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Alpha- and beta-mannan utilization by marine *Bacteroidetes*

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Summary

Marine microscopic algae carry out about half of the global carbon dioxide fixation into organic matter. They provide organic substrates for marine microbes such as members of the *Bacteroidetes* that degrade algal polysaccharides using carbohydrate-active enzymes (CAZymes). In *Bacteroidetes* genomes CAZyme encoding genes are mostly grouped in distinct regions termed polysaccharide utilization loci (PULs). While some studies have shown involvement of PULs in the degradation of algal polysaccharides, the specific substrates are for the most part still unknown. We investigated four marine *Bacteroidetes* isolated from the southern North Sea that harbour putative mannan-specific PULs. These PULs are similarly organized as PULs in human gut *Bacteroides* that digest α- and β-mannans from yeasts and plants respectively. Using proteomics and defined growth experiments with polysaccharides as sole carbon sources we could show that the investigated marine *Bacteroidetes* express the predicted functional proteins required for α- and β-mannan degradation. Our data suggest that algal mannans play an as yet unknown important role in the marine carbon cycle, and that biochemical principles established for gut or terrestrial microbes also apply to marine bacteria, even though their PULs are evolutionarily distant.

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Introduction

The remineralization of algal glycans by marine bacteria represents one of the largest heterotrophic transformations of organic matter fueling marine food webs (Doubet and Quatrano, 1982; Gerken *et al*., 2013; Williams *et al*., 2013). Algae mediate about half of the global photosynthetic primary production of reduced organic molecules (Field *et al*., 1998; Sarmento and Gasol, 2012). Photosynthesis generates primarily sugars, which are polymerized into complex energy storage compounds, cell wall and cell matrix components and secreted polysaccharides (Fredrick, 1951; Ramus, 1972; McCandless and Craigie, 1979). When released, part of these polysaccharides aggregate to particles that sink into deeper waters. However, only a minor fraction reaches the seafloor due to extensive degradation in the water column (Field and Raupach, 2004; Moran *et al*., 2016). Still, the molecular details of these degradation processes remain largely unknown.

Flavobacteriia, members of the phylum *Bacteroidetes*, are well known for their abilities to degrade polysaccharides by means of various carbohydrate-active enzymes (CAZymes). Based on sequence similarities, CAZymes have been categorized in glycoside hydrolase (GH), glycosyltransferase (GT), carbohydrate esterase (CE), polysaccharide lyase (PL) and auxiliary activity (AA) functional classes in the CAZy database (Lombard *et al*., 2014). CAZymes in *Flavobacteriia* are often encoded in polysaccharide utilization loci, or PULs (Sonnenburg *et al*., 2010).

These PULs usually contain tandem genes of SusD-like surface glycan-binding proteins and TonB-dependent receptors (TBDR) as well as glycan-specific CAZymes (e.g., Kappelmann *et al*., 2018). Accessory genes, such as transcriptional regulators, transporter genes and sulfatases, are also often encoded by PULs. Recent studies showed the remarkable repertoires of CAZymes of *Flavobacteriia* (Hehemann *et al*., 2012; Thomas *et al*., 2012; Ficko-Blean *et al*., 2017), which are frequently associated with algae blooms (Teeling *et al*., 2012; Williams *et al*., 2013; Teeling *et al*., 2016). Due to the large chemical diversity of algal polysaccharides, individual bacterial strains have adapted to utilize only selected polysaccharide subsets as energy and carbon sources (Landa *et al*., 2014).

Mannan degradation has been extensively studied in *Bacteroides thetaiotaomicron* for yeast α-mannan PULs (Cuskin *et al*., 2015) and in *Bacteroides ovatus* for a carob β-mannan (Bågenholm *et al*., 2017), but so far not in marine bacterial species. Beta-mannans are complex polysaccharides, which are important components of hemicelluloses in plant cell walls (Moreira, 2008; Hall *et al*., 2013). These mannans can be classified into four types, (i) linear mannan, (ii) galactomannan, (iii) glucomannan and (iv) galactoglucomannan (Stephen, 1983; Hegnauer and Gpayer-Barkmeijer, 1993; Petkowicz *et al*., 2001; Scheller and Ulvskov, 2010; Bento *et al*., 2013). The unifying feature of these polysaccharides is the β-1,4-linked backbone that contains mainly mannose and sometimes substitutions of glucose (Molinari, 2007; Moreira, 2008). Galactomannans, have a linear backbone of β-1,4-linked D-mannose with side chains of α-1,6-linked D-galactose branches. Galactomannans come from a variety of sources and differ in the number of D-galactose units along the mannan structure (McCleary and Matheson, 1975; Moreira, 2008).

Alpha-mannans with a α-1,6-D-mannose backbone have been found in the cell walls of yeasts, such as *Candida* sp. (Kobayashi *et al*., 1994), *Schizosaccharomyces pombe* (Horisberger and Rosset, 1977) and *Saccharomyces cerevisiae* (Cuskin *et al*., 2015) and in the fruiting bodies of the edible terrestrial fungus *Cantharellus cibarius* (Nyman *et al*., 2016). Furthermore, a galactomannan with a α-1,6- and α-1,2-D-mannose backbone was found in the edible terrestrial fungus *Cordyceps sinensis* (Kiho *et al*., 1986), and α-1,6-linked arabinomannan was detected in the cell wall of the limnic green alga *Chlorella vulgaris* (Pieper *et al*., 2012). Overall β-mannans represent structurally more conserved polysaccharides, whereas α-mannan structures are considerably more variable depending on the source.

Both α- and β-mannans seem to be also relevant in marine ecosystems. For example, a sulfated α-1,3-linked D-mannan has been reported in the red seaweed *Nothogenia fastigiata* (Matulewicz and Cerezo, 1987; Kolender *et al*., 1997) and more recently in the cell wall of the diatom *Phaeodactylum tricornutum* (Le Costaouëc *et al*., 2017). Likewise, mixed-linkage mannans were found in the marine green alga *Codium fragile* (Tabarsa *et al*., 2013) and a 1,4-linked β-mannan was found in the cuticle of the marine red alga *Porphyra umbilicalis* (Frei and Preston, 1964). Recent studies suggest that marine bacteria can degrade mannose-rich substrates, possibly including mannans. For example, using a metaproteomic approach it has been shown that bacterial GH92 family exo-α-mannosidases are expressed during a North Sea spring phytoplankton bloom (Teeling *et al*., 2012). GH92 family genes were also detected in bacterioplankton metagenome datasets during four consecutive years of North Sea spring blooms (Teeling *et al*., 2016), as well as in the open Atlantic (Bennke *et al*., 2016). Recently we could show that homologues of SusC- and SusD-like proteins from predicted bacterial β-mannan PULs are expressed during North Sea spring phytoplankton blooms (Kappelmann *et al*., 2018). All of these results suggest substantial levels of substrate-driven selection of marine bacteria by both α- and β-mannose-rich algal polysaccharides. However, so far it is neither clear to what extent genuine α- and β-mannans are produced in marine ecosystems, nor are the bacteria and their proteins known that recycle these mannans.

In this study, we investigated mannan degradation in four marine *Flavobacteriia.* For two of these strains, *Salegentibacter* sp. Hel_I_6 isolated during a spring phytoplankton bloom at the island Helgoland and *Leeuwenhoekiella* sp. MAR_2009_132 isolated near the island Sylt in the North Sea (Hahnke and Harder, 2013; Hahnke *et al*., 2015), a proteogenomic approach was used to experimentally verify the substrate-specific induction of PULs responsible for α- and β-mannan utilization. We show that, even though marine polysaccharides often differ from their land plant counterparts, marine bacteria possess mannan-inducible PULs that are highly conserved and resemble mannan-specific PULs in human gut bacteria. Based on the data gathered on the mannan-inducible proteins in this study and published homologues in human intestine *Bacteroides* (Cuskin *et al*., 2015; Bågenholm *et al*., 2017) we propose models for α- and β-mannan degradation pathways in these two marine *Bacteroidetes* strains.

Results

Salegentibacter sp. Hel_I_6 and *Flavimarina* sp. Hel_I_48 were isolated from surface seawater off Helgoland in the German Bight of the North Sea (Hahnke *et al*., 2015). Phylogenetic 16S ribosomal RNA analyses (Fig. 1, Supporting Information Table S1) revealed that both strains are closely related to each other and to

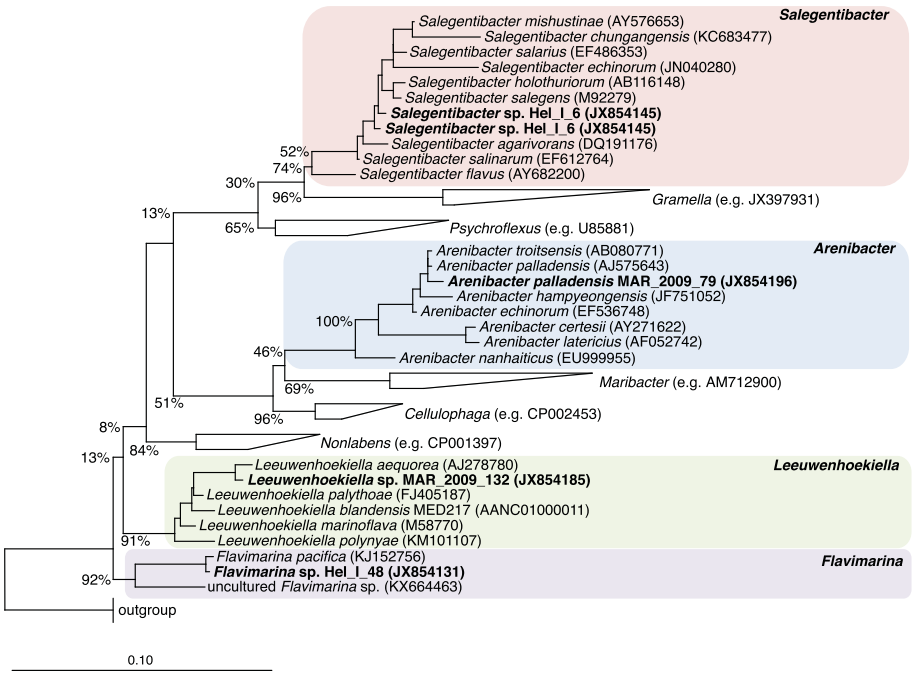

Fig. 1. Phylogenetic tree of examined isolates and related flavobacterial strains. The tree was computed with the RAxML (Stamatakis, 2006) maximum likelihood method (GTR gamma substitution model, 100 repetitions) using a 50% positional conservation filter. *Alphaproteobacteria* was used as outgroup. Bar: 0.1 substitutions per nucleotide position. The 16S rRNA gene sequence similarity matrix is summarized in Supporting Information Table S1.

Leeuwenhoekiella sp. MAR_2009_132, a strain that was isolated together with another putative mannan-degrading bacterium, *Arenibacter palladensis* MAR_2009_79, from the North Sea near the island Sylt in 2009. Annotation of the sequenced genomes revealed the presence of putative β-mannan PULs in the *Salegentibacter*, *Leeuwenhoekiella* and *Flavimarina* strains (Supporting Information Fig. S1) and an α-mannan PUL in the *Salegentibacter* strain (Supporting Information Fig. S2). The genome of the *Arenibacter* strain lacked obvious β/α-mannan PULs, but featured a PUL rich in GH92 exo-α-manosidases. A similar PUL was also present in the *Flavimarina* strain (Supporting Information Fig. S2 and Table S2).

Beta-mannan PULs

The predicted β-mannan PULs of *Salegentibacter* sp. Hel_I_6 and *Leeuwenhoekiella* sp. MAR_2009_132 showed a high synteny (Fig. 2A). Both encode enzymes of families GH5, GH9, GH26, GH27 and GH130 (Supporting Information Fig. S1). Their GH9 (P162DRAFT_1684 and FG27DRAFT_2043) and GH130 (P162DRAFT_1674 and FG27DRAFT_2054) enzymes share >70% sequence similarity, and their GH5 (P162DRAFT_1678 and FG27DRAFT_2050) and GH26 (P162DRAFT_1672 and FG27DRAFT_2056) enzymes show greater than 60% sequence similarity (Fig. 2A).

We compared the putative β-mannan PULs of our marine isolates with orthologous PULs from other habitats. Parts of the PULs of *Salegentibacter* sp. Hel_I_6 and *Leeuwenhoekiella* sp. MAR_2009_132 exhibit high syntenies to PULs of the human gut bacterium *B. ovatus* (Bågenholm *et al*., 2017) (Fig. 2A). In particular the GH130 protein sequences, which code for predicted mannobiose phosphorylases (Nihira *et al*., 2013), are highly conserved as the GH130 orthologs from the marine and human gut isolates are up to 63% identical (Supporting Information Table S3).

For the homologous PULs of *B. ovatus* a specific activity on galactomannan was recently shown (Bågenholm *et al*., 2017). In the characterized system, a pair of GH26 endo-β-mannanases cleave the β-1,4-mannose linkages with one GH26 in the periplasm and the other on the extracellular surface. The marine β-mannan PULs from *Salegentibacter* sp. Hel_I_6 and *Leeuwenhoekiella* sp. MAR_2009_132 harbour two and three GH26 enzymes respectively. The reason for multiple paralogs for a single enzyme activity might be slight differences in substrate specificity as well as the subcellular localization of these enzymes (Zhu *et al*., 2010; Bågenholm *et al*., 2017).

In a tree computed with homologues from other bacteria (Supporting Information Fig. S3) the GH26 proteins fell into three distinct clusters (GH26A, GH26B, GH26C). SpI signal peptides are clear indications for a periplasmic localization, whereas SpII signal peptides are more ambiguous and could result in periplasmic or extracellular localizations (Zuckert, 2014). Based on signal peptide predictions of the sequences in cluster GH26A and similarity to the periplasmic *B. ovatus* protein BoGH26A (Bågenholm *et al*., 2017), the Hel_I_6 and MAR_2009_132 GH26 proteins in this cluster are

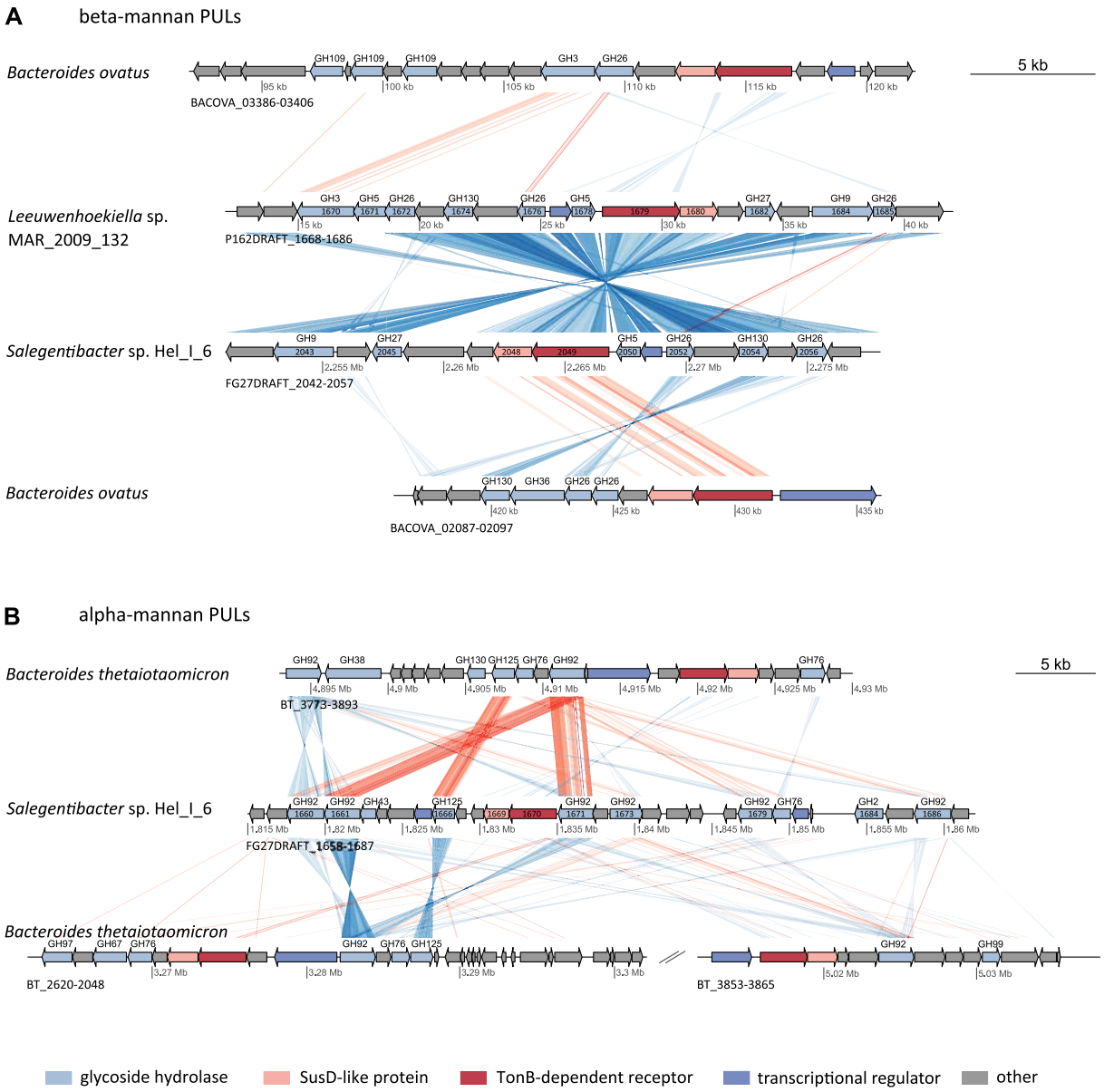

Fig. 2. A. Synteny between the putative β-mannan PULs of *Salegentibacter* sp. Hel_I_6, *Leeuwenhoekiella* sp. MAR_2009_132 and related PULs from *Bacteroides ovatus* (Bågenholm *et al*., 2017). **B**. Synteny between the putative α-mannan PUL of *Salegentibacter* sp. Hel_I_6 and PULs from *Bacteroides thetaiotaomicron* (Cuskin *et al*., 2015). The sequence comparisons were performed with bl2seq (tblastx version 2.7.1+, E-value 1E-5) (see Supporting Information Table S3). Sequence data and the BLAST comparison files were drawn with the R package genoPlotR version 0.8.4 (Guy *et al*., 2010). Sequence similarities are symbolized by red hues for direct comparisons and blue hues for reversed comparisons. Darker colours correspond to higher identities.

predicted to be periplasmic. The sequences in cluster GH26B all feature SpI signal peptides, thus the singular MAR_2009_132 sequence in this cluster is also predicted to be periplasmic. The Hel_I_6 and MAR_2009_132 sequences in the GH26C cluster feature SpII-signal peptides and are similar to the structurally resolved CjMan26C from *Cellvibrio japanicus*, which is believed to be a surface-bound extracellular enzyme (Cartmell *et al*., 2008). Based on this similarity, GH26C may reside on the extracellular surface of the cells.

In order to remove the α-1,6-galactose side chains of galactomannan rather than the GH36 of the enteric bacterial PUL of *B. ovatus* (Reddy *et al*., 2016), the marine PULs code for a conserved GH27 family enzyme. The families GH36 and GH27 share the same three-dimensional fold, substrate specificity and catalytic mechanism, but are nonetheless only distantly related at the sequence level (Comfort *et al*., 2007). The marine PUL, relative to the gut version, is also much larger and includes additional GH5 and GH9 family β-glucosidases.

This suggests that these marine PULs may have evolved to target more complex sets of polysaccharides, potentially including both glucose, mannose and galactose units. One example for such a polysaccharide is galactoglucomannan that occurs in hemicellulose of trees in a heavily acetylated form (Capek *et al*., 2002; Xu *et al*., 2010). Galactoglucomannan is highly similar to galactomannan that has β-1,4-linked glucose substitutions in the mannan backbone. Overall, the set of enzymes found in the putative β-mannan PULs of the marine *Bacteroidetes* strains presumably act in concert with the different GH26 proteins to produce a mixture of mannose, galactose, mannose-1-phosphate and eventually glucose.

Alpha-mannan and other GH92-rich PULs

The putative α-mannan PUL in *Salegentibacter* sp. Hel_I_6, encodes glycoside hydrolases belonging to families GH2 (FG27DRAFT_1684), GH43 (FG27DRAFT_1662), GH76 (FG27DRAFT_1680), GH92 (FG27DRAFT_1660, _1661, _1671, _1673, _1679) and GH125 (FG27DRAFT_1666) (Supporting Information Fig. S2). Enzymes belonging to glycoside hydrolase families GH92 and GH125 can act on α-linked mannose residues in an exo-acting manner and have been implicated in the depolymerization of α-linked mannans (Zhu *et al*., 2010; Gregg *et al*., 2011). Family GH76, in contrast, subsumes endo-α-mannanases that have been shown to specifically cut the α-1,6 backbone of yeast mannan (Cuskin *et al*., 2015).

We found high levels of synteny between the putative α-mannan PUL of *Salegentibacter* sp. Hel_I_6 and three loci in the human gut bacterium *B. thetaiotaomicron* (Fig. 2B). The GH125 (FG27DRAFT_1666) of strain Hel_I_6 shares 62% and 58% sequence similarities to the GH125 family proteins BT_2632 and BT_3781 respectively. Likewise, the GH76 endo-α-mannanase (FG27DRAFT_1680) of strain Hel_I_6 shares 38% and 33% sequence similarity to the GH76 family proteins BT_2623 and BT_3792 respectively. Three of the five GH92 family exo-α-mannosidases of strain Hel_I_6 also share considerable sequence similarity to GH92 family proteins in *B. thetaiotaomicron*, namely FG27DRAFT_1661 to BT3784 (63%) and BT2629 (62%), FG27DRAFT_1671 to BT2629 (48%) and FG27DRAFT _1660 to BT3773 (41%) (Supporting Information Table S3, Supporting Information Fig. S4). The GH2 and GH43 family proteins of *Salegentibacter* sp. Hel_I_6 are most closely related to the α-L-arabinofuranosidase BT3675 of *B. thetaiotaomicron* (Ndeh *et al*., 2017) and one from *Thermotoga thermarum* (Shi *et al*., 2014) respectively.

For PULs of *B. thetaiotaomicron* it has been shown that yeast mannan degradation proceeds via action of an extracellular GH99 (BT3862) that debranches the α-1,6-mannan backbone allowing a pair of GH76 (BT2663, BT3792) enzymes to cleave the α-1,6-glycosidic binds to degrade the α-mannan into transportable oligosaccharides (Cuskin *et al*., 2015). A variety of products is thus obtained and subsequently further digested by GH92, GH38, GH76 and GH125 family enzymes in the periplasm (Cuskin *et al*., 2015). Besides the GH99 (BT3862), the GH92 (BT2199) could also debranch yeast mannan side chains to some extent, thereby enabling the extracellular endo-α-1,6-mannanase GH76 (BT2663 and BT3792) to work on the mannan backbone (Cuskin *et al*., 2015). The mannan PULs of our tested strains all lack a GH99 and only the PUL of *Salegentibacter* sp. Hel_I_6 contains a GH76 (FG27DRAFT_1680) (Supporting Information Fig. S2). The latter harbours also two genes (FG27DRAFT_1660, _1679) that code for GH92 exo-α-mannosidases. Prediction of subcellular locations for these two proteins (Xiao *et al*., 2011) suggests extracellular locations for both, and a possible association with fimbriae or flagellae. FG27DRAFT_1679 is located adjacent to the aforementioned GH76 endo-α-1,6 mannanase encoding gene (Supporting Information Table S10). It is possible that these two GH92 exo-α-mannosidases function similar to the *B. thetaiotaomicron* GH92 (BT2199) by removing yeast mannan side chains and thereby enhancing the GH76 endo-α-1,6 mannanase's efficiency.

Flavimarina sp. Hel_I_48 and *Arenibacter palladensis* MAR_2009_79 both feature PULs that code for numerous exo-α-mannosidases of the family GH92 and, in case of the latter, also of family GH38 (Supporting Information Fig. S2). A phylogenetic tree computed from the GH92 protein sequences of all analysed putative α-mannan PULs revealed that the three GH92s from *Flavimarina* sp. Hel_I_48 (P164DRAFT_3397, P164DRAFT_3398, P164DRAFT_3391) were closely related to three of the in total six GH92s (FG27DRAFT_1660, FG27DRAFT_1661 and FG27DRAFT_1679) from *Salegentibacter* sp. Hel_I_6 (Supporting Information Fig. S4). This suggests that there may be a conserved function of the two PULs. These PULs, however, both lack a GH76 or GH99 family endo-α-mannanase, and therefore likely target mannose-rich substrates other than mannans, such as eukaryotic N-glycosylated proteins. The PUL of *Flavimarina* sp. Hel_I_48 is similar in gene content to a part of the larger α-mannan PUL of *Salegentibacter* sp. Hel_I_6. Hence it is possible that this shorter PUL variant is truncated and therefore inoperable.

Growth of the flavobacterial isolates on selected mono/di- and polysaccharides

The genomes of all four model strains harbour putative laminarin PULs (Supporting Information Fig. S5) with high levels of synteny to a known laminarin-specific PUL of

Gramella forsetii KT0803^T (Kabisch *et al*., 2014) (Supporting Information Fig. S6). These putative laminarin PULs encode glycoside hydrolases that have been previously shown to be involved in laminarin degradation by proteomics (Kabisch *et al*., 2014; Xing *et al*., 2015) and biochemical analyses (Labourel *et al*., 2014; 2015; Unfried *et al*., 2018). We therefore used laminarin degradation activity as a functionally characterized positive control.

In order to verify the predicted polysaccharide substrate spectra, we tested growth of all strains on laminarin, galactomannan (a β-mannan), yeast α-mannan and fucoidan, as well as on selected mono- and disaccharides as sole carbon sources (Supporting Information Fig. S7, Supporting Information Table S4). As predicted, all four strains grew on laminarin but not on fucoidan. *Salegentibacter* sp. Hel_I_6, *Leeuwenhoekiella* sp. MAR_2009_132 and *Flavimarina* sp. Hel_I_48 grew on galactomannan, but only *Salegentibacter* sp. Hel_I_6 could grow on yeast α-mannan. The analysis of the growth rates indicated fast growth of all strains on laminarin, maltose and glucose, and also on the mannan-specific monomers mannose and galactose (Fig. 3).

Proteomics of the β- and α-mannan utilization pathways

Proteome analyses revealed a laminarin inducible gene expression of the predicted laminarin PULs of *Salegentibacter* sp. Hel_I_6 and *Leeuwenhoekiella* sp. MAR_2009_132 (Supporting Information Fig. S8). In order to test, whether expression of the putative β-mannan PULs of these two strains and the putative α-mannan PUL in strain Hel_I_6 are also subject to substrate-specific control, we compared the proteomes of exponentially growing cells on galactomannan and yeast α-mannan to that of cultures growing on D-fructose (Supporting Information Fig. S9).

The proteome analysis of *Leeuwenhoekiella* sp. MAR_2009_132 (Supporting Information Table S5) revealed that the GH3 (P162DRAFT_1670), the GH9 (P162DRAFT_1684), the GH26A (P162DRAFT_1676, WP_035899930.1) and the GH130 (P162DRAFT_1674) of the putative β-mannan PUL are strongly upregulated with galactomannan (Fig. 4A, Supporting Information Table S6). The GH27 (P162DRAFT_1682), which is predicted to have α-galactosidase activity, could not be detected under these conditions, possibly because its abundance was below the detection limit. The proteome data for *Salegentibacter* sp. Hel_I_6 (Supporting Information Table S7) demonstrated that glycoside hydrolases of the putative β-mannan PUL are upregulated in presence of galactomannan (Fig. 4B, Supporting Information Table S8). Selected genes of this PUL revealed a weak expression also with laminarin and fructose as substrates, possibly due to the presence of 0.1 g l^{-1} yeast extract in the medium and thus a low-level baseline of mannose that might have served as an inductor. However, no such expression was observed for *Leeuwenhoekiella* sp. MAR_2009_132 with laminarin and fructose. Therefore, it is also conceivable that the PUL in *Salegentibacter* sp. Hel_I_6 has a low-level constitutive expression of some of its beta-mannan-targeting genes, including the GH9 (FG27DRAFT_2043), GH27 (FG27DRAFT_2045), TBDT (FG27DRAFT_2049), SusD (FG27DRAFT_2048), GH5 (FG27DRAFT_2050) and GH130 (FG27DRAFT_2054).

Proteome analyses with *Salegentibacter* sp. Hel_I_6 showed that the expression of the predicted α-mannan PUL was significantly upregulated on yeast α-mannan (Fig. 5, Supporting Information Table S9), as the GH43 (FG27DRAFT_1662), GH76 (FG27DRAFT_1680), GH92s

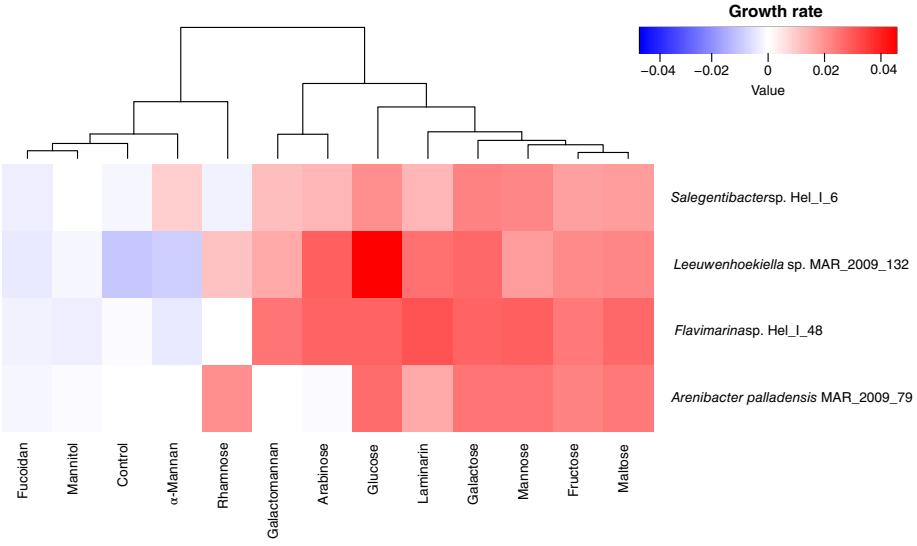

Fig. 3. Heat map illustrating growth rates of *Leeuwenhoekiella* sp. MAR_2009_132, *Salegentibacter* sp. Hel_I_6, *Flavimarina* sp. Hel_I_48 and *Arenibacter palladensis* MAR_2009_79 in marine minimal medium supplemented with selected mono/di/polysaccharides (see section on "Experimental procedures").

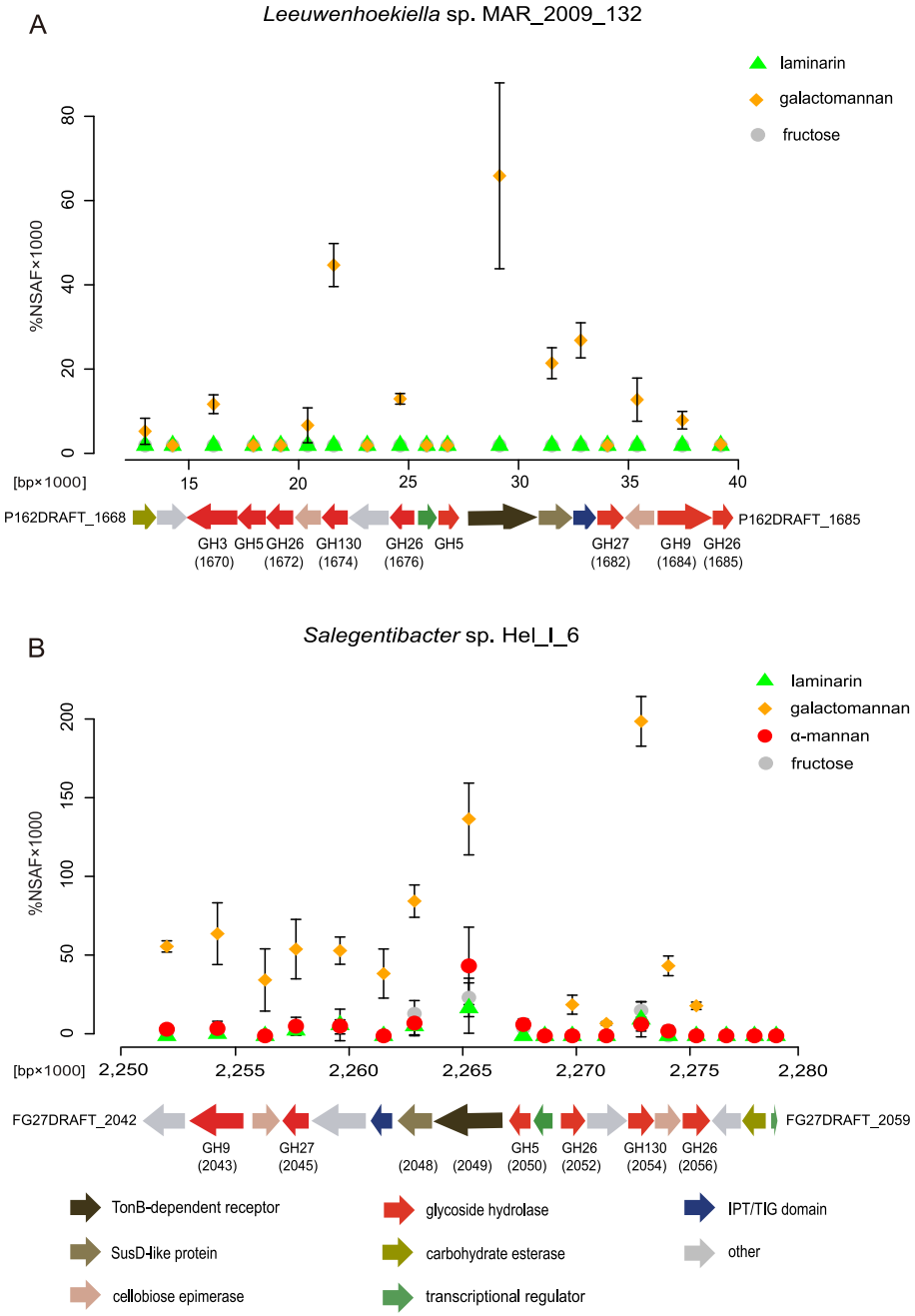

Fig. 4. Galactomannan specifically induces the expression of two related PULs in *Leeuwenhoekiella* sp. MAR_2009_132 (**A**) and *Salegentibacter* sp. Hel_I_6 (**B**). Differential expression of genes in the β-mannan PUL during growth on galactomannan, laminarin and yeast α-mannan as compared with growth on fructose. Ordinate: relative protein abundance (%NSAF); Abscissa: position in the *Salegentibacter* sp. Hel_I_6 or *Leeuwenhoekiella* sp. MAR_2009_132 genome. The potential affiliation of selected glycoside hydrolases to specific GH families and the respective locus tags are indicated.

(FG27DRAFT_1660, 1661, 1671, 1673, 1679) and GH125 (FG27DRAFT_1666) proteins of this PUL all exhibited significantly increased expression levels. The α-mannan PUL is neighbouring a PUL of unknown function, which also encodes a putative GH92 that, however, was not expressed under the conditions tested. Furthermore, expression of the α-mannan PUL in this strain was also elicited by the addition of galactomannan. Different to the expression profiles of control experiments with laminarin and galactomannan, the CBM32 protein (FG27DRAFT_1668), which has been demonstrated to have galactose-binding activities (Abbott *et al*., 2007; Ficko-Blean and Boraston, 2009), revealed the highest expression level with α-mannan. It is worth mentioning that the neighbouring genes, which were annotated as dehydrogenases (FG27DRAFT_1675) and an unknown protein (FG27DRAFT_1676) also showed remarkably high expression in the α-mannan induction experiments.

Selected proteins of the putative α-mannan PUL of *Salegentibacter* sp. Hel_I_6 (e.g., the CBM32, GH76, GH92 (FG27DRAFT_1661), GH125, the TBDR and the SusD-like protein) also responded moderately to

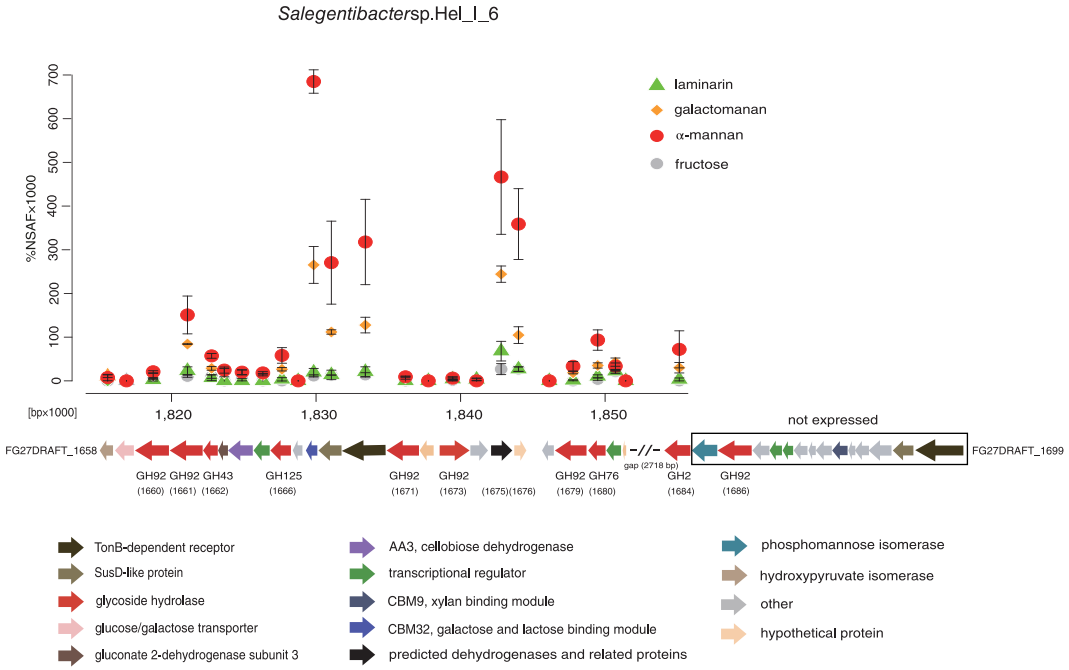

Fig. 5. Yeast α-mannan specifically stimulates the expression of an α-mannanase-encoding PUL in *Salegentibacter* sp. Hel_I_6. Differential expression of genes in the α-mannan PUL in cells supplied with yeast α-mannan, galactomannan and laminarin compared with fructose-supplied cells. Ordinate: relative protein abundance (%NSAF); Abscissa: position in the *Salegentibacter* sp. Hel_I_6 genome. The potential affiliation of selected glycoside hydrolases to specific GH families and the respective locus tags are indicated.

galactomannan, suggesting some cross reactivity between the α- and β-mannan PULs in this strain (Fig. 5, Supporting Information Tables S7 and S8). All of the predicted hydrolases of the α-mannan PUL revealed a reproducibly higher protein level (up to threefold) with galactomannan compared with fructose. This suggests that although the proteins within the identified PUL likely confer the ability to metabolize α-mannan, generally mannose-containing carbohydrates appear to activate the expression of this cluster.

Discussion

Structure and composition of microalgal glycans are still poorly understood. Functional analysis of PULs in bacterial isolates that have been linked to the growth of distinct microalgae under *in situ* conditions might thus be used to gather information on the polysaccharides that these algae produce. Only bacterial clades that possess and express corresponding PULs will be able to compete for distinct algal polysaccharide substrates in nature, in particular during rapid algae growth as in bloom situations. The reverse conclusion is that sequenced isolates with known enzyme repertoires that are cultivated on unknown organic matter could be used to reveal which types of glycans are present.

We found that the predicted β-mannan PULs of our tested marine strains are conserved. All investigated strains, except the *A. palladensis* strain MAR_2009_79, could utilize galactomannan, which indicates that β-mannans likely constitutes important and accessible algal substrates for marine *Bacteroidetes* and are therefore of environmental relevance in productive marine habitats.

By comparison, the structures of the GH92 exo-α-mannosidase-rich PULs in the tested strains were diverse. Besides the presence of different GH92 genes (Supporting Information Fig. S3), various combinations of other glycoside hydrolases were observed. For example, GH2, GH43, GH76 and GH125 genes were found in *Salegentibacter* sp. Hel_I_6, whereas combinations of genes encoding GH43, GH63, GH97, GH125 family proteins are present in *Flavimarina* sp. Hel_I_48. In contrast, the PUL of *A. palladensis* MAR_2009_79 encodes two GH38 genes. This functional diversity could indicate a specialization of these strains on different polysaccharides containing α-linked mannoses, for example, produced by different marine microalgae or other marine organisms. The commercially available α-mannan from *S. cerevisiae* enabled only growth of the *Salegentibacter* sp. Hel_I_6 strain. The other strains could not grow on this substrate, most likely due to a lack of a GH76 endo-α-mannase, which seems to be indispensable for bacterial degradation of yeast mannan (Cuskin *et al*., 2015).

Based on the proteogenomic analyses in this study we are able to propose distinct pathways for α- and β-mannan

utilization in our marine model bacteria that are similar but not identical to those proposed by Cuskin and colleagues (2015) and Bågenholm and colleagues (2017) respectively. In the putative β-mannan degradation pathways of *Salegentibacter* sp. Hel_I_6 and *Leeuwenhoekiella* sp. MAR_2009_132 (Supporting Information Fig. S10A) the GH26C (FG27DRAFT_2056, P162DRAFT_1672) is supposed to act as extracellular β-mannanase that hydrolyze galactomannan polymers into shorter oligomers (Bågenholm *et al*., 2017). The SusD (FG27DRAFT_2048, P162DRAFT_1680) and IPT/TIG-domain proteins (FG27DRAFT_2047, P162DRAFT_1681), immunoglobulin-like plexins, usually found in cell surface receptors (Bork *et al*., 1999), subsequently recognize, bind and deliver the galactomannan oligomers to the TBDR (FG27DRAFT_2049, P162DRAFT_1679), which transfers the oligomers through the outer membrane into the periplasm. Then the galactosyl branches are removed by action of the GH27 (FG27DRAFT_2045, P162DRAFT_1682), which functions as α-galactosidase. The exposed β-1,4-linked mannan backbones are further cut by the β-mannanase GH26A (FG27DRAFT_2052, P162DRAFT_1676) and GH26B (P162DRAFT_1685) in the periplasm, which releases mannobiose. Mannobiose is transformed into 4-O-β-D-mannopyranosyl-D-glucose by a cellobiose epimerase (P162DRAFT_1673, 1683; FG27DRAFT_2044, 2055) and the 4-O-β-D-mannopyranosyl-D-glucose is hydrolyzed into mannose-1-phosphate and glucose by GH130 (FG27DRAFT_2054; P162DRAFT_1674) (β-1,4-mannosylglucose phosphorylase). The mannose-1-phosphate is finally converted to fructose-6-phosphate in the cytoplasm and then channelled into the EMP pathway (Kawaguchi *et al*., 2014).

A proposed α-mannan degradation pathway in *Salegentibacter* sp. Hel_I_6 (Supporting Information Fig. S10B) involves side chain removal by two GH92 family exo-α-1,2-mannosidases (FG27DRAFT_1679, _1660) and cleavage of the α-1,6-linked mannan backbone by the GH76 family endo-α-1,6-mannanase (FG27DRAFT_1680), which produces oligo-mannans of different lengths. The SusD (FG27DRAFT_1669) recognizes, binds and delivers these oligo-mannans to the TBDR (FG27DRAFT_1670), which transfers them across the outer membrane into the periplasm. There the α-1,2-linked branches are hydrolyzed by GH92 family exo-α-1,2-mannosidases (FG27DRAFT_1661, _1671), releasing α-1,6-linked mannan backbones, α-1,3-linked mannobioses and mannose monomers. The exposed α-1,6-linked mannan backbones and α-1,3-linked mannobioses are further digested by the GH125 α-1,6-mannosidase (FG27DRAFT_1666) and the GH92 α-1,3-mannosidase (FG27DRAFT_1673), respectively, producing monomeric mannose. In the cytoplasm, mannose monomers are transformed into glucose-6-phosphate, which enters the EMP pathway.

Our study demonstrates that some marine *Flavobacteriia* feature α-mannan and β-mannan degradation capacities that resemble those in human gut *Bacteroidetes.* In previous studies we also observed strong selection and expression of genes that target mannose-rich substrates in marine bacterioplankton during algae blooms (Teeling *et al*., 2012; 2016). Taken together these findings suggest that mannans are produced in larger quantities by marine microalgae and thus constitute an important carbon and energy source for specialized marine bacteria.

Experimental procedures

Genome sequencing, annotation and bioinformatic analyses

The strains used in this study were sequenced at the Department of Energy Joint Genome Institute (DOE-JGI, Walnut Creek, CA, USA) in the framework of the Community Sequencing Project No. 988 COGITO (Coastal Microbe Genomic and Taxonomic Observatory). *Leeuwenhoekiella* sp. MAR_2009_132 and *Flavimarina* sp. Hel_I_48 were sequenced by a combination of Illumina HiSeq2000 and 2500, and Pacbio RS and RSII sequencing. *Salegentibacter* sp. Hel_I_6 and *Arenibacter palladensis* MAR_2009_79 were sequenced exclusively on the PacBio RS and RSII platforms.

All four stains are GOLD certified at level 3 (improved high-quality draft) and are publicly available at the DOE-JGI GOLD database (Mukherjee *et al*., 2017) under the GOLD Analysis Project IDs: Ga0040974 (*Salegentibacter* sp. Hel_I_6), Ga0042082 (*Leeuwenhoekiella* sp. MAR_2009_132), Ga0005363 (*Flavimarina* sp. Hel_I_48) and Ga0040982 (*Arenibacter palladensis* MAR_2009_79).

16S rRNA gene sequences of studied isolates and related *Flavobacteriia* were obtained from the Silva SSURef v.132 dataset (Quast *et al*., 2013). Sequence alignments were manually curated in Arb v.6.1 (Ludwig *et al*., 2004) using the Silva Incremental Aligner (SINA) (Pruesse *et al*., 2012). Phylogenetic analysis was done with the RAxML v.7 (Stamatakis, 2006) maximum likelihood method (GTR-GAMMA rate distribution model, rapid bootstrap algorithm, 100 repetitions) with a 50% positional conversation filter.

Sequence alignments of the PULs of *Salegentibacter* sp. Hel_I_6 and *Leeuwenhoekiella* sp. MAR_2009_132 with related PULs, for example, from *Bacteroides ovatus* and *Bacteroides thetaiotaomicron* were performed with bl2seq (tblastx version 2.7.1+, E-value 1E-5). Sequence

data and the BLAST comparison files were drawn with the R package genoPlotR version 0.8.4 (Guy *et al*., 2010).

Prediction of localization was carried out using the LipoP 1.0 server (Juncker *et al*., 2003) that detects signal peptides and differentiates between SpI or SpII driven secretion. In case of ambiguous GH92 family proteins, additional predictions were made using the iLoc-Gnec server (Xiao *et al*., 2011).

Growth experiments

Growth assays were conducted in triplicates in 10 ml modified marine mineral media (MMM) (Thomas *et al*., 2011) with a collection of different carbohydrates as only carbon source. Briefly, 1 l MMM (pH 7.5–7.8) contained 25.95 g NaCl, 2 g $MgCl_2 \cdot 6H_2O$, 3 g $MgSO_4 \cdot 7H_2O$, 0.12 g $CaCl_2 \cdot 2H_2O$, 27 ml 0.5 M Na_2EDTA, 25 ml 1 M Tris–HCl (pH 8),10 ml 1 M NH_4Cl, 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.015 g $FeSO_4 \cdot 7H_2O$, 0.03 g $H_4MoNa_2O_6$, 1 ml trace elements solution (H_3BO_3 2.86 g l^{-1}, $MnCl_2 \cdot 4H_2O$ 1.86 g l^{-1}, $ZnSO_4 \cdot 7H_2O$ 0.22 g l^{-1}, $Na_2MoO_4 \cdot 2H_2O$ 0.39 g l^{-1}, $CuSO_4 \cdot 5H_2O$ 0.08 g l^{-1}, $Co(NO_3)_2 \cdot 6H_2O$ 0.05 g l^{-1}) and 1 ml 1000 * vitamins (aminobenzoate 0.04 g l^{-1}, D(+) biotin 0.01 g l^{-1}, folate 0.03 g l^{-1}, lipoate 0.01 g l^{-1}, nicotinate 0.1 g l^{-1}, Ca-(D+) pantothenate 0.05 g l^{-1}, pyridoxamine dihydrochloride 0.11 g l^{-1}, thiamine hydrochloride 0.1 g l^{-1}, vitamin B_{12} 0.05 g l^{-1}). Media were filter-sterilized with Millipore Express filter units (0.22 μm).

Stock solutions (20 g l^{-1}) of monosaccharides [D(+)-glucose (Sigma-Aldrich, Taufkirchen, Germany), D (−)-fructose (Merck, Darmstadt, Germany), D(+)-mannose (Sigma-Aldrich, Taufkirchen, Germany), D(+)-galactose (Sigma-Aldrich, Taufkirchen, Germany), L(+)-arabinose (Sigma-Aldrich, Taufkirchen, Germany), D(−)-mannitol (Sigma-Aldrich, Taufkirchen, Germany), L(+)-rhamnose (Fluka, Germany)] and disaccharides (D(+)-maltose (Fluka, Germany)) were filter-sterilized with Millipore Express filter units (0.22 μm). Stock solutions of polysaccharides [laminarin (*Eisenia bicyclis*, OL02421, Carbosynth, Berkshire, UK), galactomannan (from carob; Low viscosity, P-GALML, Megazyme, Bray, Ireland), α-mannan (*Saccharomyces cerevisiae*, M7504, Sigma-Aldrich, Taufkirchen, Germany) and fucoidan (from *Fucus vesiculosus*)] were autoclaved at 121°C for 15 min. The laminarin used (from *Eisenia bicyclis*) is a β-1,3-glucan with β-1,6-side chains consisting of a single glucose residue with a ratio of β-1,3 and β-1,6 linkages of 3:1 (Carbosynth, Berkshire, UK). The applied galactomannan (from carob) is structured as β-1,4-linked mannan with α-1,6 linked galactose substitution (Gal: Man = 22/78) (Megazyme, Bray, Ireland). The structure of *Saccharomyces cerevisiae* yeast mannan had been reported by Cuskin and colleagues (2015). The backbone is α-1,6-linked mannan with α-1,2- and α-1,3-linked mannan branches. All carbon sources were added to MMM to a final concentration of 2 g l^{-1}. The substrate solutions were inoculated with 1 ml of the stock culture of one of the respective strains. The stock cultures of the bacterial strains were prepared by harvesting the biomass of each strain, which grew in modified HaHa_100 medium (Hahnke and Harder, 2013) with glucose. The harvested cell pellets were washed twice with MMM and then the cells were re-suspended with 50 ml MMM. Inoculated medium that was not supplemented with saccharides was used as control. Optical density measurements (600 nm) were taken every 2 or 3 days for 18 days.

Proteome analyses

Salegentibacter sp. Hel_I_6 and *Leeuwenhoekiella* sp. MAR_2009_132 were grown in modified HaHa_100 medium with 0.1 g l^{-1} yeast extract, 0.1 g l^{-1} peptone, 0.1 g l^{-1} casamino acids, 5 mg l^{-1} NH_4Cl and 0.8 mg l^{-1} KH_2PO_4. Galactomannan (β-mannan) and laminarin were used as carbon sources for both strains. Yeast mannan (α-mannan) was added only for *Salegentibacter* sp. Hel_I_6. Fructose was applied besides laminarin as another reference substrate in these experiments. Final concentrations of all supplemented saccharides were 2.0 g l^{-1}. Strains were grown in medium with the defined carbon source until mid-exponential phase. About 1 ml of culture was transferred into the same medium and again grown until mid-exponential phase. This adaptation was repeated thrice before recording the final growth curve in 100 ml medium (1% inoculum, modified HaHa_100 medium with the glycan substrate) at 20°C. All experiments were carried out in triplicates. Once the cultures reached the mid-exponential phase, cells were harvested by centrifugation (13 000*g*, 15 min, 4°C). The harvested pellets were stored at −80°C until further analysis.

Soluble proteins were extracted as described previously (Xing *et al*., 2015). Protein concentration was determined according to Bradford (1976). In brief, for each sample, 15 μg proteins were loaded onto a 10% polyacrylamide sodium dodecyl sulfate mini gel and run at 150 V for 60 min, fixed with ethyl acetate and stained with Coomassie G 250 (Candiano *et al*., 2004). Afterward, the proteins were in-gel digested for 16 h with trypsin as described by Heinz and colleagues (2012) and eluted in an ultrasonic bath for 15 min. Peptides were subjected to a reversed-phase C18 column chromatography operated on a nanoAC-QUITY-UPLC (Waters Corporation, Milford, MA, USA) and separated as described by Otto and colleagues (2010). Mass spectrometry (MS) and MS/MS data were acquired using an online-coupled LTQ-Orbitrap Classic mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). MS spectra were searched against the target-decoy protein sequence databases of *Salegentibacter* sp. Hel_I_6 and

Leeuwenhoekiella sp. MAR_2009_132, respectively, each including the respective organism's amino acid sequences in forward and reverse directions (decoys) and common laboratory contaminants. Validation of MS/MS-based peptide and protein identifications was performed with Scaffold v4 (Proteome Software Inc., Portland, OR, USA) using a maximum false discovery rate of 0.01 (1%) on the peptide level and 0.01 (1%) on the protein level. Only proteins that could be detected in at least two out of three biological replicates were considered identified. Normalized spectral abundance factors (NSAFs) were calculated (Zybailov *et al*., 2006) using Scaffold's 'total spectrum counts' for each protein, which allows comparing relative protein abundances between individual samples. The mass spectrometry proteomics data are available through the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaino *et al*., 2013) with dataset identifiers PXD009066 and 10.6019/PXD009066 for *Leeuwenhoekiella* sp. Mar_2009_132 and with the dataset identifiers PXD009080 and 10.6019/PXD009080 for *Salegentibacter* sp. Hel_I_6.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix: Supporting Information

Table S1: 16S rRNA gene sequence similarity matrix

Table S2: Predicted mannan and laminarin degradation ability of the four flavobacterial strains: *Salegentibacter* sp. Hel_I_6, *Leeuwenhoekiella sp.* MAR_2009_132, *Flavimarina* sp. Hel_I_48 and *Arenibacter palladensis* MAR_2009_79. For the isolation and sampling procedure see Hahnke and Harder (2013).

Table S3: Comparison of the sequence identity of the proteins of the β- and α-mannan PULs of *Salegentibacter* sp. Hel_I_6 with related protein sequences of *Bacteroides ovatus* and *B. thetaiotaomicron*. The sequence comparison was performed with bl2seq (tblastx version 2.7.1+, E-value 1e-5).

Table S4: Utilization of mono-, di- and polysaccharides by *Salegentibacter* sp. Hel_I_6, *Leeuwenhoekiella sp.* Hel_I_48, *Flavimarina* sp. MAR_2009_132 and *Arenibacter palladensis* MAR_2009_79.

Table S5: Overview of the proteome data of *Leeuwenhoekiella* sp. MAR_2009_132 during growth on galactomannan (β-mannan).

Table S6: List of galactomannan-induced proteins of *Leeuwenhoekiella* sp. MAR_2009_132. GO: only detected with galactomannan; FN: not detected with fructose. Domain predictions of hypothetical proteins were done with blastP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and CDD (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi), No: no putative conserved domains have been detected

Table S7: Overview of the proteome data of *Salegentibacter* sp. Hel_I_6 during growth on yeast mannan (alpha-mannan).

Table S8: List of galactomannan-induced proteins of *Salegentibacter* sp. Hel_I_6. GO: only detected with galactomannan; OGM: only detected with galactomannan and yeast mannan; FN: not detected with fructose. Domain predictions of hypothetical proteins were done with blastP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and CDD (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi), No: no putative conserved domains have been detected

Table S9: List of yeast mannan-induced proteins of *Salegentibacter* sp. Hel_I_6. MO: only detected with yeast mannan; OGM: only detected with galactomannan and yeast mannan; FN: not detected with fructose. Domain predictions of hypothetical proteins were done with blastP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and CDD (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi); No: no putative conserved domains have been detected

Table S10: Prediction of the cellular localization of PUL encoded proteins by the LipoP 1.0 server (Juncker *et al*., 2003)