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

Richard L. Hahnke, Jens Harder*

Department of Microbiology, Max Planck Institute for Marine Microbiology, Celsiusstr. 1, D-28359 Bremen, Germany

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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].

* Corresponding author. Tel.: +49 4212028750.
E-mail address: jharder@mpi-bremen.de (J. Harder).

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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