ORIGINAL ARTICLE

Niches of two polysaccharide-degrading *Polaribacter* isolates from the North Sea during a spring diatom bloom

Peng Xing[1,2,8], Richard L Hahnke[1,8,9], Frank Unfried[1,3], Stephanie Markert[3], Sixing Huang[1,9], Tristan Barbeyron[4], Jens Harder[1], Dörte Becher[5], Thomas Schweder[3,6], Frank Oliver Glöckner[1,7], Rudolf I Amann[1] and Hanno Teeling[1]

[1]*Department of Molecular Ecology, Max Planck Institute for Marine Microbiology, Bremen, Germany;* [2]*State Key Laboratory of Lake Science and Environment, Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences, Nanjing, People's Republic of China;* [3]*Institute of Marine Biotechnology e.V., Greifswald, Germany;* [4]*UPMC University Paris 6 and CNRS, UMR 7139 Marine Plants and Biomolecules, Station Biologique de Roscoff, Roscoff, Bretagne, France;* [5]*Institute of Microbiology, Ernst-Moritz-Arndt-University, Greifswald, Germany;* [6]*Pharmaceutical Biotechnology, Ernst-Moritz-Arndt-University, Greifswald, Germany and* [7]*Jacobs University Bremen gGmbH, Bremen, Germany*

Members of the flavobacterial genus *Polaribacter* thrive in response to North Sea spring phytoplankton blooms. We analyzed two respective *Polaribacter* species by whole genome sequencing, comparative genomics, substrate tests and proteomics. Both can degrade algal polysaccharides but occupy distinct niches. The liquid culture isolate *Polaribacter* sp. strain Hel1_33_49 has a 3.0-Mbp genome with an overall peptidase:CAZyme ratio of 1.37, four putative polysaccharide utilization loci (PULs) and features proteorhodopsin, whereas the agar plate isolate *Polaribacter* sp. strain Hel1_85 has a 3.9-Mbp genome with an even peptidase:CAZyme ratio, eight PULs, a mannitol dehydrogenase for decomposing algal mannitol-capped polysaccharides but no proteorhodopsin. Unlike other sequenced *Polaribacter* species, both isolates have larger sulfatase-rich PULs, supporting earlier assumptions that *Polaribacter* take part in the decomposition of sulfated polysaccharides. Both strains grow on algal laminarin and the sulfated polysaccharide chondroitin sulfate. For strain Hel1_33_49, we identified by proteomics (i) a laminarin-induced PUL, (ii) chondroitin sulfate-induced CAZymes and (iii) a chondroitin-induced operon that likely enables chondroitin sulfate recognition. These and other data suggest that strain Hel1_33_49 is a planktonic flavobacterium feeding on proteins and a small subset of algal polysaccharides, while the more versatile strain Hel1_85 can decompose a broader spectrum of polysaccharides and likely associates with algae.
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Introduction

The genus *Polaribacter* belongs to the family *Flavobacteriaceae* of the phylum *Bacteroidetes*. To date, 12 *Polaribacter* species have been described with validly published names, *P. atrinae* (Hyun *et al.*, 2014), *P. butkevichii, P. dokdonensis, P. filamentus, P. franzmannii, P. glomeratus,* *P. irgensii, P. gangjinensis, P. porphyrae, P. reichenbachii, P. sejongensis* and *P. huanghezhanensis* (Bernardet, 2010; Parte, 2014). The first described *Polaribacter* species were psychrophilic isolates from Arctic and Antarctic sea ice (Gosink *et al.*, 1998), but ever since, *Polaribacter* have also been identified in temperate marine habitats, both in seawater (Nedashkovskaya *et al.*, 2005; Yoon *et al.*, 2006; Fukui *et al.*, 2013) and sediments (Li *et al.*, 2014). Based on *in situ* detection and 16S rRNA gene sequence analysis, it has been suggested that the *Polaribacter* genus consists of cold- and warm-water-adapted phylotypes (Gómez-Pereira *et al.*, 2010; Díez-Vives *et al.*, 2013). *Polaribacter* species have also been isolated from red (Fukui *et al.*, 2013) and green macroalgae (Nedashkovskaya *et al.*, 2013) and were found to thrive during diatom-dominated phytoplankton blooms in the North Sea (Teeling *et al.*, 2012) and

the Antarctic Southern Ocean (Williams *et al.*, 2013). Association of *Polaribacter* spp. with phytoplankton was also observed in the open North Atlantic (Gómez-Pereira *et al.*, 2012) and the coastal North Sea (Bennke *et al.*, 2013) and has been inferred in a recent study on an Antarctic polynya (Kim *et al.*, 2013). Because of their presence in cold and temperate waters, in oligotrophic and copiotrophic nutrient regimes within open ocean and coastal zones, members of the genus *Polaribacter* have recently been recognized as 'widely distributed in marine habitats' (Nedashkovskaya *et al.*, 2013).

Marine *Bacteroidetes*, in general, and marine *Flavobacteriia*, in particular, have been ascribed to act as degraders of biopolymers, such as proteins and polysaccharides (for example, Thomas *et al.*, 2011). This view is supported by high *Flavobacteriia* abundances in nutrient-rich habitats, such as aggregates of particulate organic matter (Williams *et al.*, 2013) or in the microbiota of marine algae and invertebrates (Di Camillo *et al.*, 2012; Dong *et al.*, 2012). Also, so far sequenced genomes of marine *Flavobacteriia* have revealed clear adaptations towards biopolymer degradation (for example, Bauer *et al.*, 2006; González *et al.*, 2008; Woyke *et al.*, 2009; Mann *et al.*, 2013), as they feature high proportions of peptidase and glycoside hydrolase (GH) genes. Fernández-Gómez *et al.* (2013) have shown that marine *Bacteroidetes* tend to have higher numbers of peptidase than GH genes, whereas the number in non-marine *Bacteroidetes* is about even. However, depending on lifestyle, there is considerable variation in the gene numbers for peptidases and carbohydrate-active enzymes (CAZymes; Lombard *et al.*, 2014). Planktonic *Flavobacteriia* such as *Polaribacter* sp. MED152 and *Dokdonia donghaensis* MED134 are subjected to rare feast and extended famine conditions. Both strains feature relatively small genomes of around 3.0 Mbp, with almost twice as many peptidases than CAZymes, and can gain additional energy via the light-dependent proton-pumping membrane protein proteorhodopsin, as has been inferred for *Polaribacter* sp. MED152 (González *et al.*, 2008) and verified for *Dokdonia donghaensis* MED134 (González *et al.*, 2011). In contrast, macroalgae-colonizing *Flavobacteriia* such as *Zobellia galactanivorans* Dsij[T] (Barbeyron *et al.*, 2001) and *Formosa agariphila* KMM 3901[T] (Nedashkovskaya *et al.*, 2006) live under more stable conditions with ample nutrient supply. Both feature much larger genomes of 5.5 and 4.2 Mbp, respectively, and feature more CAZymes than peptidases but are notably devoid of proteorhodopsin.

Pure culture studies on *Polaribacter* strains have demonstrated growth on biopolymers, such as aesculin, casein, starch and gelatin (Gosink *et al.*, 1998; Nedashkovskaya *et al.*, 2005; Yoon *et al.*, 2006; Lee *et al.*, 2011; Fukui *et al.*, 2013; Kim *et al.*, 2013; Nedashkovskaya *et al.*, 2013). Growth on algal polysaccharides has not yet been investigated in detail, but *Polaribacter* co-occurrences with phytoplankton and metagenome data support this capability (Gómez-Pereira *et al.*, 2012; Teeling *et al.*, 2012).

So far, limited genomic data are available for members of the *Polaribacter* genus. A complete genome is present only for *Polaribacter* sp. MED152 (3.0 Mbp; González *et al.*, 2008), an isolate from northwestern Mediterranean surface water. Furthermore, draft genomes are available for two isolates from Antarctic sea ice, *P. irgensii* 23-P[T] (~2.8 Mbp; two scaffolds, seven contigs) and *P. franzmannii* 301[T] (~6.9 Mbp; 56 scaffolds, 69 contigs).

In 2010, two novel *Polaribacter* species were isolated from surface seawater nearshore the North Sea island Helgoland during a diatom-dominated spring phytoplankton bloom (Hahnke and Harder, 2013; Hahnke *et al.*, 2014). Strain Hel1_85 (formerly Hel_I_85) was isolated from agar plates and strain Hel1_33_49 in liquid culture. Strain Hel1_33_49 did not grow on agar plates, indicating distinct lifestyles of both strains.

In this study, we report almost complete genomes of these *Polaribacter* strains. Annotations were combined with growth experiments on various substrates, including a suite of different carbohydrates and polysaccharides, to elucidate the metabolic potential and associated niches of both strains. These experiments were complemented by comparative genomics with 25 additional *Flavobacteriaceae* genomes and by proteomics of Hel1_33_49 cultures grown on the non-substituted algal polysaccharide laminarin as well as the sulfated polysaccharide chondroitin sulfate.

Material and methods

Substrate tests
Various substrates (2.0 g l^{-1}; Supplementary Table S1) were tested at 12 °C in marine mineral medium (10 ml) as described previously (Mann *et al.*, 2013), including kappa- and iota-carrageenan, amorphous cellulose, glycogen, laminarin and xanthan. Volumes of 100 μl of either *Polaribacter* sp. Hel1_33_49 or Hel1_85 stock culture were added as inocula. Optical density measurements (660 nm) were taken for 2 weeks for monosaccharides and disaccharides and 8 weeks for polysaccharides and compared with inoculated control cultures without saccharide supplements and non-inoculated, saccharide-supplemented media.

DNA extraction, genome sequencing and annotation
Cells were grown on HaHa_100 medium (Hahnke *et al.*, 2014) at 12 °C in the dark until the late exponential phase and subsequently pelleted by centrifugation (2500 *g*, 60 min). Wet cell pellets of 0.5 g were dissolved in 10 ml sodium phosphate buffer (pH 7.8) and incubated with 30 U α-amylase (10065, Sigma-Aldrich, Hamburg, Germany) at 37 °C for 60 min to remove glycoconjugates. Cells were subsequently centrifuged (30 000 *g*, 20 min), and

DNA was extracted according to the protocol of Zhou *et al.* (1996).

Sequencing was performed at LGC Genomics (Berlin, Germany) using the 454 GS FLX Ti platform (454 Life Sciences, Branfort, CT, USA). The Hel1_33_49 genome was assembled from 733 179 reads from a standard shotgun library and 139 390 reads from a 3-kbp mate pair library (451.7 Mbp; $148 \times$ coverage) using Newbler v2.6. The initial assembly comprised 38 contigs, 31 of which could be joined into 10 scaffolds. The remaining gaps were closed by PCR and Sanger sequencing yielding circular assemblies of 2 894 172 bp and 118 580 bp (total: 3 012 752 bp). The latter does not carry any known plasmid genes and likely constitutes a repeat-bound integral piece of the chromosome. The Hel1_85 draft genome was created the same way with 646 110 reads from a standard shotgun library and 135 989 reads of a 3-kbp mate pair library (394.9 Mbp; $100 \times$ coverage). The initial assembly comprised 83 contigs, 63 of which could be joined into 46 scaffolds. Subsequent gap closure yielded four larger contigs of 1 642 720, 1 466 007, 752 210 and 53 131 bp (total: 3 914 068 bp). The remaining gaps consist of four almost identical 16S-23S-5S rRNA operons that could not be traversed in several targeted gap closure approaches.

Gene prediction and annotation (including the phylogeny-guided CAZyme annotations provided in Supplementary Table S2) were performed as described previously (Mann *et al.*, 2013). Annotated genome sequences were submitted to NCBI's Genbank with the accession numbers JPDI00000000 (Hel1_33_49) and JPDS00000000 (Hel1_85).

Comparative analysis
We selected a total of 27 *Flavobacteriaceae* genomes from marine or aquatic habitats for comparative analysis (Supplementary Table S3). CAZymes were predicted using searches against the CAZy (Lombard *et al.*, 2014), Pfam (Finn *et al.*, 2014) and dbCAN (Yin *et al.*, 2012) databases, and peptidases were predicted using similarity searches against the MEROPS database (Rawlings *et al.*, 2014). The Pfam database was also used for the prediction of sulfatase ($\leqslant$E-13), bacteriorhodopsin ($\leqslant$E-5) and mannitol dehydrogenase ($\leqslant$E-5) frequencies.

Phylogenetic analysis
16S rRNA gene sequences of *Polaribacter* strains Hel1_33_49, Hel1_85 and all *Flavobacteriaceae* type strains of the 'The All-Species Living Tree' project (LTPs_111_SSU; Yarza *et al.*, 2010) were aligned in ARB (Ludwig *et al.*, 2004) using the SINA aligner (Pruesse *et al.*, 2012) and manually corrected according to secondary structure information. Bootstrapped maximum likelihood trees were constructed from sequences >1300 bp with and without a 40% conservation filter using RAxML

(Stamatakis and Ott, 2008) with the GTR substitution model and 1000 repetitions. Average nucleotide identity and digital DNA-DNA hybridization were calculated using JSpecies (Richter and Rosselló-Móra, 2009) and the Genome-to-Genome Distance Calculator (Auch *et al.*, 2010), respectively.

Proteomics
Polaribacter sp. Hel1_33_49 was grown at 12 °C in modified HaHa_100 medium with 0.1 g l^{-1} peptone, 0.1 g l^{-1} casamino acids, 200 μM NH$_4$Cl and 16 μM KH$_2$PO$_4$. Laminarin (L9634, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) or chondroitin 4-sulfate (27042, Sigma-Aldrich) were used as carbon source, and D-mannose served as control (concentrations: 2.0 g l^{-1}; Supplementary Figure S1). The strain was adapted by three passages into the respective medium (1% inoculum), followed by cultivation in 100 ml medium. All growth experiments were carried out in triplicates. Cells were harvested by centrifugation (15 min; 13 000 g; 4 °C), and the resulting pellets were stored at -80 °C until use.

Cell pellets were washed in TE buffer (10 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0) containing 'cOmplete Protease Inhibitor' (Roche, Berlin, Germany). Soluble proteins were extracted by sonication (2×25 s), and the protein concentration was determined as described by Bradford (1976). Fifteen micrograms of protein were loaded onto 10% polyacrylamide sodium dodecyl sulfate mini gels, separated (60 min, 150 V) and stained with Roti-Blue quick (Carl Roth, Karlsruhe, Germany). Subsequently, entire gel lanes were excised, and proteins were digested as described by Heinz *et al.* (2012).

Peptides were subjected to a reversed-phase C18 column chromatography on a nano ACQUITY-UPLC (Waters Corporation, Milford, MA, USA) and separated as described by Otto *et al.* (2010). Mass spectrometry (MS) and MS/MS data were determined using an online-coupled LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). MS spectra were searched against a target-decoy protein sequence database, including sequences of *Polaribacter* sp. Hel1_33_49 and of common laboratory contaminants. Validation of MS/MS-based peptide and protein identifications was performed with Scaffold (http://www.proteomesoftware.com) using the SEQUEST filter settings described by Heinz *et al.* (2012). Normalized spectral abundance factors (NSAFs) were calculated (Zybailov *et al.*, 2006) using Scaffold's 'exclusive spectrum count' for each protein, which allows to compare relative protein abundances between individual samples.

Results and Discussion

Phylogeny
With three rRNA operons in Hel1_33_49 and four in Hel1_85, both genomes feature about one rRNA

operon per megabase pair (Supplementary Table S4). The Hel1_33_49 16S rRNA gene sequence was nearly identical to previously obtained 16S rRNA gene sequences from Helgoland bacterioplankton (99.8% identity), whereas the Hel1_85 16S rRNA sequence was distinct and exhibited a weaker similarity to a sequence from the North Atlantic (97.8% identity) (Figure 1). Both strains have a 16S rRNA identity of 98.3% and an average nucleotide identity of 80% (Supplementary Table S5). They constitute distinct species that are closer related to each other than to the sequenced *Polaribacter* strains MED152 and 23-P^T.

Degradation of biopolymers

GH and polysaccharide utilization loci (PUL) gene repertoires. CAZymes and, in particular, GHs are centrally important for polysaccharide decomposition. A comparison of the GH repertoires of strains Hel1_33_49 and Hel1_85 with 25 other sequenced marine *Flavobacteriaceae* comprising 133 GH families (Supplementary Table S3) revealed GH13, GH2, GH16, GH3, GH43 and GH29 as the families with the highest mean abundances (Supplementary Figure S2). No significant differences in GH abundances were detected between *Polaribacter* and other *Flavobacteriaceae* members (Supplementary Figure S3).

Polysaccharide degradation and uptake genes in *Bacteroidetes* are often co-located in operon-like PULs (Sonnenburg *et al.*, 2010). PULs comprise a characteristic gene tandem coding for a SusD-like substrate-binding protein and a TonB-dependent receptor (TBDR) together with CAZyme genes and often contain genes for transport, transcriptional regulation, peptidases and sulfatases. Strains Hel1_33_49 and Hel1_85 contain 5 and 11 SusD/TBDR gene pairs, respectively, 4 and 7 of which are part of putative PULs (Supplementary Figures S4 and S5). A single PUL in Hel1_33_49 and three in Hel1_85 contain sulfatase genes (Supplementary Figures S4C and S5E, G and H). These PULs constitute the largest in both strains with up to 49 genes.

Both strains contain genes for β-galactosidases (Hel1_33_49: 1; Hel1_85: 3) and α-glucosidases (Hel1_33_49: 1; Hel1_85: 4). Such genes are typically induced by dissolved polymeric glucose. In addition, strain Hel1_85 can likely degrade xylan as it does grow on xylose and features four β-xylosidase genes, three D-xylose proton-symporters (*xylE*) and one hexuronate transporter. In contrast, strain Hel1_33_49 is notably devoid of these genes and does not grow on xylose. Furthermore, five putative PL7 alginate lyase genes were identified in strain Hel1_85, three in a single PUL (Supplementary Figure S5C). The polysaccharide alginate is found in brown alga cell walls, and alginate lyase excreting *Polaribacter* have been described before as abundant members of epiphytic bacterial communities on *Laminaria* sp. brown alga (Dong *et al.*, 2012).

The storage polysaccharide of brown algae is laminarin, a β-glucan of β-1,3-linked glucose residues with occasional β-1,6-linked glucose side chains. These relatively short polysaccharides of ~25 residues are terminated by 1-linked D-mannitol at the reducing end (for example, Read *et al.*, 1996). Strain Hel1_85 contains a mannitol 2-dehydrogenase gene, which is absent from strain Hel1_33_49. Mannitol 2-dehydrogenase allows conversion of D-mannitol from mannitol-capped laminarin to D-fructose 6-phosphate that can subsequently be funneled into the Embden–Meyerhof–Parnas
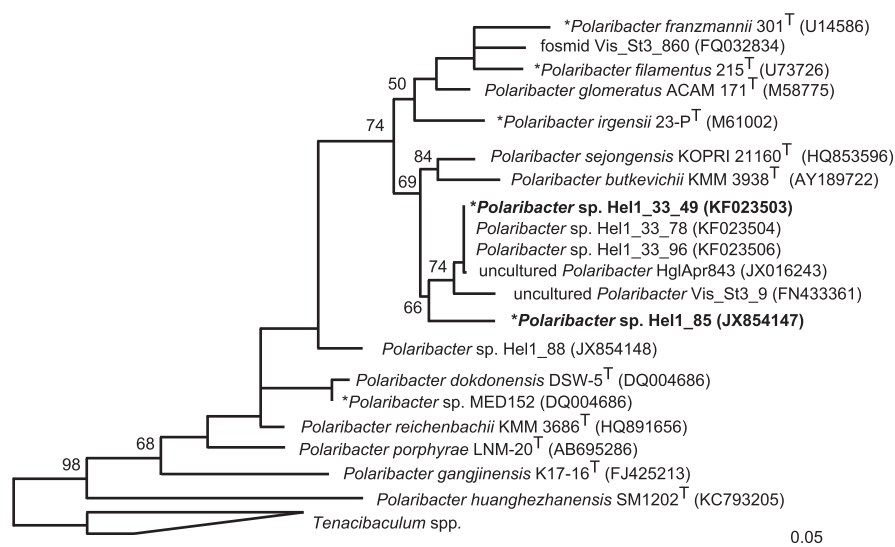
Figure 1 Maximum likelihood tree based on *Polaribacter* spp. 16S rRNA gene sequences. Strains Hel1_33_49 and Hel1_85 are depicted in bold. Bootstrap values are based on 1000 sub-samplings. *Polaribacter* species with sequenced genomes are marked by an asterisk (*). Outgroup: *Tenacibaculum* spp. Bar: 0.05 substitutions per nucleotide position.

pathway. Presence of this gene in strain Hel1_85 hence supports possible association with brown algae. Diatoms in contrast feature chrysolaminarin as main storage polysaccharides (Beattie *et al.*, 1961). Chrysolaminarin has a similar structure than laminarin, but reducing ends are not capped by mannitol residues.

Carbohydrate and polysaccharide utilization. Growth studies using a suite of different carbohydrate and polysaccharide substrates revealed that strains Hel1_33_49 and Hel1_85 both grew on D-mannose and D-maltose, but differed in the utilization of other tested monosaccharides, disaccharides and trisaccharides. As predicted, strain Hel1_85 grew on mannitol, and strain Hel1_33_49 did not. Neither strain could use D-trehalose, L-rhamnose and *N*-acetyl-D-glucosamine as is also true for their closest type strains (Supplementary Table S1). Both strains were able to grow on the polysaccharides cellulose, carboxymethyl cellulose, laminarin, agar, xanthan, kappa- and iota-carrageenan, glycogen and chondroitin sulfate. A surprising observation was that cells of both *Polaribacter* isolates increased in lengths from approximately 0.8 to 3–4 μm during growth on laminarin or chondroitin sulfate. Laminarin and chondroitin sulfate are readily metabolized by marine microbial communities (Arnosti, 2008; Arnosti *et al.*, 2011) and were thus selected for further tests with strain Hel1_33_49.

Decomposition of laminarin. Both strains Hel1_33_49 and Hel1_85 grow on laminarin and contain a putative laminarin-specific PUL (Supplementary Figures S4D and S5B) with GH16 (Labourel *et al.*, 2014), GH17 and one putative GH30 family β-1,6-glucanase but lack GH55 family β-1,3-glucanases (Ishida *et al.*, 2009).

Proteomic analysis of strain Hel1_33_49 on laminarin supplemented medium revealed a significant upregulation of the respective PUL vs D-mannose-supplied controls (Figure 2a, Supplementary Table S6). Syntenic PULs were also found in other *Flavobacteriaceae* (Figure 2b). Induction of this PUL by laminarin was recently demonstrated in a detailed proteomics approach for 'Gramella forsetii' KT0803 (Kabisch *et al.*, 2014), which has been isolated earlier from the same North Sea sampling site as strain Hel1_33_49 (Eilers *et al.*, 2001).

Decomposition of chondroitin sulfate. Previous work indicates that members of *Polaribacter* are able to degrade sulfated algal polysaccharides. First, Gómez-Pereira *et al.* (2012) obtained a fosmid (S3_860) from the North Atlantic Ocean that harbored a 16S rRNA gene affiliating with *Polaribacter* together with a partial PUL with five sulfatase genes. Additionally, *Polaribacter* sulfatase expression was found to increase during a diatom-dominated phytoplankton bloom in the North Sea (Teeling *et al.*, 2012). However, to date sequenced

Polaribacter genomes contain only two to three sulfatase genes (Supplementary Table S4). In contrast, strains Hel1_33_49 and Hel1_85 harbor 12 and 33 sulfatases, respectively, with as many as 9 (Supplementary Figure S4C) and 8 (Supplementary Figure S5G) sulfatases in a single PUL. Strain Hel1_85 furthermore features a PUL-like gene cluster without a *susD*-like gene in which 14 sulfatases are co-located with GHs (Supplementary Figure S5D). Large numbers of diverse GH genes in these sulfatase-rich PULs indicate that they target more than one type or rather heterogeneous sulfated polysaccharides. This is supported by growth of both Helgoland strains on sulfated polysaccharides, such as kappa- and iota-carrageenan, agar and xanthan (Supplementary Table S1).

The sole sulfatase-containing PUL of the North Sea isolate Hel1_33_49 (Supplementary Figure 3C) exhibits a strong synteny with the partial PUL from the Atlantic fosmid S3_860 (Figure 3). In both PULs, three GH92 α-1,2-mannosidases are clustered with five sulfatases. A recent study on the human gut microbe *Bacteroides thetaiotaomicron* indicated that its arsenal of family GH92 exo-acting enzymes decomposes α-mannoside-containing *N*-glycans and glycoproteins from land plant cell walls (Zhu *et al.*, 2010). It is possible that *Polaribacter* sp. Hel1_33_49 can utilize similarly structured but sulfated polysaccharides from marine algae. Furthermore, the presence of such highly similar PULs in distinct *Polaribacter* species from the North Sea and Atlantic Ocean (97.6% 16S rRNA sequence identity) indicates that this PUL is widespread and functionally important in marine systems.

Chondroitin sulfate is a heterogeneous polysaccharide composed of alternating *N*-acetylgalactosamine and glucuronic acid that carries sulfate moieties at various positions. It is not a common substrate in surface seawater, but it is readily metabolized by microbial communities and might mimic polysaccharides of similar composition, such as transparent exopolymers of diatoms that are known to contain high proportions of glucuronic acids and sulfated monosaccharides (for example, Zhang *et al.*, 2008). It has been shown that *Polaribacter* abundance of up to 7% of the bacterioplankton (Gómez-Pereira *et al.*, 2010) coincided with peaking potential for chondroitin sulfate decomposition in the North Atlantic Ocean (Arnosti *et al.*, 2012). However, the so far sequenced *Polaribacter* genomes do not contain any known chondroitinase genes. Therefore, chondroitin sulfate decomposition in *Polaribacter* might not be mediated by a specific PUL but rather initiated by distinct sulfatases that desulfate chondroitin sulfate to a glycosaminoglycan composed of *N*-acetylgalactosamine and glucuronic acid, which is subsequently decomposed.

Proteomic analyses (Supplementary Table S6) of chondroitin sulfate-grown Hel1_33_49 cultures revealed differential expression of one sulfotransferase,
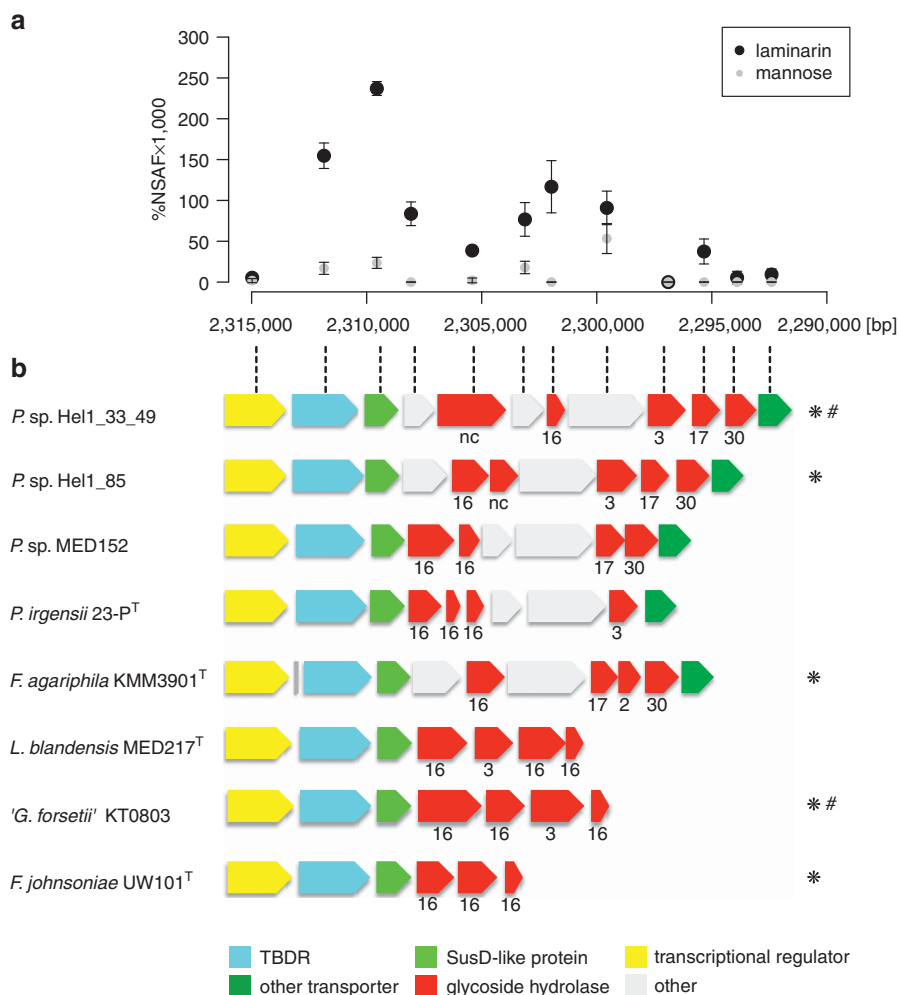
Figure 2 Laminarin-induced locus of *Polaribacter* sp. Hel1_33_49: (**a**) Differential expression of the genes in laminarin- vs mannose-supplied cells. Ordinate: NSAFs; abscissa: position in the Hel1_33_49 genome (large contig; Supplementary Figure S4D). (**b**) Gene arrangement of the locus as well as similar loci in other *Flavobacteriaceae*, including *Polaribacter* sp. Hel1_85 (see Supplementary Figure S5B); numbers indicate GH families (nc = not classified); species that have been shown to use laminarin in cultivation experiments are marked by an asterisk (*); species where laminarin usage is supported by proteomics are marked by a hash (#).
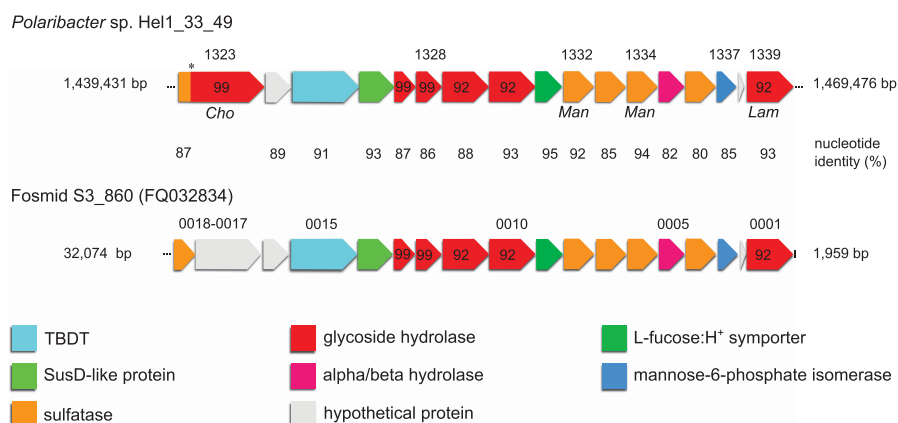

Figure 3 Synteny between a PUL in *Polaribacter* sp. strain Hel1_33_49 (Supplementary Figure S4C) and a partial PUL on fosmid S3_860 from the Atlantic Ocean identified by Gómez-Pereira *et al.* (2012). *Cho*, *Man* and *Lam* indicate proteins that were only detected in chondroitin sulfate-, mannose- and laminarin-supplied cells, respectively. Numbers above genes represent gene locus tags; numbers in genes represent CAZyme family affiliations. The asterisk (*) indicates a possible sequencing error mimicking a gene fusion.

three sulfatases and one gene with sulfatase and GH99 domains. The latter resides in the sulfatase-containing PUL with high synteny to fosmid S3_860 (Figure 3) and likely represents two distinct genes that are artificially fused due to a sequencing error. This is supported by an amino-acid identity of ca. 95% of the N-terminal sulfatase and of ca. 90% of the C-terminal GH (PHEL49_1323) with corresponding genes on the fosmid sequence. Expression of this sulfatase/GH99 locus was only detected with chondroitin sulfate (1.3%NSAF × 1000) and not with laminarin or mannose. A second sulfatase (PHEL49_1477) was detected only with chondroitin sulfate (7.0%NSAF × 1000) or laminarin (2.3%NSAF × 1000) but not with mannose. The remaining two sulfatases (PHEL49_1332/1334) were present only in mannose-supplied cultures, whereas the sulfotransferase (PHEL49_498) was higher expressed with chondroitin sulfate (12 ×) and mannose (7 ×) than with laminarin. Ten GHs (including families GH13, GH16, GH65, GH74 and GH99), eight GTs (including families GT2, GT4 and GT19) and two non-GH carbohydrate-binding proteins (CBM4 and CBM50) were more than twofold upregulated with chondroitin sulfate, whereas five GHs (including families GH13, GH16, GH31 and GH81) were more than twofold down-regulated vs mannose-supplied controls. Furthermore, glutamate synthase and glutamine synthetase were downregulated with chondroitin sulfate, and

the assimilatory nitrate/nitrite reductases were only expressed in laminarin-supplied cultures. This suggests that strain Hel1_33_49 can effectively scavenge nitrogen from the chondroitin sulfate glycosamino-glycan backbone, whereas it needs additional nitrogen when grown on nitrogen-free laminarin.

One of the two mannose-6-phosphate isomerase and a putative mannan endo-1,4-β-mannosidase (GH26) were downregulated with chondroitin sulfate vs mannose-supplied controls. Surprisingly, these enzymes were also expressed to similar extents in laminarin-grown cultures as in the controls (%NSAF × 1000: 31.2 vs 38.8 and 43.6 vs 41.7), which indicates co-regulation of the laminarin and mannose metabolism. Such co-regulations for naturally co-occurring saccharides have been described before, for example, for the β-1,3-glucan laminarin and α-1,4-glucans in 'Gramella forsetii' KT0803 (Kabisch *et al.*, 2014).

Hel1_33_49 cultures grown on chondroitin sulfate showed an unexpected upregulation of a PUL-like operon without known CAZymes (Figure 4) composed of genes for a TBDR (2.8 ×), a SusD-like protein (3.5 ×), an integrin-like protein (only detected with chondroitin sulfate), an arylsulfate sulfotransferase (1.8 ×), a methyltransferase (1.5 ×) and a conserved hypothetical protein (4.6 ×). The N-terminal half of the integrin-like protein resembles the human cartilage acidic protein CRTAC1 (Steck *et al.*, 2007), which might specifically
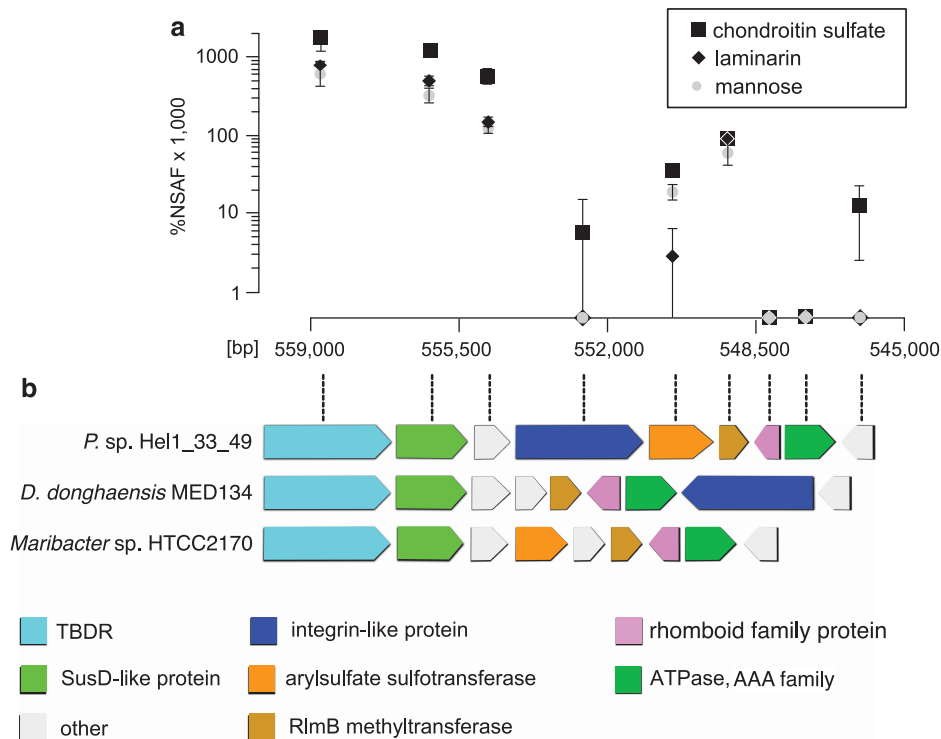
Figure 4 Chondroitin sulfate-induced locus in *Polaribacter* sp. Hel1_33_49. (**a**) Differential expression with chondroitin sulfate and laminarin vs mannose. Ordinate: NSAFs; abscissa: position in the Hel1_33_49 genome (large contig). (**b**) Gene arrangement of the locus as well as similar loci in other *Flavobacteriaceae*.

recognize chondroitin. Such integrin-like proteins are also present in strain Hel1_85 and *Dokdonia donghaensis* MED134 where they are also co-localized with TBDRs/SusD-like proteins. Conversely, the arylsulfate sulfotransferase of Hel1_33_49 has not been found in any other *Polaribacter* strain so far.

Protein utilization. Peptidases of 90 different MEROPS families (Rawlings *et al.*, 2014) were detected among the 27 investigated *Flavobacteriaceae* genomes (Supplementary Figure S6, Supplementary Table S3) dominated by cysteine (C), metallo (M) and serine (S) families (Supplementary Figure S7). Twenty-seven MEROPS families were common to all *Flavobacteriaceae*, with the highest average gene numbers in families S09 ($7.0\,Mbp^{-1}$), S33 and M38 (both $2.8\,Mbp^{-1}$). *Mesoflavibacter zeaxanthinifaciens* S86 (Oh *et al.*, 2011a) and *Flavobacteriaceae* bacterium S85 (Oh *et al.*, 2011b) had the highest ($68.0\,Mbp^{-1}$) and lowest ($28.7\,Mbp^{-1}$) peptidase density, respectively. Both were isolated from seawater in Micronesia but seem to exhibit a pronounced niche separation.

Sequenced *Polaribacter* strains (excluding the fragmentary *P. franzmannii* 301^T genome) have 161–167 peptidases. In comparison to the other investigated *Flavobacteriaceae*, *Polaribacter* have elevated proportions of serine (S) peptidases, and strain Hel1_33_49 also has an elevated proportion of metallo (M) peptidases. Members of these families mediate the degradation and uptake of extracellular proteins, suggesting that digestion of external proteins and subsequent uptake of oligopeptides and amino acids constitutes an important carbon and nitrogen source for both Helgoland strains. Kirchman (2000) estimated that ∼50% of bacterial production in the oceans is supported by amino acids as a key nitrogen source. This agrees with the finding that strain Hel1_33_49 did not grow in medium without yeast extract, casamino acids or peptone and due to a lack of detectable asparagine synthetase is likely auxotrophic for this amino acid.

There are notable differences in the peptidase profiles of *Polaribacter* sp. strain Hel1_33_49 and Hel1_85. For example, strain Hel1_33_49 contains more M38 and S09 peptidases than strain Hel1_85, which in turn has more S08 and S41 peptidases. However, MEROPS family assignments are of limited value in linking peptidase profiles to environmental processes. Distinct peptidases with varying specificities are known to act on polypeptides (for example, M01), oligopeptides (for example, S09, M20) and carboxy/aminopeptidases (for example, M14/M42). Strains Hel1_33_49 and Hel1_85 have six and eight exported family M01 aminopeptidases, respectively. Elevated aminopeptidase activities have been reported for marine bacteria that grow on high-molecular weight dissolved organic nitrogen as sole nitrogen source (Korth *et al.*, 2012; Calleja *et al.*, 2013). *Polaribacter* sp. Hel1_85 has 27 signal peptide-containing S09 prolyl oligopeptidases

that cannot degrade peptides >30 amino acids and are likely used to decompose external oligopeptides prior to uptake.

Light-dependent proteins

Bacterial rhodopsins are light sensors or light-dependent ion pumps. The latter can either translocate protons or sodium ions across the cytoplasmic membrane, which contributes to the chemiosmotic potential that can be used, for example, for ATP production, or function as chloride importers as has been shown for the marine flavobacterium *Nonlabens marinus* S1–08^T (Yoshizawa *et al.*, 2014). Rhodopsin genes have been estimated to be present in about 50% of the North Sea bacterioplankton, most of which absorbs green light (Riedel *et al.*, 2010). González *et al.* (2008) hypothesized that during oligotrophic conditions *Polaribacter* sp. MED152 uses surplus ATP from its proton-pumping rhodopsin for anaplerotic carbon dioxide fixation via pyruvate and phosphoenolpyruvate carboxylase. This is supported by recent stable isotope experiments that demonstrated active assimilation of dissolved inorganic carbon in *Polaribacter* in the northeast Pacific (DeLorenzo *et al.*, 2012).

The rhodopsin light-absorbing cofactor retinal is produced from oxidative cleavage of β-carotene by 15,15'-monooxygenases. Both rhodopsin and 15,15'-monooxygenase genes are present in *Polaribacter* strains MED152, 23-P^T and Hel1_33_49 but notably absent from strain Hel1_85. The Leu^{105} of the Hel1_33_49 rhodopsin sequence indicates a 490-nm light absorption maximum (Man *et al.*, 2003). Its sequence resembles that of the *Polaribacter* isolate SA4–10 (99.7% amino-acid identity; Supplementary Figure S8), for which rhodopsin functionality has been demonstrated (Yoshizawa *et al.*, 2012).

Strain Hel1_33_49 expressed its rhodopsin when grown on chondroitin sulfate even in the dark (Supplementary Table S6), similar to *Dokdonia* sp. PRO95, which expresses rhodopsin independent of light conditions and organic substrate supply (Riedel *et al.*, 2010). In contrast, no rhodopsin expression was observed when strain Hel1_33_49 was grown on laminarin or mannose. This suggests that rhodopsin expression in Hel1_33_49 depends on the availability of specific substrates. The effect of light on rhodopsin expression has been demonstrated for marine model bacteria (Gómez-Consarnau *et al.*, 2007; Riedel *et al.*, 2010; Kimura *et al.*, 2011; Steindler *et al.*, 2011) and in field studies (Lami *et al.*, 2009; Poretsky *et al.*, 2009). Possible effects of nutrient availability on rhodopsin expression are still not well studied, but it was recently shown by Akram *et al.* (2013) that nutrient limitation and not light exposure regulates differential rhodopsin expression in *Vibrio* sp. AND4.

Photolyases and cryptochromes are further light-dependent proteins in *Polaribacter* strain Hel1_33_49.
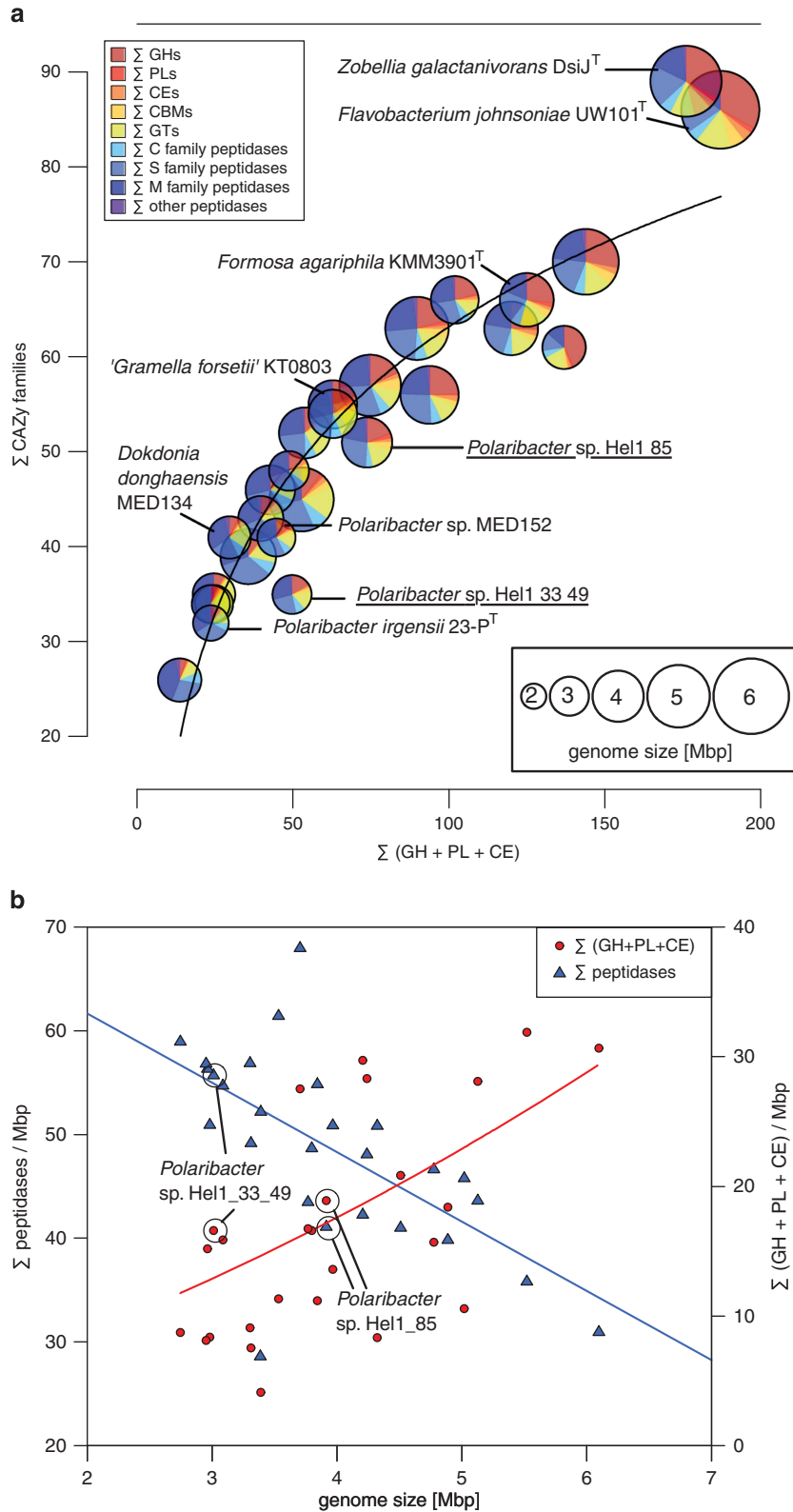
Figure 5 Comparison of 27 *Flavobacteriaceae* genomes based on automated annotations (Supplementary Table S3): (**a**) Interdependency of the number of degradative CAZymes and the number of CAZyme families (trend line: logarithmic regression). Genomes are represented by circles with radii proportional to genome sizes. Relative proportions of CAZymes vs peptidases are shown for each genome. (**b**) Densities of CAZymes with degradation functions and peptidases vs genome size (trend lines: second-order polynominal regressions).

González *et al.* (2008) identified a set of three different DNA photolyase/cryptochrome genes in the *Polaribacter* sp. MED152 genome, which might function as blue light photoreceptors and mediate gene expression levels. We found that the respective locus has a strong synteny among the three rhodopsin-containing *Polaribacter* strains Hel1_33_49, MED152 and 23-P^T, with amino-acid sequence identities >86% among all DNA photolyase/cryptochrome genes (Supplementary Figure S9).

Niche separation

Comparisons of the CAZyme and peptidase profiles of the 27 analyzed *Flavobacteriaceae* revealed pronounced differences between both North Sea *Polaribacter* strains (Figure 5a). Strain Hel1_33_49 has a smaller genome with less CAZymes representing less families and, based on manual CAZyme annotations, a peptidase:CAZyme ratio of 1.37. In contrast, strain Hel1_85 has a larger genome with more CAZymes representing more families an even peptidase:CAZyme ratio. In general, small *Flavobacteriaceae* genomes tend to have a higher peptidase density than larger genomes, while the opposite trend is true for the number of CAZymes with degradation functions (Figure 5b). Although Hel1_33_49 and Hel1_85 feature similar CAZyme densities, they exhibit notably different peptidase densities.

Flavobacteriaceae species with high peptidase:CAZyme ratios include known planktonic species such as *Polaribacter* sp. MED152 and thus are likely adapted towards carbohydrate-depleted marine habitats and preferentially feed on proteins. By contrast, CAZyme-rich species with lower peptidase proportions include known algae-associated species such as *Zobellia galactanivorans* Dsij^T and *Formosa agariphila* KMM 3901^T, reflecting that these species have an ample supply of algal polysaccharides. Further commonalities among these species are (i) the tendency for larger genomes, (ii) the absence of rhodopsins, and (iii) often presence of mannitol dehydrogenases (Supplementary Figure S10).

Although the characteristics of strain Hel1_33_49 match those of typical planktonic *Flavobacteriaceae*, Hel1_85 lies in between those of planktonic and algae-associated species (Figure 5) and might thrive under both conditions. Algae-association of *Polaribacter* sp. Hel1_85 is supported by the fact that it can use laminarin, D-mannitol and alginate as carbon sources, which are typical constituents of brown algae, its high number of sulfatases and its lack of rhodopsin genes.

In situ abundance

We used the genomes of the two North Sea *Polaribacter* strains for read-recruitment with 26 bacterioplankton metagenomes that were obtained in 2008–2012 from the North Sea Polaribacter's isolation site (Teeling *et al.*, 2012) and the English Channel (Gilbert *et al.*, 2010). The proportion of recruited reads at ⩾95% sequence identity by each genome was <1% for all metagenomes (Supplementary Table S7 and Supplementary Text). However, *Polaribacter* abundances in the North Sea are dynamic and peak during spring phytoplankton blooms (Teeling *et al.*, 2012). It is thus likely that higher abundances of either of the two *Polaribacter* strains were missed by any of the metagenome sampling dates. Read abundances were consistently higher for *Polaribacter* sp. Hel1_33_49 than for Hel1_85, which supports genomic analyses indicating that the former is better equipped for a planktonic lifestyle than the latter.

Concluding remarks

Polaribacter sp. Hel1_33_49 and Hel1_85 were isolated from the same sample but exhibit detectable differences with respect to their genomes, physiologies and inferred lifestyles. Strain Hel1_33_49 constitutes what might be considered a typical pelagic representative with a smaller genome, rhodopsin, a limited number of PULs in conjunction with a higher peptidase:CAZyme ratio and no mannitol dehydrogenase. Strain Hel1_85, in contrast, has a larger genome with more PULs in conjunction with a lower peptidase:CAZyme ratio, features a mannitol dehydrogenase but no rhodopsin and seems better equipped to cope with low oxygen conditions as they might occur in biofilms (see Supplementary Text). This species possibly constitutes a *Polaribacter* representative that can associate with algae, which, however, has to be confirmed in a future *in situ* localization study. Strain Hel1_85 also has a higher density of histidine kinases and response regulators than strain Hel1_3_49 and thus seems to be the more adaptive and versatile of the two (Supplementary Table S4).

Both strains grow on laminarin and chondroitin sulfate, and for strain Hel1_33_49 we identified (i) a laminarin-dependent PUL, (ii) chondroitin sulfate-induced CAZymes and (iii) a novel operon that might enable recognition of chondroitin sulfate-like substrates. In comparison to the genomic data from *Polaribacter* spp., it is evident that the genus *Polaribacter* is not only broad in terms of phylogeny but also in terms of genome sizes, physiological traits and associated ecological niches.

Conflict of Interest

The authors declare no conflict of interest.

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