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Phylogenetic diversity of *Flavobacteria* isolated from the North Sea on solid media

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ABSTRACT

Flavobacteria are abundant in the North Sea, an epeiric sea on the continental shelf of Europe. However, this abundance has so far not been reflected by the number of strains in culture collections. In this study, *Flavobacteria* were isolated from pelagic and benthic samples, such as seawater, phytoplankton, sediment and its porewater, and from surfaces of animals and seaweeds on agar plates with a variety of carbon sources. Dilution cultivation with a new medium, incubation at low temperatures and with long incubation times, and colony screening by a *Flavobacteria-Cytophagia*-specific PCR detecting 16S rRNA gene sequences led to a collection of phylogenetically diverse strains. Two strains affiliated with *Flammeovirgaceae* and seven strains affiliated with *Cyclobacteriaceae*, whereas within the *Flavobacteriaceae* 20 isolated strains presumably represented seven novel candidate genera and 355 strains affiliated with 26 of 80 validly described marine *Flavobacteriaceae* genera, based on a genus boundary of 95.0% 16S rRNA gene sequence identity. The majority of strains (276) affiliated with 37 known species in 16 genera (based on a boundary of 98.7% 16S rRNA gene sequence identity), whereas 79 strains likely represented 42 novel species in 22 established *Flavobacteriaceae* genera. Pigmentation, iridescence, gliding motility, agar lysis, and flexirubin as a chemical marker supported the taxonomy at the species level. This study demonstrated the culturability on solid medium of phylogenetically diverse *Flavobacteria* originating from the North Sea.

Introduction

Flavobacteria are common in epipelagic oceanic and coastal waters as well as in benthic habitats, accounting for 10–30%, and sometimes up to 70%, of the bacterial populations [14,20,55]. Together with *Alphaproteobacteria*, *Flavobacteria* have been shown to be more abundant in the particle-associated fraction, whereas *Gammaproteobacteria* were dominantly free floating [1]. *Flavobacteria* are known to attach to phytoplankton [20] and to participate in the initial degradation of complex organic matter, thus playing an important part in the carbon cycle [28]. In a decaying phytoplankton spring bloom in the North Sea, *Flavobacteria* populations dominated the initial degradation process [49]. The German Bight in the North Sea is a shallow coastal area with high tidal dynamics [38] whose seafloor is a major sink of organic matter and nitrogen species [2,19]. In this coastal region, *Flavobacteria* were a dominating population in the microbial community in surface seawater, accounting for up to 55% of bacterioplankton cells [14]. In the benthos, *Flavobacteria* were the most abundant phylogenetic group, accounting for 15–25% of all cells [31]. In 2006, the *Flavobacteriaceae* comprised 168 species in 53 genera [6]. However, this family has increased to 393 species in 95 genera (www.bacterio.cict.fr, June 2012) [15], and marine strains represented 210 *Flavobacteriaceae*-type strains in 80 genera (Table S1).

In contrast to the population size, previous attempts to cultivate representatives of bacterial communities from the Wadden Sea obtained a low number of *Flavobacteriaceae* strains, irrespective of whether they originated from seawater [14] or intertidal sediment [47]. In both cases, polymeric carbohydrates (e.g. chitin, cellulose and agar) did not support an increase in culturability. The authors concluded that (I) frequently isolated bacteria were of low abundance in nature [14], and (II) *Flavobacteria* did not grow well on solid agar [47]. Nevertheless, seven novel species of *Flavobacteriaceae* have been isolated and described from the North Sea in recent years. *Leeuwenhoekiella marinoflava* [35] was cultivated from the seawater of the coast of Aberdeen [12], whereas *Maribacter forsetii* [4] and '*Gramella forsetii*' [5] were isolated from the seawater of Helgoland, an island in the German Bight. *Muricauda ruestringensis* was isolated from the intertidal sediment near the former village of Rüstringen [9]. *Tenacibaculum ovolyticum* was isolated from the epiflora of halibut eggs from Bergen, Norway [22,48]. *Tenacibaculum skagerrakense* was isolated from the seawater of Skagerrak, Denmark [18], and *Cellulophaga fucicola* from the brown alga *Fucus* of Hirsholm Island, Denmark [24].

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Therefore, the aim of the current study was to collect phylogenetically diverse *Flavobacteriaceae* from different locations and sample types of the German Bight in the North Sea. Improved techniques were explored for isolating marine *Flavobacteria* using suitable medium components, and PCR with a *Flavobacteria-Cytophagia*-specific primer for the 16S rRNA gene enabled fast identification of *Flavobacteria* colonies.

Materials and methods

Sampling

Samples were collected with Niskin bottles, 20 μm- or 80 μm-plankton nets, sterile syringes or tubes at Helgoland, Harlesiel, Janssand and the Königshafen, Hausstrand/List and Weststrand sites on Sylt (Table S1). Samples were stored at *in situ* temperature, transported to the laboratory within one to three hours and directly processed.

Medium preparation

Artificial seawater (ASW) and all media were prepared with sterile filtered (0.2 μm polycarbonate filter) ultra pure water (Aquintus system, membraPure, Germany) with a resistivity of 18.3 MΩ m. For dilutions and washing steps, ASW was prepared following the recipe of Widdel and Bak [52], as described by Winkelmann and Harder [53]. Basal salts: 26.37 g NaCl, 5.67 g $MgCl_2 \times 6H_2O$, 6.8 g $MgSO_4 \times 7H_2O$, 0.19 g $NaHCO_3$, 1.47 g $CaCl_2 \times 2H_2O$, 0.72 g KCl, 0.10 g KBr, 0.02 g H_3BO_3, 0.02 g $SrCl_2$ and 0.003 g NaF were dissolved in 1 L water. After autoclaving at 121 °C for 25 min and cooling, the ASW was slowly adjusted to pH 7.5 with autoclaved 1 M NaOH or 1 M HCl. Autoclaved water was used to replace the evaporated water. The ASW had a salinity of 34‰ S, which was comparable to the euhaline (>30‰ S) sampling sites.

ZoBell [56,57] suggested the marine medium 2216 with yeast extract (=2216E) for the cultivation of most marine bacteria, which is nowadays sold as marine agar 2216, and it was prepared following the manufacturer's instructions (Difco Laboratories, Detroit, USA). The evaporated water was replaced by autoclaved water. Other solid media with defined carbon sources required the preparation of twofold concentrated ASW and purification of Bacto Agar (Difco Laboratories, Detroit, USA). Agar (18 g L^{-1}) was washed three times with 700 mL ultra pure water, in order to remove soluble substances that may inhibit bacterial growth [23,52]. Solid HEPES (50 mM) and 500 mL twofold ASW were added to the agar suspension. After autoclaving, the medium was cooled to 55 °C and supplemented with 5 mL NH_4Cl (50 g L^{-1}, autoclaved), 10 mL KH_2PO_4 (50 g L^{-1}, autoclaved), 2 mL trace element solution (per L: $FeSO_4 \times 7H_2O$, 2.1 g; Na_2-EDTA, 5.2 g; H_3BO_3, 30 mg; $MnCl_2 \times 4H_2O$, 100 mg; $CoCl_2 \times 6H_2O$, 190 mg; $NiCl_2 \times 6H_2O$, 24 mg; $CuCl_2 \times 2H_2O$, 10 mg; $ZnSO_4 \times 7H_2O$, 144 mg; $Na_2MoO_4 \times 2H_2O$, 36 mg; pH adjusted to 6.0 with 5 M NaOH [37]), and 0.7 mL SeW solution [52]. Carbon sources for the SYL media were 2 g L^{-1} of yeast extract, peptone tryptone, casamino acids, glucose, cellobiose, N-acetylglucosamine, xylose, galactose, malate, arabinose or rhamnose, for the HAR medium 0.3 g L^{-1} of casamino acids and 0.5 g L^{-1} of glucose, xylose and N-acetylglucosamine, and for the HaHa medium 0.5 g L^{-1} of yeast extract, peptone tryptone, casamino acids, glucose, and cellobiose. The SYL media received, per litre, 1 mL 7-vitamin solution [53], 1 mL vitamin B_{12} solution [52], 1 mL thiamine solution [53], and 1 mL riboflavin solution [53]. The pH was slowly adjusted to 7.5 with autoclaved 1 M NaOH. Evaporated water was replaced with autoclaved water before the plates were poured.

Isolation and cultivation

To enrich sediment-attached bacteria, 5 mL of the sediment from Harlesiel were sampled with a sterile cut-off syringe. The sediment was washed successively five times with 40 mL sterile artificial sea water in a 50 mL polypropylene tube, which resulted in approximately 5.5×10^4 cells mL^{-1} sediment. Sediments were allowed to settle for 30 min and supernatants were decanted. The washed sediment was incubated in HAR liquid medium at 25 °C for 24 h. The sediment was mixed with the medium in an overhead rotator at 25 rpm (Reax 2, Heidolph, Schwabach, Germany). The next day, the sediment was washed five times with artificial seawater (40 mL) and afterwards incubated for 48 h and 96 h in HAR liquid medium. The supernatants were decanted and collected in fresh, sterile 50 mL polypropylene tubes. Samples of the sediment or the supernatant were incubated on solid HAR medium.

Kanamycin was reported to select for *Flavobacteria* [17]. Therefore, surface intertidal sediments from Königshafen of Sylt, Janssand or Harlesiel were incubated on SYL agar, optionally supplemented with 50 μg mL^{-1} kanamycin [17] and incubated at 25 °C for 3–4 weeks.

For inoculation, seawater aliquots were spread on solid agar plates using sterile glass beads, and sediment was spread on agar plates with an inoculating loop. Algae were chopped and washed with sterile artificial seawater. Animal specimens were washed with seawater and sterile artificial seawater. A 96-pin replicator enabled the transfer of 1 μL per pin onto 96 defined positions on the agar surface in a 150 mm Petri dish [53]. HAR and HaHa agar plates were incubated at 11 °C and SYL agar plates at room temperature (22 °C) for at least two months. Single colonies were examined and transferred three times to new plates in order to obtain pure strains. Colonies were characterized by phenotypic characteristics as well as *Flavobacteria-Cytophagia*-specific 16S rRNA gene amplification and sequence analysis. Strains were maintained as viable cultures on 2216 marine agar or on HaHa agar plates at 4 °C and also cryopreserved at −80 °C in artificial seawater supplemented with 30% (v/v) glycerol.

Phenotypic characterization

The bathochromic shift test with 20% (w/v) KOH was performed to detect flexirubin-type pigments [16] (Fig. S6). Cell shapes were visualized with phase contrast microscopy. Shape and colour of colonies on the agar plate were visualized with a binocular microscope.

16S rRNA gene analysis

Two protocols were applied to release DNA from cells. A tiny amount of a colony was dissolved from a sterile wooden toothpick in 20 μL PCR water. After three freeze/thaw cycles (−20 °C/+4 °C), one microlitre served as PCR template. Alternatively, the smallest separable part of the colony was squashed in 100 μL PCR water and lysed by three freeze/thaw cycles. The frozen sample finally received 100 μL PCR water and was thawed without mixing. Ten μL of supernatant served as PCR template. The 16S rRNA gene was amplified with the general bacterial primers GM3F (5′-AGA GTT TGA TYM TGG CTC AG-3′) (positions 8–27 according to *Escherichia coli* numbering) and 907R (5′-CCG TCA ATT CCT TTR AGT TT-3′) [34], as well as with the primers GM3F and CF1489R. The *Flavobacteria-Cytophagia*-specific reverse primer CF1489R (5′-TAC CTT GTT ACG ACT TAG C-3′, positions 1489–1507) was designed and validated with the ARB software [32] on the dataset SILVA ref108_NR99 [39] and with SILVA TestPrime [29]. PCR amplifications were performed in 25 μL with 96 °C for 4 min, 35 cycles of 96 °C for 1 min, 55 or 62 °C for 1 min – for primer pair GM3F, 907R

and GM3F, CF1489R, respectively – 72 °C for 3 min and a final elongation at 72 °C for 10 min. The sequencing reaction applied the ABI Dye Terminator technology and an Applied Biosystems 3130xl DNAsequencer (Applied Biosystems). As an exception to standard conditions, GM3F-CF1489R amplicons were sequenced with an elongation temperature of 62 °C, the optimal annealing temperature of CF1489R. The 16S rRNA gene sequences were analysed with Applied Biosystems Sequencing Analysis 5.2 (Applied Biosystems, Foster City, USA) and assembled with Sequencer 4.6 (Gene Codes, Ann Arbor, MI).

The initial phylogenetic affiliation was assigned using the Ribosomal Database Project [11]. After alignment of sequences in ARB, evolutionary distances were calculated by the method of Jukes and Cantor [25] and a phylogenetic consensus tree was constructed with neighbour-joining [42] using a 0% and 40% base frequency filter in ARB.

The 16S rRNA gene sequences were deposited in GenBank under Acc. Nos. JX854056–JX854433.

Results and discussion

A total of 375 strains affiliated to *Flavobacteriaceae* were isolated from all samples investigated: seawater of Helgoland, sediment of Harlesiel and Jansand, and seawater, sediment and its porewater, phytoplankton, seaweed and animal specimens of Sylt in the German Bight. The affiliation was based on the current nomenclature of *Flavobacteriaceae* (Table S2). A novel species was defined by a 16S rRNA gene sequence identity of between 95.0% and 98.7% (Table S3), and a novel genus was defined as <95.0% 16S rRNA gene sequence identity with validly described *Flavobacteriaceae* (Table S4) [45,54]. The strains represented 7 novel genera, 42 novel species, and 37 validated species, including four species previously isolated from the North Sea: '*G. forsetii*' [5], *M. forsetii* [4], *M. ruestringensis* [9] and *C. fucicola* [24]. Detailed information on the strains is presented in Fig. 1 and Tables S3 and S4.

Selection criteria for isolation were initially the yellow colony colour and a short rod-shaped to filamentous cell morphology (Fig. S2) [6,7]. These criteria yielded a bias towards strains of the genera *Arenibacter*, *Cellulophaga* and *Maribacter*. A colour-independent screen for the presence of *Flavobacteria* colonies was developed with the new *Flavobacteria-Cytophagia*-specific reverse primer CF1489R. This primer covered 86% of all *Bacteroidetes* sequences present in the SILVA ref108_NR99 database. The primer exclusively amplified, in combination with the *Bacteria*-forward primer GM3F at 62 °C, a nearly full-length 16S rRNA gene of *Flavobacteria* or *Cytophagia*. The *Bacteria*-specific primers GM3F and 907R revealed the presence of *Actinobacteria*, *Firmicutes*, *Alphaproteobacteria* and *Gammaproteobacteria* among the non-*Flavobacteria-Cytophagia* colonies. Thus, the new primer was used for aerobic marine samples, and it was highly specific for *Flavobacteria*, *Sphingobacteria* and *Cytophagia*. Among the non-intensive yellow colonies detected as *Flavobacteria* were strains of *Zunongwangia*, 30 of 42 novel species, and 6 of 7 novel candidate genera.

Variations in media and cultivation conditions

All strains were cultured as chemoheterotrophic bacteria on (I) ZoBell's 2216 marine agar or (II) a defined artificial seawater medium supplemented with ammonium, phosphate, trace elements, and for carbon and energy sources with 2 g L^{-1} of complex carbon sources (yeast extract, peptone, casamino acids), defined carbohydrates (glucose, galactose, ramnose, xylose, cellobiose, malate, or N-acetylglucosamine), or a mixture of both. HaHa medium was more suitable than marine agar 2216, partly because colonies of *Vibrio*, *Alteromonas* and *Pseudoalteromonas* were very large on 2216 and covered small adjacent colonies, which rarely formed colonies on HaHa medium. This may have been due to the absence of vitamins in the HaHa medium.

For a spring sample with a temperature of 6.4 °C, isolation and cultivation were performed at 11 °C. This experiment yielded strains representing 11 of 42 novel *Flavobacteriaceae* species and 3 of 7 novel candidate genera in our study, but only one of 37 known species. Even if the environmental temperature during the year reached mesophilic temperatures (20 °C), the observed shift towards novel species observed in cultivations at low temperature, near the *in situ* temperature, highlighted temperature as an important variable in isolation experiments.

Inoculation on plates was performed with traditional spreading techniques. Alternatively, one microlitre was spotted on a plate with a 96-pin replicator [53]. Twofold dilution series yielded plates with high numbers of single colonies per inoculation spot. To determine the time for colony formation, a spring pelagic water sample from Helgoland with an *in situ* temperature of 6.4 °C was diluted and 1152 spots of a 0.1 μL original seawater sample were observed for growth at 11 °C for 300 days (Table 1). The CFU increased during the incubation time, and was comparable to a growth curve. After a lag phase of 10 days, the number of colony forming units (CFU) exponentially increased to 153 CFU until day 23 and reached 208 CFU on day 110. Besides *Actinobacteria* (e.g. *Rhodococcus*, *Nocardioides*), *Alphaproteobacteria* (*Erythrobacter*, *Sulfitobacter* and *Brevundimonas*) and *Gammaproteobacteria* (e.g. *Marinobacter*, *Pseudoalteromonas*), 88 yellow–orange to brownish pigmented colonies were obtained and they included 43 *Flavobacteriaceae*, which corresponded to 273 CFU per mL seawater. The first *Flavobacteriaceae* colonies were strains of *Krokinobacter*, *Croceibacter*, *Maribacter* and *Salegentibacter*. Also, strains affiliated to *Gillisia*, *Stenothermobacter*, *Arenibacter* and *Marixanthomonas* required less than 20 days to form visible colonies. In contrast, strains of *Cellulophaga*, *Flavobacterium* and *Nonlabens* required at least 20 days for colony formation (Fig. 2). Several strains of novel species required long incubation times of several weeks, whereas over 80% of viable cells needed only three weeks to grow to visible colonies. The incubation time seemed to be an important factor for the cultivation of novel species.

Flint [17] suggested kanamycin as an effective agent for enhancing the culturability of "*Flavobacterium*" species because of growth inhibition of other bacteria. Therefore, plates with 2 g L^{-1} casamino acids were supplemented with 50 μg mL^{-1} kanamycin and inoculated with phytoplankton or sediment samples. In comparison with control plates, the number of white colonies was reduced on average by 50%, whereas the number of yellow colonies remained nearly constant (Fig. S3). The *Flavobacteria-Cytophagia*-specific PCR was positive for 90% of the yellow colonies. This experiment confirmed the observations of Flint [17] and the resistance of many *Flavobacteria* to the aminoglycoside antibiotic kanamycin. Strains obtained from kanamycin-containing media affiliated with *Arenibacter* (1 strain), *Cellulophaga* (7), *Gramella* (6), *Kriegella* (1), *Lutibacter* (15), *Maribacter* (1), *Mesonia* (1), *Muricauda* (2), *Saligentibacter* (1), *Sediminicola* (1), *Tenacibaculum* (2), *Winogradskyella* (1), and *Zobellia* (9). Thus, selective isolation of certain genera was not observed by the application of kanamycin.

Biogeography and culturability

The genera *Cellulophaga*, *Maribacter*, *Gramella*, *Arenibacter*, *Lutibacter*, *Zunongwangia*, *Olleya*, *Zobellia*, and *Muricauda* were isolated frequently, with more than ten strains per species. *Zunongwangia profunda* (15 strains) and *Gramella echinicola* (13 strains) were exclusively isolated from one sampling site, in this case, from the porewater 1.5 m below the surface of West Beach, Sylt. Strains

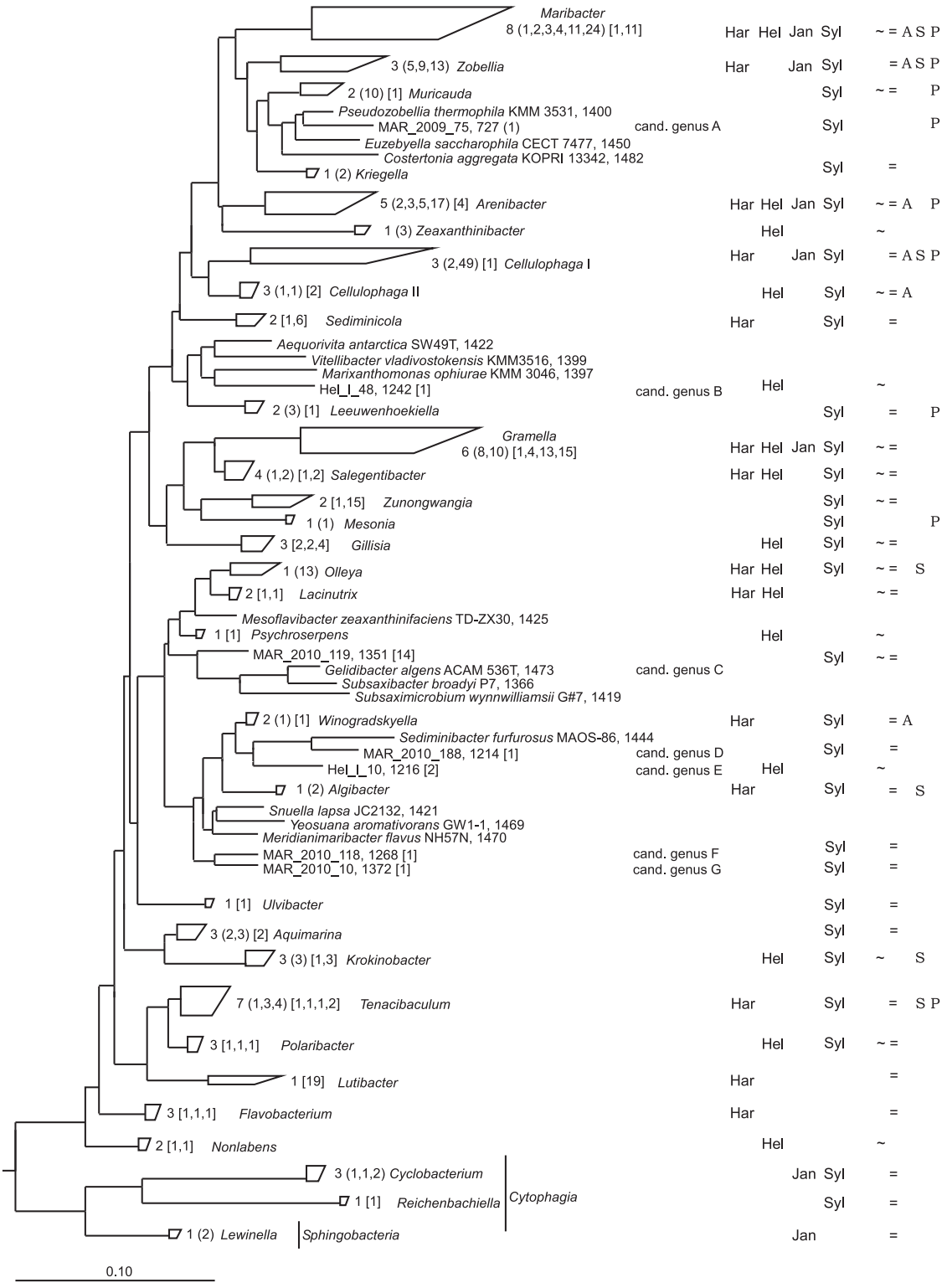

Fig. 1. Neighbour-joining tree of *Flavobacteria*, based on nearly-complete 16S rRNA gene sequences (>1100 bp) with parsimony addition of partial 16S rRNA gene sequences (<1100 bp). Isolated strains originated from Harlesiel (Har), Helgoland (Hel), Janssand (Jan) or Sylt (Syl) and from seawater (~), sediment (=), surfaces of animals (A), seaweed (S) or phytoplankton (P). The number preceding the bracket indicates the total number of species in the genus represented by isolated strains. The numbers in the round and square brackets indicate the number of strains affiliated to each species in the branch, separated by a comma. Square brackets indicate strains first identified by the *Flavobacteria*-*Cytophagia*-specific PCR. The scale bar represents 10 nucleotide substitutions per 100 nucleotides.

affiliating with *Lutibacter litoralis* (19 strains) or *Maribacter stanieri* (11 strains) were only isolated from the sediment of Harlesiel or the seawater of Helgoland, respectively. All other strains affiliating with one species – as defined by the 16S rRNA gene sequence identity – were isolated from two or more sampling sites and types. This reflected either the low resolution of the 98.7% 16S rRNA gene sequence identity used as a boundary or the lack of a biogeography for many *Flavobacteria* in the German Bight.

In this study, the culturability of *Flavobacteria* from the North Sea on solid media was approximately one colony forming

Table 1
Colony formation (CFU 1 mL^{-1} seawater) of a Helgoland spring seawater sample (April 4th, 2010) on HaHa medium incubated at 11 °C.

Time (days)	15	20	23	34	38	44	52	60	100	110	300
Total CFU	227	533	1020	1100	1160	1213	1267	1320	1347	1387	1387
Pigmented CFU	167	333	507	547	567	573	587	587	587	587	587
Flavobacteria CFU	60	160	260	267	267	267	273	273	273	273	273

unit per thousand flavobacterial cells, and strains of 26 known *Flavobacteriaceae* genera were cultivated. Previous studies had isolated strains of seven genera, *Flavobacterium*, *Gillisia*, *Krokinobacter*, *Nonlabens*, *Polaribacter*, *Tenacibaculum* and *Winogradskyella* [14,36,40,46,50,51], and corresponding 16S rRNA gene sequences were detected in cultivation-independent studies [3,41,44,49,58]. Furthermore, strains of the genera *Algibacter*, *Aquimarina*, *Arenibacter*, *Cellulophaga*, *Gramella*, *Leeuwenhoekiella*, *Maribacter* and *Zobellia* had been cultivated, but were not present in cultivation-independent studies [5,14,21,36,43,46,47,51]. The 16S rRNA gene sequences of *Marixanthomonas* and *Psychroserpens* were only found in cultivation-independent studies [8,33]. These observations showed that the diversity of culturable *Flavobacteria* from the North Sea has increased and, in contrast to previous reports [14,46], a wide range of *Flavobacteria* was shown to grow well on solid agar media, although many important taxa still await cultivation.

Physiological and chemotaxonomic observations

The known types of gliding motility were observed: (I) spreading as a thin film or as waves (*Cellulophaga*, *Tenacibaculum*), (II) an outwards push even around the colony (*Leeuwenhoekiella*, *Polaribacter*, *Zobellia*) or (III) a flame-like pattern (*Aquimarina*, *Krokinobacter*, *Pseudozobellia*, *Zobellia*), and (IV) a rhizoid spreading along the streaking (*Algibacter*, *Gramella*, *Maribacter*, *Zeaxanthinibacter*). For *Krokinobacter* spp., movement by gliding was not determined, but putative gliding-related proteins were encoded in the genome of *Krokinobacter* sp. 4H-3-7-5 [30]. Strain SRO_11 that affiliated with *Krokinobacter eikastus* glided on marine agar 2216.

Iridescence [13] was briefly described among *Flavobacteria* [27], but intensively for *Cellulophaga* [26]. In this study, iridescence was observed in strains affiliated with the genera *Cellulophaga*, *Algibacter* and *Maribacter*. Flexirubin-type pigments [16] were detected in coherence with the species description in strains affiliating with *Aquimarina*, *Kriegella*, and *Zobellia*. Unexpectedly, strains MGE_SAT_544_1 and MAR_2010_101 among 31 strains of *Arenibacter* showed the bathochromic shift after KOH treatment. In contrast, the *Ulvibacter* strain MAR_2010_11 had no flexirubin-type pigment. Besides these exceptions, pigmentation, iridescence, gliding motility, agar lysis, and flexirubin as a chemical marker supported the taxonomy at the species level.

Maribacter

Fifty-seven strains were assigned to eight species within *Maribacter*, including *M. stanieri*, *Maribacter dokdonensis*, *Maribacter sedimenticola*, and *M. forsetii* [4] – a species previously isolated from Helgoland – and four novel species. Two strains of *M. dokdonensis* showed a glitter-like iridescence on the surface of the colony and gliding that followed the streaking in a rhizoid spreading pattern. None of the strains produced flexirubin-like pigments.

Zobellia, candidate genus A, Muricauda and Kriegella

A total of 28 strains were isolated that affiliated with three *Zobellia* species, *Zobellia uliginosa*, *Zobellia amurskyensis* and *Zobellia russellii*. All strains were positive for flexirubin-type pigments. Three strains of the species Z. *russellii* and one strain of Z. *uliginosa* lysed agar. Seven strains of all three species showed gliding motility in a flame-like pattern or even around the colony (Fig. 3). Ten strains affiliated with *M. ruestringensis* were flexirubin-type pigment negative and did not glide or lyse agar, except for strain MAR_2009_54. Both strains of *Kriegella* were flexirubin-type pigment positive, but showed no gliding. Strain MAR_2009_75 had a 16S rRNA gene sequence identity of 94.7% to the closest relative *Pseudozobellia thermophile*, thus representing the candidate genus A. Iridescence, agar lysis and flexirubin-type pigments were not observed. This strain was isolated from phytoplankton of Sylt and showed a flame-like gliding pattern on agar (Fig. 3).

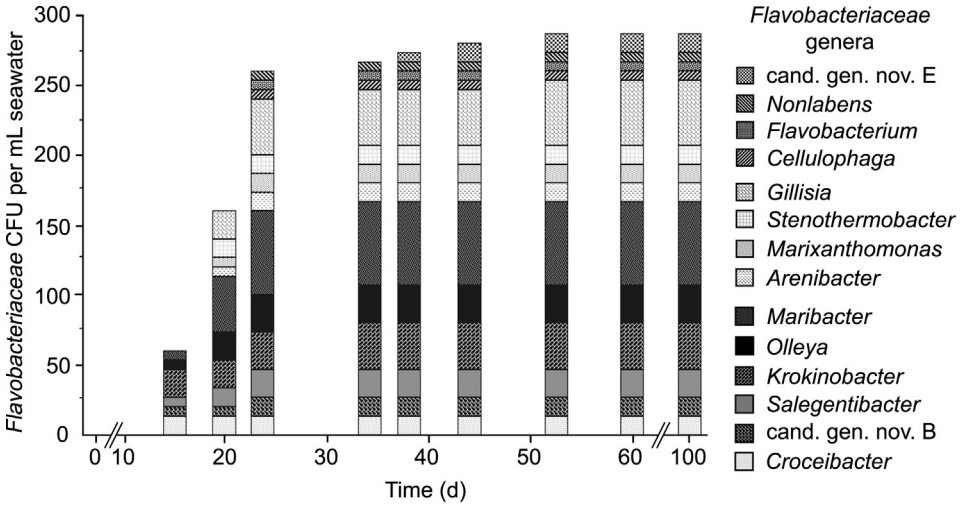

Fig. 2. Colony formation (CFU per mL seawater) of strains affiliating with *Flavobacteriaceae* genera. The seawater of Helgoland Roads on April 4th, 2010 was incubated on HaHa medium at 11 °C for 300 days.

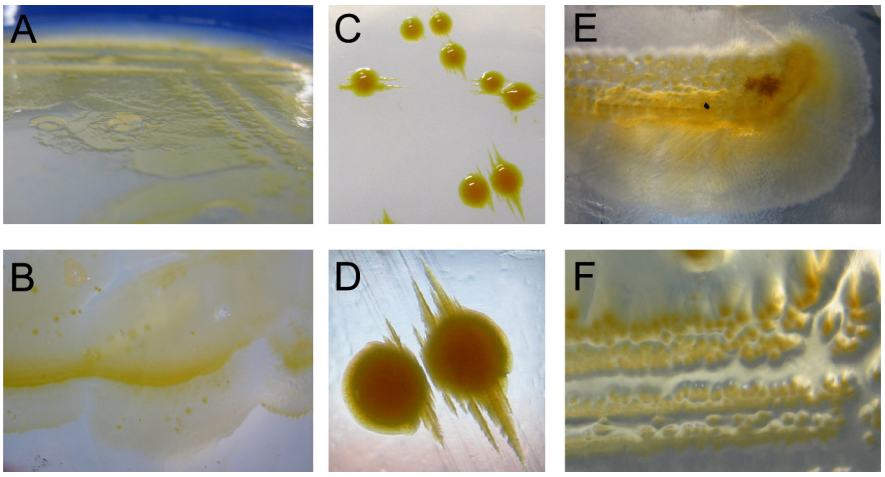

Fig. 3. Gliding modes among *Flavobacteriaceae* strains isolated from North Sea samples. Cultures of *Cellulophaga* produced a thin film on the agar surface (A, *Cellulophaga* sp. RHA_28) or biofilm waves (B, *Cellulophaga* sp. MAR_2009_44). Cultures of *Gramella* glided a little away from the colony along the streak (C,D, *Gramella* sp. MAR_2010_21). Cultures of *Zobellia* pushed outwards from around the colony (E, *Zobellia* sp. MAR_2009_186) or in a flame-like pattern (F, *Zobellia* sp. RHA_40) to new areas of the medium.

Arenibacter and Zeaxanthinibacter

A total of 31 strains were assigned to *Arenibacter*, including *Arenibacter troitsensis*, *Arenibacter palladensis*, *Arenibacter echinorum* and two novel *Arenibacter* spp.. Gliding, agar lysis and iridescence were not observed. Among the 31 strains, strains MAR_2010_101 and MGE_SAT_544_1 were flexirubin-type pigment positive. Three strains affiliating with *Zeaxanthinibacter enoshimensis* did not show iridescence, flexirubin-type pigments or agar lysis, but glided along the streaking (Fig. 3).

Cellulophaga

A total of 56 strains of *Cellulophaga* were isolated from sediment, seawater, biofilm, seaweed and animals of all sampling sites, and formed a monophyletic branch of two distinct subgroups in the 16S rRNA gene tree, as described by Bernardet [6]. In the first subgroup, 52 strains were affiliated with *Cellulophaga lytica* or represented two novel *Cellulophaga* spp.. In the second subgroup, four strains were affiliated with *Cellulophaga baltica*, *Cellulophaga pacifica* or represented another novel *Cellulophaga* sp.. Gliding movement was observed for *C. lytica* and *Cellulophaga* sp. nov. I strains, with a faster spreading on the agar plate than colony formation, resulting in a thin film on the surface of the plate (Fig. 3). These cultures were isolated by serial dilution in artificial seawater followed by homogenous distribution on an agar plate. Twelve strains of *C. lytica* and strain RHA_19 showed a glitter-like iridescence.

Sediminicola, candidate genus B, and Leeuwenhoekiella

A total of seven strains isolated from sediment were assigned to *Sediminicola luteus* and a novel *Sediminicola* sp.. No flexirubin-type pigments, gliding, agar lysis or iridescence were observed. Four strains of two novel species of *Leeuwenhoekiella* showed no flexirubin-type pigments, gliding, agar lysis or iridescence, but strain SRO_3 exhibited gliding motility. One strain isolated from the seawater of Helgoland only had a 90.4% 16S rRNA gene sequence identity, and its closest relative was *Marixanthomonas ophiurae* which represented candidate genus B. Agar lysis, flexirubin-type pigments or iridescence were not observed, but gliding cells were observed around colonies.

Gramella

A total of 32 strains were isolated from sediment that affiliated with *G. echinicola*, *Gramella gaetbulicola*, *Gramella marina* and a novel species of *Gramella*. Nineteen strains isolated from seawater and sediment affiliated with *Gramella portivictoriae* and '*G. forsetii*' [5]. Gliding was observed for three strains which moved from the colony along the streak (Fig. 3). Iridescence, agar lysis and flexirubin-type pigments were not observed.

Salegentibacter, Zunongwangia, Mesonia, and Gillisia

A total of seven strains were assigned to *Salegentibacter mishustinae*, *Salegentibacter salarius*, and two novel *Salegentibacter* spp.. Sixteen strains with a cell size of less than 1.5 μm and a faint yellow colony colour were assigned to *Z. profunda* and one novel *Zunongwangia* sp.. *Mesonia algae* was represented by one strain. In *Gillisia*, eight strains were affiliated to *Gillisia mitskevichiae*, *Gillisia myxillae*, or depicted a novel species of *Gillisia*. The strains of *Salegentibacter*, *Zunongwangia*, *Mesonia*, and *Gillisia* were negative for flexirubin-type pigments, iridescence, agar lysis and gliding.

Olleya, Lacinutrix, Psychroserpens and candidate genus C

Thirteen strains represented a novel species of *Olleya*, two strains were assigned to *Lacinutrix copepodicola* and *Lacinutrix* sp. nov., and one strain to a novel *Psychroserpens* sp.. Thirteen strains isolated from the porewater 1.5 m below the sand surface at the driftline of Sylt West Beach represented the candidate genus C, with a 16S rRNA gene sequence identity of 94.0% with *Gelidibacter algens.* The strains of *Olleya*, *Lacinutrix*, *Psychroserpens*, and the candidate genus C were negative for flexirubin-type pigments, iridescence, agar lysis and gliding.

Winogradskyella, Algibacter and candidate genera D, E, F, and G

A total of four strains represented two novel *Winogradskyella* spp. and two novel *Algibacter* spp.. Three strains were assigned to the candidate genera D and E, with a 16S rRNA gene sequence identity of 94.0% and 91.4% to the next relative *Sediminibacter furfurosus*, respectively. Two strains represented the candidate genera F and G, with the next relative being *Meridianimaribacter flavus* with a 16S rRNA gene sequence identity of 94.7% and 95.0%, respectively. Gliding, agar lysis, iridescence and flexirubin-type pigments were not observed for strains of *Winogradskyella* and the candidate genera D, E, F, and G. For *Algibacter* strains, iridescence and gliding along the streaking (Fig. 3) were observed.

Ulvibacter, Aquimarina and Krokinobacter

One strain represented a novel species of *Ulvibacter* but it did not produce flexirubin-type pigments, in contrast to the current description of the genus *Ulvibacter*. Seven strains with cells of >10 μm in length grouped into three species of *Aquimarina*, *Aquimarina macrocephali* and two novel *Aquimarina* spp.. The flexirubin test was positive for strains of two novel species of *Aquimarina*, but not for strains of *A. macrocephali*. Gliding motility was observed for strains of *A. macrocephali* and *Aquimarina* sp. nov. I, isolated from sediment, but not for strains of *Aquimarina* sp. nov. II, isolated from seawater. Seven strains affiliated with *Krokinobacter*, including *K. eikastus* and two novel *Krokinobacter* spp.. Strain SRO_199 performed flame-like gliding and agar lysis was observed for strain SRO_18.

Tenacibaculum, Polaribacter and Lutibacter

A total of 13 strains were isolated from seawater, sediment, phytoplankton and algae affiliating with *Tenacibaculum gallaicum* and *Tenacibaculum litoreum*, or they represented five novel species of *Tenacibaculum*. Strains of *T. gallaicum* and *Tenacibaculum* sp. nov. II performed gliding with faster gliding than colony formation, resulting in a thin film on the agar plate (Fig. 3). Three novel species of *Polaribacter* were isolated and strain Hel1_85 performed gliding, even around the colony. Nineteen strains from the sediment of Harlesiel affiliated with *L. litoralis*. In contrast to the cell size of less than 1.5 μm for *Polaribacter* and *Lutibacter* strains, the *Tenacibaculum* strains formed filaments of more than 100 μm in length.

Flavobacterium and Nonlabens

Three strains were isolated from sediment that affiliated with three species of *Flavobacterium*, *Flavobacterium gelidilacus*, and two *Flavobacterium* sp. nov.. Two strains isolated from seawater of Helgoland represented novel species of the genus *Nonlabens*. Gliding, iridescence, agar lysis and flexirubin-type pigments were not observed.

Cytophagia and Sphingobacteria

An orange to brown colony colour and rod-shaped cells characterized non-motile strains affiliating to *Reichenbachiella* (family *Flammeovirgaceae*), *Lewinella* (family *Saprospiraceae*) and *Cyclobacterium* (family *Cyclobacteriaceae*).

Conclusion and future perspectives

In this study, cultivation of marine *Flavobacteriaceae* was demonstrated on agar plates from diverse habitats. A broad phylogenetic diversity was obtained by different cultivation approaches for pelagic and benthic *Flavobacteria*, a *Flavobacteria-Cytophagia* specific PCR, and a suitable medium. This collection of *Flavobacteriaceae* from the German Bight of the North Sea provided model organisms of marine aerobic heterotrophic bacteria and will give access to a variety of carbohydrate active enzymes [10,49].

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2013.06.006.

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