bacteroidetes are underrepresented in culture collections when compared with other abundant phyla such as the Proteobacteria. However, in recent years there has been a surge of publications describing novel genera within the phylum 'Bacteroidetes'; this has been mainly within the family Flavobacteriaceae including *Aquimarina* (Nedashkovskaya *et al.*, 2005b), *Gillisia* (Van Trappen *et al.*, 2004), *Kordia* (Sohn *et al.*, 2004), *Ulvibacter* (Nedashkovskaya *et al.*, 2004b) and *Formosa* (Ivanova *et al.*, 2004). There have also been some novel genera described recently outside the family Flavobacteriaceae, including *Adhaeribacter* (Rickard *et al.*, 2005), *Hongiella* (Yi & Chun, 2004), *Arcicella* (Nikitin *et al.*, 2004) and *Belliella* (Brettar *et al.*, 2004). In addition, the novel family Cryomorphaceae within the phylum 'Bacteroidetes'

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Epilithonimonas tenax* EP105^T and *Persicivirga xylanidelens* SW256^T are AF493696 and AF493688, respectively.

was recently described containing five new genera, namely *Brumimicrobium*, *Cryomorpha*, *Crocinitomix*, *Owenweeksia* and *Fluviicola* (Bowman *et al.*, 2003; Lau *et al.*, 2005; O'Sullivan *et al.*, 2005).

The overall aim of this study was to investigate and compare the phylogenetic diversity of isolates within the phylum 'Bacteroidetes' from the relatively high-nutrient freshwater and marine habitats of river epilithon (River Taff, Cardiff, UK) and coastal sea water (Hope Cove, Plymouth, UK). A cultivation-based approach was employed involving standard spread-plate isolation techniques to determine whether this traditional approach still yielded novel and interesting strains of bacteroidetes. This study is the first in which a large selection of isolates obtained on agar plates were initially screened with 'Bacteroidetes'-specific 16S rRNA-gene-targeted oligonucleotide probes (Manz *et al.*, 1996; O'Sullivan *et al.*, 2002). This ensured accurate identification of colonies belonging to the 'Bacteroidetes' before undertaking an assessment of culturability diversity based on 16S rRNA gene sequence phylogeny.

Polyphasic characterization of one freshwater epilithon isolate (EP105^T) and one coastal sea-water isolate (SW256^T) enabled the description of two novel aquatic members of the *Flavobacteriaceae*: *Epilithonimonas tenax* gen. nov., sp. nov. and *Persicivirga xylanidelens* gen. nov., sp. nov.

METHODS

Samples. River epilithon samples were collected from the River Taff in Cardiff, UK, in January 2000 (Ordnance Survey map reference SO783165). Five stones were collected in sterile plastic bags and processed within 1 h of collection. Epilithon samples were obtained by scrubbing each stone with a sterile toothbrush in 30 ml sterile deionized water containing 1 µl Tween 20 l⁻¹ (Burton *et al.*, 1982). Samples from all five stones were combined and homogenized with 5 min treatment in a stomacher (O'Sullivan *et al.*, 2002). Coastal sea-water samples were obtained from Hope Cove near Plymouth, UK, in March 2000 (Ordnance Survey map reference SX675395). Sea water was collected by wading into the sea and opening sterile Duran bottles under the surface. The bottles were transported to the laboratory in a cool box and processed within 5 h.

Bacterial isolation. Bacterial strains were isolated from river epilithon and coastal sea water. Epilithon samples were serially diluted in sterile deionized water containing 1 µl Tween 20 l⁻¹ (Christensen & Cook, 1972). The spread-plate technique was used with six different agar media containing 20 µg cycloheximide ml⁻¹: plate count agar (PCA; Oxoid), PCA plus 50 µg kanamycin ml⁻¹ (Flint, 1985), medium M1 (Weeks, 1955), PMYA II (Christensen & Cook, 1972), casein-yeast-tryptone (CYT; Holmes, 1992) and casein-peptone-starch (CPS; Staples & Fry, 1973). Sea water samples were serially diluted in aged sea water from a circulating marine aquarium that had been filter-sterilized through a sterile 0.2 µm pore membrane after adjustment to 36 ‰ salinity by dilution with distilled water. Three different solid media were made with this filter-sterilized sea water: PCA plus 50 µg kanamycin ml⁻¹, CYT and R2A (Difco Bacto R2A agar). All media were adjusted to pH 7.2 before sterilization by autoclaving at 121 °C for 15 min. Agar plates were incubated at 20 °C for 10 days. Colonies showing characteristic spreading and/or yellow, orange, pink or red pigmentation were selected as

putative members of the 'Bacteroidetes'. Individual colonies were subcultured twice on their original isolation medium, before being purified on PCA (containing aged sea water for marine isolates). After isolation and purification, marine isolates were cultured on media prepared with artificial sea water (24.7 g NaCl, 0.7 g KCl, 6.3 g MgSO₄·7H₂O, 4.6 g MgCl₂·6H₂O, 1 g anhydrous CaCl₂ and 0.2 g NaHCO₃ per litre distilled water; Dawson *et al.*, 1969). Strains were maintained as viable cultures on low-nutrient media at 4 °C using CYT agar for epilithon strains and SAP2 agar (Holmes, 1992) for sea-water strains. Strains were also cryopreserved at -80 °C in FXAG liquid medium (epilithon isolates) or SP5 liquid medium (sea-water isolates) containing 30 % glycerol (Holmes, 1992). Epilithon isolates were designated 'EP' and coastal sea-water isolates were designated 'SW.'

Molecular analysis. 16S rRNA gene PCR amplification with general bacterial primers 27F and 1392R, sequencing and phylogenetic tree construction were performed as described previously (O'Sullivan *et al.*, 2004). DNA base composition and DNA-DNA hybridization experiments were conducted at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). DNA isolation and DNA G+C content analysis were performed as described previously (O'Sullivan *et al.*, 2005). DNA-DNA hybridization was carried out as described by De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983), using a Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian).

Selection of isolates for phylogenetic analysis. The 16S rRNA gene PCR product from each isolate was immobilized on nylon membranes using a slot-blot manifold (O'Sullivan *et al.*, 2002). The membranes were hybridized against the 'Bacteroidetes'-specific 16S rRNA gene-targeted oligonucleotide probes CF319a/b (Manz *et al.*, 1996) and CFB560 (O'Sullivan *et al.*, 2002). 'Bacteroidetes' strains with positive hybridization signals were grouped according to their colony morphology on PCA (containing artificial sea water for marine isolates). Features investigated were colour, transparency, spreading and texture (Smibert & Krieg, 1994). A selection of strains with representative colony morphologies was selected for phylogenetic analysis based on 16S rRNA gene sequence.

Fatty acid analysis. Strain EP105^T, *Chryseobacterium indoltheticum* NCIMB 2220^T and *Elizabethkingia meningoseptica* NCTC 10016^T were cultured on tryptone soy agar (TSA; Oxoid) for 24 h at 25 °C. Strain SW256^T was grown on marine agar 2216 (MA2216; Difco) for 3 days at 20 °C. Biomass was collected by washing with PBS (Sigma) and freeze-dried. Fatty acid analyses were carried out as described previously (O'Sullivan *et al.*, 2005).

Phenotypic analysis. The majority of phenotypic tests utilized in this study were performed as described previously (O'Sullivan *et al.*, 2005). Prior to experimental investigation, bacteria were revived from -80 °C freezer stocks at 20 °C on either PCA or nutrient agar (NA; Oxoid). Media were supplemented with artificial sea water for cultivation and testing of marine bacteria. All 'Bacteroidetes' isolates were tested for the presence of flexirubin pigments and for the ability to form spreading colonies on PCA, CYT and VY2 agar (the latter has been noted to stimulate spreading; Reichenbach, 1992). Additional phenotypic tests (see Tables 1 and 2) were performed on strains EP105^T and SW256^T, with some also carried out on the reference bacteria, *E. meningoseptica* and *C. indoltheticum*. Gliding motility was detected by phase-contrast microscopy of suspensions from the edges of colonies; strain EP105^T was grown on TSA and strain SW256^T was grown on MA2216 and both were incubated under high moisture conditions for 3 days. Strains were also characterized with Biolog GN2 Microplate (Biolog) kits processed according to the manufacturer's instructions, with the exceptions

Table 1. Characteristics that differentiate strain EP105^T from the type strains of phylogenetically related species in the genera *Bergeyella*, *Chryseobacterium*, *Elizabethkingia*, *Kaistella*, *Riemerella* and *Sejongia*

Taxa: 1, *Epilithonimonas tenax* sp. nov. EP105^T; 2, *B. zoohelcum*; 3, *C. balustinum*; 4, *C. daecheongense*; 5, *C. defluvii*; 6, *C. formosense*; 7, *C. gleum*; 8, *C. indologenes*; 9, *C. indoltheticum*; 10, *C. joostei*; 11, *C. scopthalmum*; 12, *E. meningoseptica*; 13, *E. miricola*; 14, *K. korensis*; 15, *R. anatipestifer*; 16, *R. columbina*; 17, *S. antarctica*. Data were obtained from this study or were cited by other studies (Kim *et al.*, 2004, 2005a; Yi *et al.*, 2005; Young *et al.*, 2005). All species were positive for catalase and oxidase activities, but did not show gliding motility and were Gram-negative. +, Positive; –, negative; W, weakly positive; ND, no data; V, variable between strains.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Flexirubin-type pigments	+	–	+	+	+	+	+	+	+	+	+	–	–	ND	–	–	–
Reduction of:																	
Nitrate	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Nitrite	–	–	–	–	+	ND	+	–	–	–	–	+	–	–	ND	ND	–
Indole production	–	+	+	–	+	+	+	+	+	+	–	+	+	+	–	–	+
Growth at:																	
30 °C	+	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+	V
37 °C	–	+	+	+	+	–	+	+	+	–	–	+	+	+	+	+	–
Growth on:																	
Cetrimide agar	–	–	–	ND	ND	ND	–	+	–	+	–	+	+	ND	ND	ND	–
MacConkey agar	+	–	+	–	–	–	+	–	+	+	–	+	+	–	–	–	–
Enzyme activity:																	
β-Galactosidase	–	–	–	ND	ND	–	W	V	–	ND	ND	+	+	–	–	ND	–
Urease	–	W	–	–	–	–	–	+	–	+	+	–	+	–	V	V	–
Hydrolysis of:																	
Aesculin	+	–	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+
DNA	–	–	+	ND	ND	ND	+	+	+	+	+	+	+	ND	ND	ND	+
Starch	W	–	–	+	+	ND	+	+	–	+	–	–	–	–	+	ND	+
Tween 80	–	–	+	ND	ND	ND	+	+	+	+	+	–	+	ND	ND	ND	+
Acid production from glucose	–	–	+	–	+	W	+	+	+	+	–	+	+	–	+	+	+

that incubation was at 20 °C and strain SW256^T was inoculated in artificial sea water containing Casamino acids and trace elements (O'Sullivan *et al.*, 2005).

RESULTS AND DISCUSSION

Phylogenetic diversity of 'Bacteroidetes' isolates

A collection of 382 isolates exhibiting spreading colonies or yellow, orange, pink or red pigmentation was isolated and purified from River Taff epilithon (145) and Hope Cove coastal sea water (237). Overall, 74.3 % (284/382) of the isolates hybridized with at least one of the 'Bacteroidetes'-specific 16S rRNA gene-targeted oligonucleotide probes. These 'Bacteroidetes'-positive isolates were then grouped into 17 types according to colony morphology and 59 representative isolates from these morphologically similar types were selected for phylogenetic analysis. Fig. 1 shows examples of most of the typical colony morphologies encountered in this study. The 16S rRNA gene sequences of 55 of these isolates were affiliated with the phylum 'Bacteroidetes' (55/59 or 93.2 %; 37 epilithon, 18 sea-water isolates) and 52 of the isolates belonged to the family *Flavobacteriaceae* (52/55 or 94.5 %; Fig. 2). The three 'Bacteroidetes' isolates which did not belong to the *Flavobacteriaceae* were isolates

EP211, EP316 and EP293 (GenBank accession numbers AF493691–AF493693). EP211 and EP293 had 96–97 % sequence similarity to *Pedobacter heparinus* ATCC 13125^T (GenBank accession number M11657) and EP293 had 93 % sequence similarity to *Flectobacillus major* ATCC 29496^T (GenBank accession number M62787).

Interestingly, there was no relationship between phylogeny and the original isolation medium. Some isolates with > 97 % 16S rRNA gene sequence similarity were isolated on different media and vice versa (data not shown). In addition, there was no relationship between phylogeny and colony morphology, with some closely related bacteria producing distinct colonies (data not shown). This suggests that colony morphology in the 'Bacteroidetes' is not a good indicator of phylogeny and so, in future, a sequence-based screening method would be better for diversity studies. The ability of colonies to spread on the agar media was sporadically distributed. This was particularly obvious within the genus *Flavobacterium*, where 23 isolates could spread and 11 isolates could not. Spreading can be tentatively attributed to gliding motility which was proposed to be an ancestral property lost by some members of the 'Bacteroidetes' (Woese *et al.*, 1990). The presence of flexirubin-type pigments was also much more prevalent in freshwater epilithon isolates

Table 2. Characteristics that differentiate strain SW256^T from phylogenetically related genera of the family *Flavobacteriaceae*

Taxa: 1, *Persicivirga xylanidelens* sp. nov. SW256^T; 2, *Aquimarina*; 3, *Cellulophaga*; 4, *Gillisia*; 5, *Kordia*; 6, *Mesonina*; 7, *Polaribacter*; 8, *Psychroflexus*; 9, *Salegentibacter*; 10, *Stanierella*; 11, *Tenacibaculum*. Data are from this and other studies (Dobson *et al.*, 1993; Bowman *et al.*, 1998; Gosink *et al.*, 1998; Johansen *et al.*, 1999; McCammon & Bowman, 2000; Suzuki *et al.*, 2001; Nedashkovskaya *et al.*, 2003, 2005b; Sohn *et al.*, 2004; Van Trappen *et al.*, 2004). All genera were Gram-negative. +, Positive; −, negative; W, weakly positive; ND, no data; V, variable between strains.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Gliding motility	−	+	+	−	−	−	−	V	−	−	+
Flexirubin type pigments	−	+	−	−	−	−	−	−	−	−	−
Nitrate reduction	−	−	V	−	+	−	−	−	+	−	V
Growth at:											
4 °C	+	+	+	−	+	+	+	+	+	+	V
20 °C	+	+	+	+	+	+	−	−	+	+	+
30 °C	−	+	+	W	+	+	−	−	+	+	V
37 °C	−	−	−	−	+	−	−	−	−	−	V
Growth on NaCl (%):											
0	−	−	−	−	−	−	−	−	+	−	−
2.5	+	+	+	+	+	+	+	−	+	+	+
15.0	−	−	−	−	−	+	ND	V	+	−	V
Enzyme activity:											
Catalase	−	+	+	+	−	+	−	+	+	−	+
Oxidase	−	+	−	+	+	+	−	+	+	+	+
Hydrolysis of:											
Aesculin	−	ND	ND	+	−	ND	V	V	+	ND	−
Agar	−	−	+	−	−	−	−	−	−	+	−
Starch	−	+	+	−	+	−	+	+	+	−	V
Tween 80	+	+	V	−	ND	+	ND	+	+	+	+
Acid production from glucose	−	−	+	−	−	−	+	+	+	−	V

(73 % or 27/37) than in sea-water isolates (11 % or 2/18), a feature previously recognized by Reichenbach *et al.* (1980).

Putative taxonomy of *Flavobacteriaceae* isolates

The isolates retrieved from this study can be tentatively compared with the current taxonomy of the family *Flavobacteriaceae* based on 16S rRNA gene sequence analysis. However, a full polyphasic characterization would be required for certainty (Vandamme *et al.*, 1996).

Thirty-four isolates, mainly from river epilithon, were closely related to members of the genus *Flavobacterium* and potentially represented strains of existing or novel species (Fig. 2). For example, the 16S rRNA gene sequence of *Flavobacterium johnsoniae* DSM 425 was almost identical

Fig. 1. Colony morphologies of a selection of strains belonging to the ‘*Bacteroidetes*’ isolated from River Taff epilithon and Hope Cove coastal sea water. Growth was on PCA plus artificial sea water for marine strains) for 1 week at 20 °C. (a) EP101; (b) EP248; (c) SW254; (d) *Epilithonimonas tenax* EP105^T; (e) EP240; (f) SW268; (g) SW58; (h) EP252; (i) SW265; (j) EP286; (k) EP134; (l) EP233; (m) EP293; (n) EP211; (o) *Persicivirga xylanidelens* SW256^T; (p) SW62. Note that colour rendition in these photographs does not always match that observed by eye, but gives an idea of the range of colours observed for these isolates. Bar, 2 cm.

to those of isolates EP286, EP300 and EP246; *Flavobacterium saccharophilum* NCIMB 2072^T was identical to isolate EP251; but isolate SW254 was only 96.5 % similar to *Flavobacterium frigidarium* ATCC 700810^T. EP333 and EP131 branch deeply within the genus and so might represent novel species.

Most of the sea-water isolates were most closely related to genera other than *Flavobacterium* (Fig. 3). For example, SW258 and SW274 potentially belonged to the genus *Tenacibaculum* (Suzuki *et al.*, 2001), showing 98 % 16S rRNA gene sequence similarity to *Tenacibaculum ovolyticum* IAM 14318^T (GenBank accession number AB032506) and other strains of this species (see Fig. 3). Strains SW7, SW19 and SW146 possibly represented novel species of the genus *Polaribacter* (Gosink *et al.*, 1998), exhibiting 96–97 % 16S rRNA gene sequence similarity to each other and 94–96 % similarity to the four recognized *Polaribacter* species. Sea-water isolates SW72, SW265 and SW285 were potentially members of the genus *Cellulophaga* (Johansen *et al.*, 1999). Strain SW72 was most similar to *Cellulophaga fucicola* NN015860^T (GenBank accession number AJ005973; 94 %;

Fig. 2. Phylogenetic tree of 16S rRNA gene sequences illustrating relationships between River Taff epilithon and Hope Cove coastal sea-water 'Bacteroidetes' isolates and members of the genus *Flavobacterium*. Sequences were obtained from GenBank at the National Centre for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>). Sequences were aligned using CLUSTAL W and the alignments were trimmed to 1250 bp. The trees were reconstructed with the neighbour-joining and Jukes–Cantor algorithms and were rooted with *Bacteroides fragilis* ATCC 25285^T. Bootstrap values from 100 replicate trees are shown at the nodes. Bar, 10% difference in nucleotide sequence.

data not shown), while strains SW265 and SW285 were 99% related to each other and to *Cellulophaga baltica* NN015840^T. Strains SW58, SW62, SW152 and SW334 potentially represented novel species of the genus *Aquimarina* (Nedashkovskaya *et al.*, 2005b) since they formed a monophyletic lineage with *Aquimarina muelleri* KMM 6020^T (95–98%). Strain SW268 formed a monophyletic lineage with *Maribacter sedimenticola* KMM 3903^T (97%) and possibly represented a novel species of the genus *Maribacter* (Nedashkovskaya *et al.*, 2004a). Strain SW269 had 95–96% 16S rRNA gene sequence similarity to members of the genus *Winogradskyella* (Nedashkovskaya *et al.*, 2005a), whilst SW325 was closely related to *Psychroserpens burtonensis* ACAM 188^T (95%).

Phylogenetic differentiation between members of the *Flavobacteriaceae* from freshwater and marine habitats

The *Flavobacteriaceae* isolates showed phylogenetic relationships which strongly correlated with environmental origin. Freshwater epilithon strains were affiliated almost exclusively to the genus *Flavobacterium* (Bernardet *et al.*, 1996; Fig. 2), the only exception being isolate EP105^T (Fig. 4). Nearly all sea-water strains belonged to a monophyletic branch typified by marine genera such as *Aequorivita*, *Cellulophaga*, *Polaribacter*, *Tenacibaculum* and *Zobellia* (Fig. 3). The only exception was isolate SW254, which was probably within the genus *Flavobacterium*. This phylogenetic

Fig. 3. Phylogenetic tree of 16S rRNA gene sequences illustrating relationships between Hope Cove coastal sea-water 'Bacteroidetes' isolates and members of the genera *Aequorivita*, *Algibacter*, *Arenibacter*, *Aquimarina*, *Bizionia*, *Cellulophaga*, *Formosa*, *Gelidibacter*, *Gillisia*, *Kordia*, *Leeuwenhoekiella*, *Maribacter*, *Mesonina*, *Muricauda*, *Pibocella*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Robiginitalea*, *Salagentibacter*, *Stanierella*, *Tenacibaculum*, *Ulvibacter*, *Vitellibacter*, *Winogradskyella* and *Zobellia*. The tree was constructed as described for Fig. 2. Bootstrap values from 100 replicate trees are shown at the nodes. Bar, 10% difference in nucleotide sequence.

distinction between freshwater and marine members of the *Flavobacteriaceae* was originally noted by Reichenbach *et al.* (1980) and Paster *et al.* (1985). However, this distinction is not found in all genera within the family. For example, some species of the genera *Flavobacterium*, *Chryseobacterium* and *Sejonia* can grow on media with and without added salt.

Overall, the coastal sea-water isolates exhibited greater 16S rRNA gene sequence diversity than the freshwater epilithon isolates. Sea-water isolates had 88.9% (16/18) unique sequence similarity groups (a sequence or group of sequences with >97% sequence identity), compared with only 43.2% (16/37) for the epilithon isolates. A greater proportion of sea-water isolates (13/19; 68.4%) were ≤ 97% similar to their closest BLAST match compared

with epilithon isolates (18/37; 48.6%). Bowman *et al.* (1997) conducted a similar cultivation-based study of heterotrophic Antarctic sea-ice bacteria and retrieved many extremely novel members of the *Flavobacteriaceae*. Suzuki *et al.* (1997) also cultured many novel marine heterotrophic bacteria on high-nutrient agar. They concluded that either this marine bacterial cultivation approach had not yet been exhausted or these novel bacteria had previously been isolated but their 16S rRNA gene sequences had not been submitted to public databases.

These cultivation studies suggest that the marine environment contains greater 'Bacteroidetes' diversity than the freshwater epilithon. However, these differences might in part be due to sampling epilithon in the freshwater site

Fig. 4. Phylogenetic tree of 16S rRNA gene sequences illustrating relationships between River Taff epilithon 'Bacteroidetes' isolate EP105^T and members of the genera *Chryseobacterium*, *Bergeyella*, *Elizabethkingia*, *Kaistella*, *Riemeirella* and *Sejongia*. The tree was constructed as described for Fig. 2. Bootstrap values from 100 replicate trees are shown at the nodes. Bar, 10% difference in nucleotide sequence.

and sea water in the coastal site. There was very little overlap between the epilithic 'Bacteroidetes' diversity estimated from this study and a molecular-based 16S rRNA gene clone library study of River Taff epilithon (O'Sullivan *et al.*, 2002). Only one epilithon clone was > 97% similar to a cultivated cellular isolate (clone TAF-B87 and isolates EP209 and EP235). The cultivation approach successfully retrieved isolates from the genus *Flavobacterium*, and to a lesser extent *Chryseobacterium*, *Pedobacter* and *Flectobacillus*, but did not isolate the majority of members of the 'Bacteroidetes' found in the 16S rRNA gene libraries, such as the large group of 'Taxeobacter' and *Hymenobacter* clones.

In general, the standard agar-based cultivation approach utilized in this study successfully isolated bacteria from the family *Flavobacteriaceae*, but appeared to be inappropriate for isolating other members of the 'Bacteroidetes'. The reasons for this are likely to be numerous: the cultivation technique may discriminate against the isolation of anaerobic, facultatively aerobic, psychrophilic, oligotrophic, filamentous or specialized cellulose-degrading bacteroidetes. Consequently, there is a need for more innovative cultivation methods to isolate the most abundant and ecologically significant aquatic members of the 'Bacteroidetes', but no single technique can be expected to cultivate all of the physiological diversity within the phylum (Suzuki *et al.*, 1997). However, this study does illustrate that routine isolation on high-nutrient agar still yields novel isolates of the *Flavobacteriaceae* worthy of further investigation. More targeted approaches have successfully isolated novel members of other families within the 'Bacteroidetes'. For example, Bowman *et al.* (2003) used a mixture of broth culture and agar plates incubated at 2–4 °C for at least 4–6 weeks to

isolate three new genera within the novel family designated *Cryomorphaceae*. However, isolates from two other genera in this family were subsequently isolated on agar incubated at 20–30 °C for 3–6 days (Lau *et al.*, 2005; O'Sullivan *et al.*, 2005). One of these studies used hybridization with a group-specific, phylogenetic probe to screen many isolates for bacteria related to the novel target group (O'Sullivan *et al.*, 2005). These and other targeted approaches that have been successful within other phyla (Fry, 2004) might well be applied for isolating novel, abundant members of the 'Bacteroidetes'.

Polyphasic characterization of isolates EP105^T and SW256^T

One River Taff epilithon strain (EP105^T) and one Hope Cove coastal sea-water strain (SW256^T) were selected for polyphasic characterization, in accordance with the proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). These isolates were selected because they both branched deeply between genera within 16S rRNA gene sequence-based phylogenetic trees (Figs 3 and 4). Sequence analysis of their 16S rRNA genes revealed that both strains were affiliated with the family *Flavobacteriaceae* (Figs 3 and 4).

Strain EP105^T belonged to a monophyletic cluster containing the genera *Bergeyella*, *Chryseobacterium*, *Elizabethkingia*, *Kaistella*, *Riemeirella* and *Sejongia* (commonly termed the *Chryseobacterium*–*Bergeyella*–*Riemeirella* branch; Vandamme *et al.*, 1994). EP105^T formed a discrete lineage positioned between the genus *Elizabethkingia* and the other genera (Fig. 3; bootstrap 75%). This separate branch

Table 3. Major whole-cell fatty acid content of *Epilithonimonas tenax* EP105^T, *Persicivirga xylanidelens* SW256^T, *Elizabethkingia meningoseptica* and *Chryseobacterium indoltheticum*

Taxa: 1, EP105^T (this study); 2a, *E. meningoseptica* NCTC 10016^T (this study); 2b, *E. meningoseptica* LMG 12279^T (Kämpfer *et al.*, 2003); 3a, *C. indoltheticum* NCIMB 2220^T (this study); 3b, *C. indoltheticum* LMG 4025^T (Kämpfer *et al.*, 2003); 4, SW256^T (this study). Two structural unspecified compounds (ECL 13:566 and ECL 16:580) identified by Kämpfer *et al.* (2003) are not included in this table. ECL 13:566 may be identical to iso 14:0. tr, Trace <0.1%.

Fatty acid	Content (% of total)					
	1	2a	2b	3a	3b	4
Saturated fatty acids:						
15:0						5.5
16:0	8.0	1.8	tr	0.9	1.3	tr
Sum	8.0	1.8	0.0	0.9	1.3	5.5
Saturated branched-chain fatty acids:						
iso 13:0	0.9	0.9	1.9		tr	tr
iso 14:0	4.3	5.8		6.0		3.2
anteiso 14:0	1.2			1.3		2.6
iso 15:0	20.0	40.2	42.1	27.1	34.1	18.1
anteiso 15:0	14.5	4.5	1.9	5.4	5.3	21.6
iso 16:0						3.3
iso 17:0	tr	tr	tr	tr	1.0	tr
Sum	41.0	51.4	45.9	39.8	40.4	48.8
Unsaturated branched-chain fatty acids:						
iso 13:1*						1.9
iso 16:1*						1.4
iso 17:1 ω 9c	1.0	6.8	4.6	30.0	21.7	8.5
Sum	1.0	6.8	4.6	30.0	21.7	11.8
Monosaturated fatty acids:						
13:1*	2.3	1.0				tr
15:1*						1.4
16:1 ω 7c/iso 15:0 2-OH	21.1	17.3	17.3	9.8	10.2	5.3
Sum	23.5	18.3	17.3	9.8	10.2	6.7
Hydroxy fatty acids:						
iso 15:0 3-OH	1.6	2.7	3.7	1.8	2.0	6.9
anteiso 15:0 3-OH						2.2
iso 16:0 3-OH	1.9	1.6	1.1	1.3	tr	4.7
16:0 3-OH	5.9	2.8	2.3	0.9	1.1	tr
iso 17:0 3-OH	9.8	13.2	17.6	11.0	15.4	7.1
anteiso 17:0 3-OH	2.5			2.2		4.9
Sum	21.6	20.3	24.7	17.4	18.5	25.8

* Unspecified position and configuration of double bond.

provides strong support for this strain representing a novel genus. Some members of the *Chryseobacterium*–*Bergeyella*–*Riemerella* branch have also been isolated from freshwater environments. For example, *Chryseobacterium daecheonense* and *Kaistella koreensis* were isolated from freshwater lake sediment and a freshwater stream, respectively (Kim

et al., 2004, 2005a). However, many members of this branch seem to have a propensity for colonizing higher animals, having been isolated from human clinical samples, birds and fish. This branch may typically contain free-living bacteria which can colonize higher animals opportunistically and cause disease, in much the same way as members of the *Burkholderia cepacia* complex (Mahenthiralingam *et al.*, 2005).

The sea-water strain SW256^T affiliated with the large marine *Flavobacteriaceae* branch and formed a low bootstrap grouping with members of the genus *Cellulophaga* (Fig. 3). This isolate was not easy to place phylogenetically, as its position varied according to the other sequences in the analysis. In other trees, it was more closely associated with the genera *Psychroflexus*, *Gillisia*, *Mesonina* and *Salegentibacter* or formed a separate branch on its own (not shown). However, SW256^T exhibited <90% 16S rRNA gene sequence similarity to any recognized species within the *Flavobacteriaceae*, indicating that this strain also represents a new genus.

Strain EP105^T was originally isolated on M1 medium and produced bright-orange, opaque, smooth, raised colonies (3–6 mm diameter) with an entire edge and a viscous consistency on PCA after 1 week at 20 °C (Fig. 1d). The cells of EP105^T were short, rounded rods which did not form chains or filaments and were 1–2.5 µm long and 0.6–0.7 µm wide. Strain SW256^T was isolated on CYT plus aged sea water and produced peach-coloured, opaque, smooth, raised colonies (1–2 mm diameter) with an entire edge and creamy consistency (Fig. 1o). The cells of SW256^T were long, irregularly straight to curved rods with rounded ends, which were 2.2–7.5 µm in length and 0.5–0.6 µm in width. Neither strain produced gliding cells or spreading colonies on any agar tested.

The physiological and biochemical characteristics of strains EP105^T and SW256^T are listed in the species descriptions. Table 1 lists the phenotypic characteristics that distinguish strain EP105^T from some phylogenetically related species of the genera *Bergeyella*, *Chryseobacterium*, *Elizabethkingia*, *Kaistella*, *Riemerella* and *Sejongia*. EP105^T could be distinguished from members of the aforementioned genera by three to 11 known phenotypic characteristics. In addition, the members of genera described in Table 1 can usually be distinguished from EP105^T and each other by the following observations. Absence of yellow pigment distinguishes *Elizabethkingia*, *Bergeyella* and *Riemerella* strains. Growth on marine agar is variable for species of *Chryseobacterium*, positive for *Elizabethkingia* and *Sejongia* and negative for *Riemerella*, *Bergeyella* and *Epilithonimonas*. Capnophilic metabolism occurs in *Riemerella* species only. Table 2 lists the phenotypic characteristics that differentiate strain SW256^T from phylogenetically related marine genera of the family *Flavobacteriaceae*. SW256^T required NaCl for growth and was unable to utilize any of the substrates included in the API 20NE or Biolog GN2 commercial arrays.

SW256^T could be distinguished from related marine genera by three to nine known phenotypic characteristics.

The fatty acid profiles of SW256^T, EP105^T and two phylogenetically related species of the family *Flavobacteriaceae* are shown in Table 3. The uncertain, deep-branching phylogenetic position of SW256^T made it difficult to identify suitable cultures for comparison. Although SW256^T contained a wider range of fatty acids than EP105^T, both bacteria are characterized by large amounts of branched as well as hydroxy fatty acids, contributing over 60% to the total fatty acid content. In comparison with the phylogenetically related species, the novel strains show large relative proportions of the anteiso compounds. Fatty acid profiles obtained for reference strains in this study were in agreement with previously published profiles (Kämpfer *et al.*, 2003). There were clear differences between EP105^T, *E. meningoseptica* and *C. indoltheticum* for fatty acids 16:0, iso 17:1ω9c and the combined peak 16:1ω7c/iso 15:0 2-OH (usually called summed feature 3). These and some other differences useful in taxonomic assignment are consistent with other data for related strains presented recently (Kim *et al.*, 2005b).

Strains EP105^T and SW256^T had DNA G+C contents of 37.5 and 34.7 mol%, respectively, which are consistent with the values observed in the family *Flavobacteriaceae* (27–44 mol%; Bernardet *et al.*, 2002). Mean DNA–DNA hybridization values for EP105^T and *E. meningoseptica* and for EP105^T and *C. indoltheticum* were 35.3 and 20.8%, respectively, demonstrating that EP105^T is not closely related to the genera *Chryseobacterium* or *Elizabethkingia*.

Description of *Epilithonimonas* gen. nov.

Epilithonimonas [Ep'i.lith.on.i.mo'nas. N.L. n. *epilithon* -is (or *epilithonum* -i) epilithon; L. fem. n. *monas* a unit, monad; N.L. fem. n. *Epilithonimonas* a monad isolated from epilithon].

Short, rod-shaped, non-flagellated, Gram-negative cells. Strictly aerobic. Carbohydrates are utilized for growth. Cytochrome oxidase- and catalase-positive. Fatty acids contain a large proportion of saturated branched-chain, mono-saturated and hydroxy fatty acids. The most abundant individual fatty acids are iso 15:0 and summed feature 3 (16:1ω7c/iso 15:0 2-OH). Isolated from freshwater environments and do not require the presence of Na⁺ ions. Analysis of the 16S rRNA gene sequences indicate that the genus *Epilithonimonas* is a member of the family *Flavobacteriaceae* of the phylum 'Bacteroidetes'. The type species is *Epilithonimonas tenax*.

Description of *Epilithonimonas tenax* sp. nov.

Epilithonimonas tenax (ten'ax. L. fem. adj. *tenax* sticky, holding firm, referring to the organism's viscous colonies).

Exhibits the following characteristics in addition to those properties described for the genus. Colonies are 3–6 mm in

diameter, bright orange, opaque, smooth, with an entire edge and a viscous consistency (PCA after 1 week at 20 °C). Cells are short, rounded rods which do not form chains or filaments and are 1–2.5 μm long and 0.6–0.7 μm wide. Cells do not exhibit gliding motility. Cell mass is pigmented orange and flexirubin pigments are synthesized. Growth occurs between 4 and 30 °C, but not in the presence of NaCl. Nitrate is reduced, but nitrite is not reduced. Growth occurs on TSA, NA, MacConkey agar and DNase agar, but not on MA2216 or cetrimide agar. Aesculin and starch are hydrolysed. Agar, arginine, DNA, Tween 80 and gelatin are not hydrolysed. Indole is not produced. No β-galactosidase, urease or xylanase activities. Acid is not produced from glucose. Resistant to chloramphenicol, streptomycin, kanamycin and tetracycline, but susceptible to penicillin G, ampicillin and rifampicin. Growth occurs on glucose, mannose, maltose, α-cyclodextrin, dextrin, glycogen, Tween 40, gentiobiose, α-D-glucose, sucrose, D-trehalose, acetic acid, α-ketovaleric acid, L-alanyl-glycine, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-ornithine, L-proline, L-serine, L-threonine, inosine, uridine and thymidine. Growth does not occur on arabinose, mannitol, N-acetylglucosamine, gluconate, caprate, adipate, malate, citrate, phenylacetate, Tween 80, N-acetyl-D-galactosamine, adonitol, D-arabitol, cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, myo-inositol, α-D-lactose, lactulose, D-melibiose, methyl β-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, turanose, xylitol, methyl pyruvate, monomethyl succinate, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, alaninamide, D-alanine, L-alanine, L-asparagine, L-aspartic acid, L-histidine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-pyroglutamic acid, D-serine, DL-carnitine, γ-aminobutyric acid, urocanic acid, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, D,L-α-glycerol phosphate, glucose 1-phosphate or glucose 6-phosphate. The DNA G+C content of the type strain is 37.5 mol%.

The type strain, EP105^T (= NCIMB 14026^T = DSM 16811^T), was isolated from epilithon-covered stones from the River Taff in Cardiff, UK.

Description of *Persicivirga* gen. nov.

Persicivirga (Per.si.ci.vir'ga. L. neut. n. *persicum* peach; L. fem. n. *virga* rod; N.L. fem. n. *Persicivirga* peach-coloured rod).

Long, irregularly straight to curved, Gram-negative rods with no visible flagella. Strictly aerobic. Do not utilize carbohydrates for growth. Oxidase- and catalase-negative. The most abundant fatty acids are saturated branched-chain,

unsaturated branched-chain and hydroxy fatty acids; iso 15:0 and anteiso 15:0 are the most abundant individual fatty acids. Isolated from marine environments and require Na^+ ions or natural sea water for growth. 16S rRNA gene sequence analysis indicates that the genus *Persicivirga* is a member of the family *Flavobacteriaceae* of the phylum 'Bacteroidetes'. The type species is *Persicivirga xylanidelens*.

Description of *Persicivirga xylanidelens* sp. nov.

Persicivirga xylanidelens (xy.lan.i.del'ens. N.L. n. *xylanum* xylan; L. part. adj. *delens* destroying; N.L. part. adj. *xylanidelens* xylan-destroying).

Exhibits the following characteristics in addition to those properties described for the genus. Colonies are 1–2 mm in diameter, peachy-orange, opaque, smooth with an entire edge and creamy consistency (PCA plus artificial sea water for 1 week at 20 °C). Cells have rounded ends and are 2·2–7·5 µm long and 0·5–0·6 µm wide. Cells do not exhibit gliding motility. Cell mass is pigmented orange and flexirubin pigments are synthesized. Growth occurs at 4 and 20 °C and on 5%, but not 10%, NaCl. Growth occurs on MA2216, but not on TSA, NA, MacConkey agar, cetrimide agar or DNase agar containing 2·5% NaCl. Xylanase activity is present, but urease activity is absent. Tween 80 and gelatin are hydrolysed, but agar, arginine, aesculin or starch are not hydrolysed. Nitrate and nitrite are not reduced. Indole is not produced. Acid is not produced from glucose. Resistant to chloramphenicol, penicillin G, streptomycin, kanamycin, ampicillin and tetracycline, but sensitive to rifampicin. Growth is not detected for any substrates included in the API 20NE and Biolog GN2 commercial arrays. The DNA G+C content of the type strain is 34·7 mol%.

The type strain, SW256^T (=NCIMB 14027^T=DSM 16809^T), was isolated from coastal sea water, Hope Cove, near Plymouth, UK.

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