A marine bacterial enzymatic cascade degrades the algal polysaccharide ulvan

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27 **Abstract**

28 Marine seaweeds increasingly grow into extensive algal blooms, which are detrimental to
29 coastal ecosystems, tourism, and aquaculture. However, algal biomass is also emerging as
30 sustainable raw material for bioeconomy. The potential exploitation of algae is hindered by
31 our limited knowledge of the microbial pathways – and hence the distinct biochemical
32 functions of the enzymes involved – that convert algal polysaccharides into oligo- and
33 monosaccharides. Understanding these processes would be essential, however, for
34 applications like the fermentation of algal biomass into bioethanol or other value-added
35 compounds. Here we describe the metabolic pathway that enables the marine
36 flavobacterium *Formosa agariphila* to degrade ulvan, the major cell wall polysaccharide of
37 bloom-forming *Ulva* species. The pathway involves 12 biochemically characterized
38 carbohydrate-active enzymes, including two polysaccharide lyases, three sulfatases and
39 seven glycoside hydrolases that sequentially break down ulvan into fermentable
40 monosaccharides. This way, the enzymes turn a previously unexploited renewable into a
41 valuable and ecologically sustainable bioresource.

42

43 **Introduction**

44 Algal photosynthesis provides half of the global primary production¹. Carbon dioxide is
45 converted into carbohydrates, which are polymerized into polysaccharides to store energy,
46 build cell walls, and perform other biological functions. Algae are furthermore considered as
47 a promising renewable carbon source, due to their competitive growth rates and unique cell
48 walls. Unlike plants that are rich in woody tissue, comprising the insoluble polysaccharides
49 cellulose and the aromatic polymer lignin, which increases recalcitrance against enzymatic
50 digestion, algal cell walls are rich in gel-forming polysaccharides that are highly hydrated².
51 Hydration and the absence of lignin make harsh chemical and physical pretreatment of cell
52 walls unnecessary, and allow for easy access of enzymes that can digest the

53 polysaccharides into fermentable monosaccharides. Accordingly, recent studies showed that
54 bioengineered microbes equipped with agarases and alginate lyases can efficiently digest
55 and rapidly convert polysaccharides from brown and red algae into bioethanol³.

56 Sessile macroalgae, such as brown algae that form kelp forests, are ecologically valuable
57 because they provide nutrition and habitats for fish and other organisms and, consequently,
58 harvesting them would exacerbate pressure on natural populations. However, the planktonic
59 macroalgae *Ulva armoricana*, *Ulva rotunda* and other *Ulva* spp. that thrive in eutrophic,
60 nutrient-rich coastal waters, grow into expansive blooms that occur with increasing frequency
61 in recent years. They pose ecological but also economical threats when they accumulate on
62 beaches used for recreation⁴⁻⁶. Fertilized by nitrate from agriculture that is washed into the
63 ocean by rivers, *Ulva* blooms during summer produce up to 50–100 000 tons of biomass
64 every year, which must be removed at high expense from the northern and western coast of
65 France⁶. Even larger blooms occur in China⁵. Blooms of *Ulva* are thus a global phenomenon
66 that is bound to increase with farming activities, rendering the polysaccharide ulvan, which
67 accounts for up to 30 % of the algal dry weight⁷, an emerging yet untapped resource.

68 Ulvan is a branched polysaccharide composed of repeating disaccharide units, in which D-
69 glucuronic acid (GlcA) is β -1,4-linked or L-iduronic acid (IdoA) is α -1,4-linked to L-rhamnose-
70 3-sulfate (Rha3S), which is α -1,4-linked within the main chain. Some of the uronic acids are
71 replaced by β -1,4-linked D-xylose (Xyl), which can be sulfated at position 2 (Xyl2S).
72 Furthermore, Rha3S can be modified by β -1,2-linked GlcA side chains and the GlcA-Rha3S
73 or IdoA-Rha3S pattern can be interrupted by consecutive GlcA residues⁷⁻⁹. Increased interest
74 in the enzymatic degradation of ulvan recently led to the description of several ulvan-active
75 enzymes¹⁰⁻¹⁵. So far, and to the best of our knowledge, only two types of enzymes from
76 different carbohydrate-active enzyme (CAZyme) families showed activity on ulvan. Ulvan
77 polysaccharide lyases of the families PL24, PL25 and PL28 catalyze the initial cleavage
78 between Rha3S and GlcA or IdoA, resulting in the formation of unsaturated uronic acid
79 residues at the end of the formed oligosaccharide. Unsaturated uronic acid residues are

80 removed by glycoside hydrolases (GHs) from the family GH105^{15,16}. Ulvan-specific
81 degradation-related gene loci ('polysaccharide utilization loci', PULs) such as PUL H from
82 *Formosa agariphila* encode PL28 and GH105 together with over 10 additional, putative
83 enzymes, which were predicted to be involved in ulvan utilization. While PL28 and GH105
84 degrade ulvan, the other enzymes that were produced in *Escherichia coli* did not show
85 activity¹⁵. This result suggested that a complex cascade of sequential enzymatic reactions is
86 required for complete ulvan degradation^{15,17}.

87 Here, we experimentally established the complex ulvan degradation pathway of *F. agariphila*
88 KMM 3901^T, a marine flavobacterium, which was isolated from a green alga in the Sea of
89 Japan¹⁸. These degradation-related enzymes are encoded in an ulvan-specific PUL in the
90 bacterial genome¹⁵.

91

92 **Results**

93 **Bacterial ulvan-specific PULs**

94 To decipher the ulvan degradation pathway, we first searched microbial genomes hosted at
95 NCBI for potential ulvan-specific PULs using the known ulvan lyase PL28 as query. We
96 identified 12 putative ulvan PULs in 12 Bacteroidetes genomes (Fig. 1a), including the
97 recently discovered PUL H of *F. agariphila*^{15,18}, a more than 75 kb long genomic region
98 consisting of 39 genes (Fig. 1b). We verified the boundaries of PUL H with a comparative
99 global proteome analysis of *F. agariphila* cells fed with ulvan and with control substrates
100 (rhamnose and fructose), respectively, as sole carbon source. Ulvan promoted bacterial
101 growth (Supplementary Fig. 1) and elicited quantitative changes of most proteins that are
102 encoded by PUL H (Table 1, Fig. 1b, and Supplementary Fig. 2). Besides ulvan, also the
103 monosaccharide rhamnose induced, albeit less strongly, the expression of PUL H genes. For
104 a few proteins (P2_SusD, P3_TBDR, P8_GH2) even higher protein amounts were detected
105 with rhamnose, compared to ulvan. The increased abundance of enzymes involved in the

degradation of ulvan-derived monosaccharides indicated a co-regulation of genes for the metabolization of ulvan and its corresponding monosaccharides (Table 1, Supplementary Figs. 2 and 3, Supplementary Data Sets 1 and 2). PUL H includes 17 potential carbohydrate-active enzymes (CAZymes) from different GH and PL families (<http://www.cazy.org/>¹⁹) and eight sulfatases from five S1 subfamilies (<http://abims.sb-roscoff.fr/sulfatlas/>²⁰). For most of these enzymes, their role in ulvan depolymerization remains unknown. A co-occurrence analysis of putative enzymes and associated genes within the set of 12 PULs from marine Bacteroidetes identified conserved CAZymes in the putative ulvan pathways (Fig. 1c). This analysis allowed us to focus our biochemical experiments on a smaller subset of CAZymes and sulfatases, whose involvement in ulvan utilization was suggested by our proteomic results (Fig. 1b).

In addition to the two already known¹⁵ ulvanolytic enzyme activities (ulvan lyase and unsaturated glucuronyl hydrolase, GH105) we uncovered eight so far unknown enzyme functions for the complete depolymerization of ulvan. Besides a novel PL family, we identified and characterized six GH families (GH2, GH3, GH39, GH43, GH78, GH88) and three sulfatases.

Activity-based screenings of these enzymes were used to identify their function in the ulvan degradation pathway. The selection of putative CAZymes and sulfatases for cloning, heterologous expression and characterization was guided by the co-occurrence analysis of genes in the diverse ulvan PULs (Fig. 1c).

Sulfatases active on ulvan

Ulvans feature a large structural variability, with substitution by sulfate esters at various positions. This chemical diversity is influenced by several factors such as the algal species, the environmental conditions or the seasons⁷. The studied PUL of *F. agariiphila* encodes 8 formylglycine-dependent sulfatases belonging to 5 subfamilies of the SulfAtlas S1 family

(Table 1): S1_7: 3 genes; S1_8: 2 genes; S1_16: 1 gene; S1_25: 1 gene; S1_27: 1 gene (<http://abims.sbroscoff.fr/sulfatlas>)²⁰. With such a diversity of S1 subfamilies, these sulfatases likely display significant differences in substrate recognition, even though they are all predicted to act on ulvans. We expressed 7 sulfatases in soluble form in *E. coli*. After purification, these recombinant sulfatases were incubated with ulvan polymers from three different sources (Agrival, Elicityl, and one extracted from an Atlantic *Ulva* sp. collected in Roscoff, France). As shown by the HPAEC analyzes of released sulfate ions, 6 sulfatases are clearly active on ulvan polymers, although their activity varies depending on the polysaccharide sources (Supplementary Fig. 4). The sulfatase P18_S1_7 (for numbering/nomenclature see Table 1) was most active on ulvan polymers, particularly on the xylose-rich ulvan (Supplementary Figs. 4 and 5) and can desulfate oligosaccharides containing the motif Rha3S-Xyl2S-Rha3S. Thus, this sulfatase likely proceeds in an endolytic mode of action. This assumption is consistent with the “open groove” topology of the active site unraveled by the P18_S1_7 crystal structure (Fig. 2a and 2g). Interestingly, P14_S1_7 (predicted as exolytic, since this sulfatase is almost inactive on ulvan, Supplementary Fig. 4) and P18_S1_7 (predicted as endolytic) belong to the same subfamily (S1_7). Such dissimilar modes of action within the same (sub)family have been described in glycoside hydrolase and polysaccharide lyase families^{21,22}. In comparison to P18_S1_7, the S1_25 sulfatase module of P36 (referred to as P36_S1_25) presents moderate activities on polymers. On oligosaccharides, P36_S1_25 was the most active enzyme. This enzyme specifically desulfates L-rhamnose at the 3-position and can act on the motif Rha3S-Xyl-Rha3S in an exolytic mode of action.

Sequence analyses revealed that P18_S1_7 (485 residues) and the S1_25 sulfatase module of P36_S1_25 (443 residues) are only distantly related (25% identity) and thus belong to two different SulfAtlas S1 subfamilies²⁰, S1_7 and S1_25, respectively. We determined the crystal structure of these two sulfatases, with higher resolution for P18_S1_7 (1.23 Å) and lower resolution for the sulfatase module of P36_S1_25 (2.91 Å). P18_S1_7 and P36_S1_25 adopt a similar fold with two α/β -structural domains, an *N*-terminal catalytic domain SD1

(Ser25-Asp388; P18_S1_7) separated by a structured linker (Arg389-Val397) from a C-terminal domain SD2 (Ala398-Pro483). Nonetheless, the sulfatase module of P36_S1_25 is a smaller protein and lacks some secondary elements, which are present in P18_S1_7 (the β -strands $\beta 6$ and $\beta 8$, the α -helices $\alpha 5$, $\alpha 7$, $\alpha 8$ and several short 3:10 helices). Notably, the helix $\alpha 7$ and the loops connecting it to the main part of SD1 constitute a protruding extension, which overhangs the active site (Fig. 2d). The active site of P18_S1_7 is a large, open groove with a strong basic character (Fig. 2a and 2g). This type of active site topology is consistent with the endo-character and its efficiency on polymeric ulvan (Supplementary Fig. 4). In contrast, the active site of P36_S1_25 is a pocket (Fig. 2c), which is consistent with its activity on oligosaccharides (Supplementary Fig. 4). The most similar protein in the Protein Data Bank (PDB) is the human iduronate 2-sulfatase (IDS, 31% sequence identity; PDB: 5FQL; Fig. 2b and 2e)²³. Interestingly, IDS also displays a pocket active site topology (Fig. 2b). Therefore, different active sites (and subsequently different modes of action) can exist within the same S1 subfamily. Such differences in topology likely explain the varying efficiencies at the polymer level observed for P11_S1_7, P14_S1_7 and P18_S1_7 although they all belong to the S1_7 subfamily (Supplementary Fig. 4).

The catalytic machinery of the S1 family sulfatases²⁴ is well conserved in P18_S1_7 and P36_S1_25. We find the catalytic nucleophile (Cys74 and Cys58, respectively), residues involved in Ca^{2+} coordination (Asp35, Asp36, Asp312 and His313; Asp18, Asp19, Asp284 and Asn285), residues stabilizing the catalytic nucleophile (Arg78 and His128; Arg62 and Gly110), and residues of the sulfate-binding S subsite, as defined in the recent nomenclature for sulfatase-binding subsites²⁵ (Lys125, His213 and Lys325; Lys108, His182 and Lys297) (Fig. 2g, Supplementary Figs. 6-8). His313 in P18_S1_7 is not the most frequent residue for the coordination of the calcium ion (usually an asparagine), but a histidine at this position is found in a minority of sulfatases and is part of the updated PROSITE signature "Calcium-binding site 2"²⁰. Most surprising is the replacement of His128 in P18_S1_7 by Gly100 in the sulfatase module of P36_S1_25. Indeed, a histidine at this position is supposed not only to stabilize the catalytic formylglycine, but also to abstract its O γ 2 proton at the end of the

188 catalytic cycle to induce the sulfate elimination and the aldehyde regeneration²⁴.
189 Nonetheless, this glycine is strictly conserved in the closest homologs of the sulfatase
190 module of P36_S1_25 (105 sequences with >50% identity; Supplementary Fig. 7),
191 suggesting that the function of the histidine at this position may not be essential in this ulvan
192 sulfatase subgroup.

193 While some sulfatases were not quantified in our metabolic labeling approach (Fig. 1b, Table
194 1), they were detected by subproteome analysis in the membrane-enriched fraction. In five
195 cases, lipoprotein signal peptides were predicted and P18_S1_7 and P36_S1_25 were
196 highly abundant in the intracellular soluble fraction (Supplementary Data Set 3). Taken
197 together, these results indicate a periplasmic localization of sulfatases, with some of them
198 putatively membrane-bound. Notably, the sulfatase P36_S1_25 activity is found in a
199 multimodular enzyme that contains also a GH78 domain. Comparative genome analyses
200 indicated multimodular enzyme structures in the ulvan PUL H of *F. agariphila*¹⁵ and other
201 putative ulvan-degrading Bacteroidetes strains (Supplementary Data Set 4).

203 **Enzymatic ulvan degradation**

204 In brief, the distinct function of each enzyme was established by activity testing on ulvan and
205 on defined enzymatically produced ulvan oligomers using photometric assays, fluorophore-
206 assisted carbohydrate electrophoresis (FACE) and carbohydrate polyacrylamide gel
207 electrophoresis (C-PAGE), high performance anionic exchange chromatography with pulsed
208 amperometric detection (HPAEC-PAD) and mass-spectrometry. Detailed procedures of
209 these steps are outlined in the Online Methods section. Structures of all important
210 carbohydrate intermediates were confirmed by 1D and 2D nuclear magnetic resonance
211 (NMR) spectroscopy together with mass-spectrometry analysis.

212 We performed an initial photometric screening, which detects the unsaturated uronic acid
213 moiety (Δ) introduced by the lytic mechanism of lyases. We show that P10_PLnc and
214 P30_PL28 are both endo-acting ulvan lyases generating the same product pattern, implying

that they have a similar specificity (Supplementary Fig. 9). P30_PL28 accepts GlcA and IdoA at the cleavage site and generates the dimer Δ -Rha3S and the tetramer Δ -Rha3S-Xyl-Rha3S as main products¹⁴. Both ulvan lyases, P30_PL28²⁶ and P10_PLnc, appear to initiate ulvan depolymerization outside of the bacterial cell. P30_PL28 contains an additional ulvan-binding module¹³ and a type IX secretion system signal that drives secretion²⁷, corroborating the proteomic results (Supplementary Fig. 10, Supplementary Data Sets 2 and 3). P10_PLnc might be associated to the outer membrane (Supplementary Data Set 3), although a periplasmic localization is also possible (Supplementary Fig. 10, Supplementary Data Set 2).

Two variants of ulvan lyase with distinct localizations indicate synergistic functions: while P30_PL28 is an extracellular enzyme catalyzing rapid dissolution of insoluble ulvan, P10_PLnc most likely dissolves soluble ulvan oligomers at the cell surface, where uptake proceeds through the expressed TonB-dependent receptor system into the periplasm. Here, the unsaturated uronyl residue (Δ) at the non-reducing end of oligomers is removed by the exo-acting unsaturated glucuronyl hydrolases (outer membrane P1_GH88 and periplasmic P33_GH105) (Supplementary Figs. 11-13), thus forming 5-dehydro-4-deoxy-D-glucuronate. The resulting Rha3S was purified and the structure was confirmed by NMR (Supplementary Figs. 14 and 15, Supplementary Table 1). This monosaccharide is desulfated by the S1_25 sulfatase domain of P36_S1_25 yielding rhamnose, which can enter the cellular sugar metabolism (Fig. 3, Supplementary Fig. 16). Rha3S-Xyl-Rha3S was another major intermediate which was isolated (Supplementary Figs. 17 and 18, Supplementary Table 2). Rha3S-Xyl-Rha3S was desulfated by the sulfatase P36_S1_25 to yield Rha-Xyl-Rha3S, which was isolated to confirm the desulfation site at the non-reducing end (Supplementary Figs. 19-21, Supplementary Table 3). Next, Rha-Xyl-Rha3S is converted by the periplasmic P20_GH78 to Rha and Xyl-Rha3S (Fig. 3, Supplementary Fig. 22). The CBM67 domain of P20_GH78 likely elevates specificity for rhamnose and contributes to substrate recognition²⁸. Finally, the dimer Xyl-Rha3S is further cleaved by P24_GH3 or P27_GH43 to yield Xyl and Rha3S, making these the first identified β -xylosidases that are active on ulvan oligosaccharides (Fig. 3, Supplementary Figs. 22 and 23). Notably, only the P24_GH3 was

previously found to be active on 4-methylumbelliferyl- β -D-xylopyranoside (MUX) showing that the two enzymes have different substrate specificity at the aglycone site¹⁵.

Besides ulvan lyases, the endo-active α -1,4-L-rhamnosidase GH39 cleaves rhamnose sections interspersed between xylose residues within the polymer. Such a function has, to the best of our knowledge, not been described in this family before. Accordingly, larger oligomers with consecutive Xyl-Rha3S units that are resistant to the ulvan lyases P30_PL28 and P10_PLnc were efficiently degraded by P31_GH39 (Supplementary Fig. 24). The catalytic order of ulvan lyases and P31_GH39 was interchangeable as the larger degradation products of P31_GH39 were prime substrates for both ulvan lyases (Supplementary Fig. 25). The dimers Xyl-Rha3S and Xyl2S-Rha3S were isolated as the smallest products and the structure was elucidated by NMR, identifying GH39 as an α -rhamnosidase active on ulvan (Supplementary Figs. 26-29, Supplementary Tables 4 and 5). While Xyl-Rha3S is further degraded as described above, Xyl2S-Rha3S was resistant to P24_GH3 or P27_GH43 and needs to be desulfated by the P32_S1_8 sulfatase prior to enzymatic conversion by these enzymes (Supplementary Fig. 30). Desulfation of Xyl2S within the trimer Rha3S-Xyl2S-Rha3S, released by P30_PL28 and P33_105 digestion (Supplementary Figs. 31 and 32, Supplementary Table 6), was catalyzed by the P18_S1_7 sulfatase (Supplementary Fig. 33).

GlcA side chains present on some O2 residues of Rha3S⁷ are removed by P17_GH2. When P17_GH2 was added to untreated ulvan, it produced a single band in FACE with the same mobility as a GlcA (Supplementary Fig. 34a) while not decreasing the overall molecular weight of the raw ulvan as seen by C-PAGE (Supplementary Fig. 9). To confirm this activity, defined oligomers with GlcA side chains were produced from ulvan with P30_PL28 and P31_GH39 with or without P33_GH105. The structure of Δ -Rha3S[2GlcA]-Xyl-Rha3S and Rha3S[2GlcA]-Xyl-Rha3S, was confirmed by NMR (Supplementary Figs. 35–38, Supplementary Tables 7 and 8) and these products were used as substrates for P17_GH2. This enzyme was also active on these smaller oligomers (Fig. 3, Supplementary Fig. 34b). This result indicates that the GlcA side chains were removed from polymeric ulvan or from

smaller intermediates (Supplementary Fig. 34c), although in *F. agariphila* we predict P17_GH2 to be localized in the periplasm and thus to be active on oligomers, which also applies to P31_GH39.

GlcA side chains partially shielded the main chain against hydrolysis by P31_GH39. When the GlcA residues were removed by P17_GH2, a higher degree of degradation was observed with P31_GH39 (Supplementary Fig. 39). The newly determined crystal structure of P17_GH2 (Supplementary Fig. 40) contains a pair of *N*-terminal β -sandwich domains, a TIM-barrel with the active site, two more β -sandwich domains and a C-terminal putative carbohydrate-binding module connected by an extended flexible linker at the C-terminus that places the CBM over the active site (Supplementary Fig. 40a). The active site pocket is at the surface of the catalytic domain; its size provides just enough space to accommodate one GlcA residue. The catalytic site of this enzyme, obscured by the aforementioned CBM, further deviates from other members of the GH2 family. In most GH2 the nucleophile and acid/base catalytic residues are approximately 200 residues apart at the C-terminal ends of strands 4 and 7 of the conserved $(\alpha/\beta)_8$ -TIM barrel fold. In P17_GH2, the nucleophile is conserved (Glu509) but the acid/base position has a tryptophan (Trp447) (Supplementary Fig. 40d). Two alternative possibilities exist for the acid/base of P17_GH2 Glu411 found on strand 3 and Asp908 from the C-terminal domain (CTD) are both approximately 6.8 Å from Glu509 and could contribute to catalyzing hydrolysis as acid/base residues (Supplementary Fig. 40c).

Monosaccharide metabolism

Ulvan degradation releases different monosaccharides to be further utilized by *F. agariphila*. Many of the enzymes involved in monosaccharide metabolism had significantly higher relative abundances with ulvan compared to fructose or rhamnose as substrate (Table 1, Supplementary Fig. 3, Supplementary Data Set 1). Based on this result and on the MetaCyc database²⁹, pathways for monosaccharide utilization were deduced, which are consistent with previously proposed pathways¹⁵. Unlike the PUL H-encoded polysaccharide-degrading

proteins, these monosaccharide-utilizing proteins are randomly distributed across the *F. agariphila* genome (Supplementary Fig. 3).

The spontaneous conversion of α - to β -anomer (mutarotation) of free α -L-rhamnose is a relatively slow process. This rate-limiting step affects growth of L-rhamnose-utilizing bacteria^{30,31} because the first metabolic enzyme rhamnose isomerase (EC 5.3.1.14) is specific for the β -anomer³². Various bacteria, such as *E. coli* and *Rhizobium leguminosarum*, contain the L-rhamnose mutarotase, accelerating the rate of mutarotation of α - to β -L-rhamnose^{37,38}. In contrast to the proteobacterial L-rhamnose mutarotase genes, which are part of small operons dedicated to the uptake and use of free L-rhamnose^{30,31}, the P21_mutarotase gene is localized in PUL H. We solved the crystal structure of the P21_mutarotase at 1.47 Å (Fig. 4, Supplementary Table 9) with one molecule in the asymmetric unit. P21_mutarotase adopts a ferredoxin-like fold with an antiparallel β -sheet of 4 β -strands flanked by a bundle of 3 α -helices. The P21_mutarotase structure superimposed with the characterized L-rhamnose mutarotases YiiL (PDB: 1x8d) and RhaU (PDB: 2qlw) with rmsd on C α of 0.76 Å and 0.73 Å, respectively^{30,31}. Similar to these, the P21_mutarotase (Fig. 4a and 4b) formed a dimer with a large hydrophobic dimeric interface antiparallel β -sheets from each monomer (Fig. 4c). All key residues of the active site are well conserved in the P21_mutarotase (Fig. 4d and 4e).

F. agariphila further metabolizes the β -L-rhamnose via L-rhamnulose-1-phosphate, which is then cleaved by an aldolase (putatively NP3_ or/and NP6_aldolase, Table 1) into L-lactaldehyde and dihydroxyacetone phosphate (Fig. 5)¹⁵. The corresponding genes are located directly upstream of PUL H (Supplementary Figs. 2 and 3). Glucuronic and unsaturated uronic acids are stepwise converted into KDG (2-dehydro-3-deoxy-D-gluconate), which enters the central metabolism via D-glyceraldehyde 3-phosphate and pyruvate (Fig. 5). Corresponding genes are encoded within PUL H, PUL A or elsewhere in the genome (Supplementary Figs. 3 and 41). NP8_isomerase and NP7_kinase convert D-xylose to D-xylulose-5P, which is an intermediate of the pentose phosphate pathway. In addition, putative monosaccharide transporters were identified (Fig. 5). A D-xylose transporter

(NP16_Xyle) was quantified in the membrane fraction in the subproteome experiments (Supplementary Data Set 2). Four ATP-binding proteins of ABC transporters were more abundant with ulvan or with rhamnose in the metabolic labeling experiments (Table 1, Supplementary Data Set 1), indicating that ABC-transporters are involved in monosaccharide uptake. Specific mono- or oligosaccharides generated by the above described enzymatic steps were also verified by HPLC-ELS-ESI-MS (Supplementary Figs. 42-48).

Discussion

Using the DNA sequence of the known ulvan polysaccharide lyase PL28 as query, 12 ulvan PULs were extracted from the NCBI-GenBank, including the biochemically characterized *F. agariphila* ulvan PUL. All PULs were from Bacteroidetes, indicating that our procedure was selective for this phylum since ulvan PULs also exist in Gammaproteobacteria³³. Interestingly, although four ulvan PULs were from the genus *Polaribacter*, they did not cluster on the heatmap (Figure 1c) indicating that ulvan PULs are diverse at the genus level. Also, within different ulvan PULs, PL28 or PLs from PLnc are over 50% identical at the pairwise amino acid sequence level. Conservation and invariable presence suggest that the first steps of the ulvan degradation cascade proceed through similar enzymes in these organisms. On the other hand, the GH88 enzyme was only present in ulvan PULs of *Flammeovirga pacifica* and *F. agariphila*. GH88 is an exo-acting, unsaturated glucuronyl hydrolase. Its absence in other ulvan PULs could be compensated for by the presence of a GH105, which has the same function. Thus, the later steps in ulvan degradation proceed in dissimilar ways in bacteria.

As shown in the protein domain distribution analysis, the most abundant proteins are sulfatases, which catalyze the removal of sulfate from ulvan. Sulfatase copy numbers ranged from 4-12. At the same time, PLs or GHs such as GH2, GH78 and GH39 in the *F. agariphila* ulvan PUL were also abundant and have several copies in the other predicted ulvan PULs. Notably, some of the proteins of the ulvan PUL, such as the sulfatase P36_GH78/S1_25, are

multimodular enzymes. Our analyses indicated similar domain structures of ulvan-degrading enzymes in other marine Bacteroidetes strains. However, the cursory inspection of gut *Bacteroides* genomes revealed no multimodular GH78 and sulfatase fusion proteins. This suggests that some gene fusions involved in polysaccharide degradation could be more abundant in the marine environment³⁴.

Our biochemical analyses demonstrated that six of the putative sulfatases (P11_S1_7, P12_S1_8, P18_S1_7, P19_S1_27, P32_S1_8 and P36_S1_25) are ulvan-active indeed sulfatases (Supplementary Fig. 4). However, the sulfatase P14_S1_7 was inactive on both, ulvan from Elicityl and a xylose-rich ulvan from Atlantic *Ulva* spp. and displayed only faint activity on an ulvan from Agrival. This apparent inactivity may be due to a strict exolytic character of P14_S1_7. Consequently, activity maxima are not the same for different types of ulvans. Substrate diversity may cause the variable enzyme content in Bacteroidetes (Figure 1c). This diversity may reflect an adaptation to the different types of ulvans present in *Ulva* spp. Such fine scale adaptation points towards the exploration of PUL microdiversity as a promising avenue for enzyme discovery and for the biocatalytic elucidation of ulvan structures.

Our elucidation of the enzymatic ulvan degradation cascade and characterization of 12 of its enzymes has major implications. Firstly, the conservation of CAZyme- and sulfatase-encoding genes in ulvan PULs of different bacteria underlines their importance and provides a mean to reliably predict new ulvan degradation pathways for bioengineering. Secondly, the substantially extended knowledge of the specific substrate scope of each enzyme enables the targeted use of these enzymes for the production of a variety of novel defined, tailor-made ulvan oligomers, representing useful products, e.g., for pharmaceutical or cosmetic applications. Moreover, these enzymes provide a way to deconstruct ulvan cell walls, which may facilitate the extraction of marine poly- or oligosaccharides and other valuable molecules such as proteins from *Ulva* spp. Finally, the enzymatic cascade allows for the production of

bulk monomeric sugars from the abundant, so far underexplored renewable, the green tide
Ulva.

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Author contributions

J.-H.H., T.S., G.M. and U.T.B. initiated the study and directed the project. L.R., A.P., R.L. and M.B. cloned the genes and expressed and purified the enzymes for the degradation reactions. M.B., J.-H.H. and L.R. isolated ulvan and purified oligomers. Metabolites were analyzed by C.S. via NMR and HPLC-ELS-MS for which M.D.M. provided resources. L.R. and M.B. performed biocatalysis for the analyses in gel-based assays whereas A.P. together

with M.B. performed HPAEC-PAD analyses. M.-K.Z. with support from S.M., F.U. and A.T.-S. performed the proteome analyses for which D.B. provided the resources. N.G., C.S.R. and T.R. performed crystallographic experiments and solved the protein structures. G.M. analyzed the crystal structure of the L-rhamnose mutarotase and of the sulfatases. S.T. performed the computational analyses of PUL predictions. J.-H.H. and L.R. wrote the paper with input from U.T.B., G.M., S.M., M.-K.Z. and T.S. All authors read and approved the final manuscript and declare that there is no conflict of interest.

Competing financial interests

The authors declare no conflict of interest.

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Figure legends

Fig. 1 | Genomic overview of putative ulvan PULs in marine Bacteroidetes and the proteomic response of the *F. agariphila* PUL to ulvan and rhamnose. **a**, Comparative genomics of ulvan PULs that contain the known PL28 ulvan lyase (connected with blue lines when over 50% identical) revealed that the enzymes are encoded by conserved genes in diverse marine Bacteroidetes genomes, including the model organism of this study, *F. agariphila* shown as #1; the complete list of all analyzed strains is provided in panel 1c. SusD and TBDR proteins are colored as 'other' in this panel. **b**, Ulvan and rhamnose as sole carbon source elicit quantitative changes in proteins encoded in the putative ulvan PUL in *F. agariphila*. Bars indicate relative changes between both conditions. A positive $\Delta\log_2$ value corresponds to higher protein abundance with ulvan, while a negative value corresponds to higher protein abundance with rhamnose. Stars mark proteins that were exclusively quantified in either ulvan- or rhamnose-grown cells (see Supplementary Fig. 2, Table 1 and Supplementary Data Set 1). Arrows refer to the orientation of genes that encode the respective proteins. Proteins encoded by the ulvan PUL were numbered (P1-P39) and protein function was indicated (see Table 1). In the case of glycoside hydrolases (GH) and sulfatases (S), families and subfamilies were specified^{19,20}, e.g. GH2 (family) or S1_7 (family and subfamily). **c**, Co-occurrence analysis of genes in the predicted 12 putative bacteroidetal ulvan PULs highlights a conserved set of ulvan-degrading enzymes. The dendograms shown above and to the left of the similarity heat map depict the pairwise similarities between rows and columns, respectively.

Figure 2 | Structural analyses of ulvan specific sulfatases. **a-c**, Molecular surface of P18_S1_7 (**a**) and of the human iduronate 2-sulfatase (PDB: 5FQL) (**b**) both of which belong to the S1_7 subfamily, as well as of the S1_25 sulfatase module of P36 (P36_S1_25) (**c**). These molecular surfaces are colored according to electrostatic potential ranging from deep blue, +, to red, -. **d-e**, Fold representation of P18_S1_7 (**d**), of the human iduronate 2-sulfatase (**e**) and of P36_S1_25 (**f**). The structures are shown in cartoon style. The α -helices and the β -strands are colored in cyan and magenta, respectively. **g**, Stereo view of the key conserved residues in the catalytic groove of P18_S1_7. The amino acids are presented as sticks. The calcium ion is shown as a yellow sphere. The molecular surface of P18_S1_7 is shown as semi-transparent background. **h**, Electron density around the catalytic calcium binding site of P18_S1_7. The coordination residues (Asp35, Asp36, Asp312 and His313) and the catalytic residue Cys74 are shown as sticks. Interactions with the calcium are represented by black dashed lines. The map shown is σA -weighted $2mF_o - DF_c$ maps contoured at 1.2σ ($0.07 \text{ e}/\text{\AA}^3$).

Figure 3 | Zooming into the degradation of ulvan fragments. The experimental procedure to uncover the order of enzymes for ulvan degradation is shown exemplarily for an

ulvan pentamer. All other investigated enzyme activities are shown in the Supplementary Information. All intermediate products were purified and their structures were confirmed by NMR and MS. MS spectra for individual oligomers are shown on the left next to the respective oligomer. Full spectra for all purified oligomers are shown in the Supplementary Information together with the corresponding NMR spectra. Red arrows indicate cleavage points of the following step. FACE gels for the analysis of the enzymatic interconversion steps are displayed on the right next to the respective enzyme. Full gel images including standards are shown in the Supplementary Information. The desulfation of Rha3S was detected by HPAEC-PAD and the full chromatograms are shown in the Supplementary Information. Numbers with “S” attached to the sugar symbols indicate the position of sulfate groups.

Figure 4 | Structure of the L-rhamnose mutarotase P21_mutarotase. **a**, Stereo view of the P21_mutarotase dimer shown in cartoon style. **b**, Stereo view of the molecular surface of the P21_mutarotase dimer color coded according to electrostatic potential ranging from deep blue, +, to red, -. **c**, Electron density around the inter-subunit β -sheet in the mutarotase P21_mutarotase dimer. The β 4-strand found at the C-terminal extremity of the subunit B is involved in β -sheet formation with the subunit A through hydrogen bonding with the β 2-strand. Subunits A and B are green and yellow, respectively. Hydrogen bonds between β 2 and β 4 are shown as black dashed line. The map shown is σ A-weighted $2mF_o - DF_c$ maps contoured at 1.2σ ($0.12 \text{ e}/\text{\AA}^3$). **d-e**, Stereo view of the active site of P21_mutarotase (**d**) and of YiiL bound to an L-rhamnose (**e**). The amino acids are presented as sticks. The carbon atoms are colored in yellow and in cyan in P21_mutarotase and YiiL, respectively. The small red spheres are water molecules in the P21_mutarotase structure.

Figure 5 | Model of the ulvan degradation pathway in *F. agariphila* as suggested by the proteogenomic, biochemical and structural biological analyses in this study. Redundant pathways are omitted to maintain clarity. The ulvan molecule on top represents a part within the larger ulvan chain where rhamnose and iduronate are α - while xylose and glucuronate are β -configured. The formed products – at both ends of the initial ulvan molecule after cleavage with P30_PL28 – are not shown in the downstream degradation pathway. Activity of ulvan lyases P30_PL28 and P10_PLnc will form an unsaturated uronic acid residue from glucuronic acid or iduronic acid at the non-reducing end of the products. Numbers with “S” attached to the sugar symbols indicate the position of sulfate groups. Black arrows indicate pathways elucidated by proteogenomic, biochemical and structural biological analyses, while grey arrows only refer to proteome analyses or additional structural analyses in the case of P21_mutarotase. For numbering/nomenclature see Table 1. For reasons of simplicity, the linkage of the TBDRs to the TonB-ExbBD complex or a putative membrane association of certain enzymes were not included. KDG: 2-dehydro-3-deoxy-D-gluconate; DKII: 5-dehydro-4-deoxy-D-glucuronate; DKII: 3-deoxy-D-glycero-2,5-hexodiulosonate.

Table 1 | List of PUL H-encoded and relevant non-PUL H-encoded proteins with abbreviations used in the text, corresponding locus tags and functional annotation as well as their relative abundance (mean log₂ ratio) with the respective carbon source. Empty/white squares refer to non-quantified proteins while grey squares indicate OFF-proteins that could not be quantified due to a lack of ¹⁴N signals (see Online Methods)

PUL H-encoded proteins (for ulvan and ulvan-derived monosaccharide utilization)			log ₂ ratio		
Abbreviation	Locus tag	Functional annotation	fru	rha	ulv
P1_GH88	*21900	unsaturated glucuronyl hydrolase (GH88)			
P2_SusD	*21910	SusD-like protein			
P3_TBDR	*21920	TonB-dependent receptor			
P4_HK	*21930	histidine kinase			
P5_isomerase	*21940	4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase			
P6_dehydrogenase	*21950	2-deoxy-D-gluconate 3-dehydrogenase			
P7	*21960	conserved hypothetical protein			
P8_GH2	*21970	beta-galactosidase (GH2)			
P9_lactonase	*21980	6-phosphogluconolactonase			
P10_PLnc	*21990	ulvan lyase (PLnc)			
P11_S1_7	*22000	iduronate-2-sulfatase (S1_7)			
P12_S1_8	*22010	arylsulfatase (S1_8)			
P13_S1_16	*22020	arylsulfatase (S1_16)			
P14_S1_7	*22030	arylsulfatase (S1_7)			
P15_GH2	*22040	glycoside hydrolase (GH2)			
P16_GH2	*22050	beta-galactosidase (GH2)			
P17_GH2	*22060	beta-galactosidase (GH2)			
P18_S1_7	*22070	arylsulfatase (S1_7)			
P19_S1_27	*22080	sulfatase (S1_27)			
P20_GH78	*22090	alpha-L-rhamnosidase (GH78)			
P21_mutarotase	*22100	L-rhamnose mutarotase			
P22	*22110	conserved hypothetical protein			
P23	*22120	conserved hypothetical protein			
P24_GH3	*22130	beta-glucosidase (GH3)			
P25_SusD	*22140	SusD-like protein			
P26_TBDR	*22150	TonB-dependent receptor			
P27_GH43	*22160	beta-xylosidase (GH43)			
P28_GH78	*22170	alpha-L-rhamnosidase (GH78)			
P29	*22180	conserved hypothetical protein			
P30_PL28	*22190	ulvan lyase (PL28)			
P31_GH39	*22200	glycoside hydrolase (GH39)			
P32_S1_8	*22210	arylsulfatase (S1_8)			
P33_GH105	*22220	glycoside hydrolase (GH105)			
P34_GH3	*22230	beta-glucosidase (GH3)			
P35_oxidoreductase	*22240	oxidoreductase			
P36_GH78/S1_25	*22250	alpha-L-rhamnosidase/sulfatase (GH78/S1_25)			
P37	*22260	hypothetical protein			
P38_SusD	*22270	SusD-like protein			
P39_TBDR	*22280	TonB-dependent receptor			
Non-PUL H-encoded proteins (for ulvan-derived monosaccharide utilization)			log ₂ ratio		
Abbreviation	Locus tag	Functional annotation	fru	rha	ulv
NP1_dehydrogenase	*21840	aldehyde dehydrogenase A			
NP2_dehydrogenase	*21850	L-lactate dehydrogenase			
NP3_aldolase	*21860	class II aldolase/adducin family protein			
NP4_kinase	*21870	pentulose/hexulose kinase			
NP5_isomerase	*21880	rhamnose isomerase ^a			
NP6_aldolase	*21890	rhamnulose-1-phosphate aldolase			
NP7_kinase	*160	xylulose kinase			
NP8_isomerase	*170	xylose isomerase			
NP9_oxidoreductase	*9410	D-mannonate oxidoreductase			
NP10_dehydratase	*9420	mannonate dehydratase			
NP11_isomerase	*9430	uronate isomerase			
NP12_kinase	*9800	2-dehydro-3-deoxygluconate kinase			
NP13_aldolase	*9820	aldolase ^b			
NP14_kinase	*11640	2-dehydro-3-deoxygluconate kinase			
NP15_kinase	*16400	2-dehydro-3-deoxygluconate kinase			
NP16_XylE	*180	D-xylose transporter XylE ^c			
NP17_ABC	*11090	ABC transporter, ATP-binding protein			
NP18_ABC	*25150	ABC transporter, ATP-binding protein			
NP19_ABC	*7480	ABC transporter, ATP-binding protein			

NP20_ABC	*12820	ABC transporter, ATP-binding protein	
Proteins were numbered (P1 - P39: PUL H-encoded proteins, NP1 - NP20: non-PUL H-encoded proteins) and protein function was indicated. In the case of glycoside hydrolases (GH) and sulfatases (S), families and subfamilies were specified ^{19,20} , e.g. GH2 (family) or S1_7 (family and subfamily). *BN863_ e.g. **21900 refers to locus tag BN863_21800; ^a identified by BLAST against the Uniprot database, previously annotated as xylose isomerase-like TIM barrel domain protein, ^b 4-hydroxy-2-oxoglutarate aldolase / 2-dehydro-3-deoxyphosphogluconate aldolase, ^c only captured by subproteome analysis of ulvan-grown cells (Supplementary Data Set 2), fru: fructose, rha: rhamnose, ulv: ulvan			log ₂ ratio 1.5 -7

Online Methods

Prediction of ulvan PULs

118,981 bacterial genomes were downloaded from the NCBI-GenBank using an in-house script (updated in 2018.09.10). Hmmer 3.0 was used to identify proteins with a PL28 or sulfatase domain, using a cut-off value of $1e-10^{35}$. Hidden Markov models of PL28 and sulfatase were obtained from dbCAN2 and the pfam database, respectively^{36,37}. Models for the new PLnc family have not been released, thus blastp was used to identify its homologs, using 1e-50 and 30% sequence identity as cut-off values³⁸. In each bacterial genome, if the adjacent 50 proteins to the afore-mentioned marker genes contained three marker genes (PL28, PLnc and sulfatase), this locus was considered as a potential ulvan PUL hit. To further determine PUL boundaries, 100 proteins surrounding the predicted ulvan PUL were collected and then locally annotated using pfam and dbCAN Hidden Markov models (cut value 1e-10). Firstly, PL28 or PLnc families were set as boundaries, which were extended if adjacent genes are annotated as sugar utilization proteins, such as GH, PL, sugar transporter and transcription factors. In cases where five continuous genes were not related to sugar utilization or ulvan degradation, the last functionally relevant protein was taken as the putative ulvan PUL boundary. Protein sequences within putative ulvan PULs were collected for further analysis. Circos was used to visualize the different ulvan PULs³⁹. Blastp was used to calculate the identity between PL28 sequences from different ulvan PULs (cut-off value: 1e-10, over 50% identity). To simplify and reduce non-conserved proteins, domains with less than 80% presence among the predicted ulvan PULs were excluded. Domain numbers in each PUL were counted, summarized and displayed in R studio.

Proteome analyses

Whole cell proteome – metabolic labeling

A $^{14}\text{N}/^{15}\text{N}$ relative quantification approach, based on metabolic labeling, was used for protein quantification as described previously⁴⁰. For this purpose, *F. agariphila* KMM 3901^T was cultivated in MPM salts⁴¹ containing either ^{14}N - or ^{15}N -ammonium chloride, supplemented with 0.2% of the individual carbon source: ulvan, rhamnose or fructose. Cultivation (21°C, 170 rpm) comprised three steps: (i) 24 h of marine broth 2216-cultivation and subsequent (ii) pre-cultures as well as (iii) main cultures in the above-described minimal medium. At an $\text{OD}_{600\text{nm}}$ of 0.5, cells were harvested from main cultures by centrifugation (30 min, 9,384 x g, 4°C). Cell pellets were suspended in TE-buffer (10 mM Tris, 10 mM EDTA) and cells were disrupted by sonication (4 cycles of 25 s at 5 m/s). Cell debris and protein extract were separated by centrifugation (10 min, 21,460 x g, 4 °C). In case of the ^{15}N -labeled samples, protein extracts of all samples from all 3 carbon sources were combined to form the ^{15}N -labeled reference pool, which served as an internal standard. Protein concentration was determined using the PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific). 12.5 µg of protein of each ^{14}N -sample was combined with 12.5 µg protein of the ^{15}N -labeled pool. These mixtures were separated by 1D-SDS-PAGE. Protein lanes were cut into ten equal-sized pieces, destained and proteins were in-gel-digested with a 1 µg mL⁻¹ trypsin solution⁴². Peptides were separated by RP chromatography and analyzed in an LTQ-Orbitrap Classic mass spectrometer equipped with a nanoelectrospray ion source⁴³. Data represent three independent experiments (n=3).

MS data were searched with Sorcerer SEQUEST v.27, rev.11 (Thermo-Finnigan, Thermo Fisher Scientific, Germany) against a target decoy database including all *F. agariphila* KMM 3901^T protein sequences, corresponding reversed sequences (decoys) as well as common laboratory contaminants (total 7224 entries) as described previously⁴³, but using a false positive rate of 0.05. In brief, peak intensities of the ^{14}N -peptide ions of a protein versus its ^{15}N -peptide ions were compared to calculate a regression ratio. Only unique peptides and

peptides with an R^2 above 0.7 were taken into account. Non-quantified peptides were manually checked. Average regression ratios were then exported. Proteins with at least two quantified peptides were considered for the following calculations: ratios were median-centered and log-transformed, termed as $\log_2$ ratios, per sample. If proteins were quantified in at least two of the three replicates, means and standard deviations (SD) were calculated from these values. In order to identify relative changes between the different carbon sources, $\log_2$ ratios of fructose- or rhamnose-cultivated cells were subtracted from $\log_2$ ratios of ulvan-cultivated cells, termed $\Delta\log_2$. Fold-changes correspond to the exponentials of these $\Delta\log_2$ values. Statistical analyses were performed with Welch's two-sided t-test (permutation-based false discovery rate 0.01) using Perseus v1.6.0.7⁴⁴, based on the $\log_2$ ratios. Putative ON/OFF proteins were marked with 15N (OFF) or 14N (ON) in Supplementary Data Set 1, but were not included in any of the calculations. Only if a protein was identified as an ON/OFF protein in all three replicates, it was assigned to a fixed value (10/-10), to highlight these proteins.

Subproteome fractionation

F. agariphila KMM 3901^T was cultivated as described above, except that no ¹⁵N-labeling was performed and only ulvan was applied as a carbon source.

For the surface proteome (trypsin-shaving approach), 1.5 mL of cell suspension was removed from the culture and centrifuged (5 min, 5,867 x g, 4 °C). Cells were washed with 50 mM triethylammoniumbicarbonate-buffer (TEAB) and finally resuspended in 45 μ L TEAB-buffer. In order to cleave proteins from the cell surface, 5 μ L of a 1 μ g mL⁻¹ trypsin solution was added. The solution was transferred onto a 0.22 μ m cellulose-acetate spin-column and incubated for 15 min at 900 rpm and 37 °C. The flow-through was collected by centrifugation (10 min, 4,000 x g, 4 °C), another 1 μ L of trypsin was added and the sample was incubated at 900 rpm and 37 °C overnight. The peptide mixture was desalted using C18 StageTips. The following solutions were used: 0.1% (v/v) acetic acid in ultra-pure water (buffer A) and 0.1% (v/v) acetic acid in acetonitrile (buffer B). Before the sample was added, C18 material

was rinsed and equilibrated with buffer A and washed with buffer B in between these steps. After the sample was added, buffer A was used for washing and buffer B for elution.

In the case of cytosolic, membrane-associated and extracellular protein fractions, 100 mL of cell suspension was harvested by centrifugation (30 min, 9,384 x g, 4 °C). Cell pellets and supernatants were processed separately as previously described^{45,46}. 1D-SDS-PAGE, in-gel-digestion and LC-MS/MS analysis were performed as described above. Experiments were carried out in triplicates (n=3).

Database searches were done with Sorcerer SEQUEST v.27, rev.11 (see above). Results were summarized and filtered using Scaffold 4.4.1.1 (Proteome Software, Portland, OR, USA): protein and peptide false discovery rate was set to 0.01 and protein identification required two peptides minimum. For protein quantification, the normalized spectral abundance factor was calculated for each protein giving the percentage (%NSAF) of all proteins in the same sample⁴⁷. If proteins were identified in at least two of the three replicates, they were considered for further calculations.

Gene cloning and expression

Expression constructs were prepared using the FastCloning strategy⁴⁸ with genomic DNA from *F. agariphila* KMM 3901^T (collection number DSM15362 at DSMZ, Braunschweig, Germany) as template for the amplification of the inserts. Generally, the pET28 constructs were prepared as described previously¹⁴ with the gene primers shown in Supplementary Table 10. To clone the gene for the formylglycine-generating enzyme (FGE) from *F. agariphila*, the vector backbone was amplified with the primers 5'-AATA GCGC CGTC GACC ATCA TCAT CATC ATCAT-3' and 5'-CATG GTTA ATTG CTCC TGTT AGCC CAAA AA-3' from pBAD/myc-his A. For the pFA, constructs were cloned and overexpressed as previously described⁴⁹. Briefly, genes were PCR-amplified using the NEB Q5 High-Fidelity DNA Polymerase system. PCR reactions were done with 30 cycles (denaturation: 95 °C; annealing: 60 °C; elongation: 72 °C) using 0.5 units of enzyme in a total reaction of 50 µL

using the primers shown in Supplementary Table 10. Amplicons were cleaned up using the QIAquick PCR Purification Kit (Qiagen) and digested with the appropriate restriction endonucleases. All ligations were done in the linearized T7 system vector pFO4.

Genes encoding the sulfatases P18_S1_7, P19_S1_27, P32_S1_8 and P36_S1_25 were ordered codon-optimized for *E. coli* and sub-cloned into pET28 with NheI and XhoI from Genscript. The optimized nucleotide sequences are shown in the Supplementary Information.

Escherichia coli BL21(DE3) was transformed with pET28-based plasmids. For the overexpression, 50 mL ZYP-5052⁵⁰ with 100 µg mL⁻¹ kanamycin were inoculated from an overnight culture in LB containing 50 µg mL⁻¹ kanamycin. The culture was grown at 30 °C and 180 rpm until the OD_{600nm} reached 1.0 and was then cooled to 20 °C for 48 h. In the case of sulfatases, the formylglycine-generating enzyme (FGE) from *F. agariphila* was co-expressed. LB medium with 100 µg mL⁻¹ ampicillin and 50 µg mL⁻¹ kanamycin was inoculated from an overnight culture in the same medium and incubated at 37 °C and 180 rpm until the OD_{600nm} reached 0.3 to 0.5. After the addition of 1.5 mM L-arabinose and incubation for 90 min at 37 °C, the culture was cooled to 18 °C for 2 h before 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and the culture was incubated overnight at 18 °C. Alternatively, sulfatases were expressed from the pFA constructs in *E. coli* BL21(DE3) cells grown in LB medium supplemented with 15 µg mL⁻¹ ampicillin, at 37 °C, until reaching an OD_{600 nm} of 0.8. Expression was induced with 0.1 mM IPTG overnight at 18 °C. For crystallization screening, *E. coli* BL21(DE3) cells were transformed with the plasmids containing the gene fragment of interest, then grown in the autoinduction Zyp-5052 medium (200 µg mL⁻¹ ampicillin, 20 °C, 72 h). Cells were harvested by centrifugation (10,000 x g, 4 °C, 20 min) and the cell pellets were stored at -20 °C until further use.

Samples from the cultivations equivalent to 1 mL of culture with an OD_{600nm} of 7 were taken before harvest and the cells were collected by centrifugation (13,000 x g, 4 °C, 2 min). Pellets were resuspended in 500 µL 50 mM HEPES with 100 mM NaCl (pH 7.4). After

chemical lysis with BugBuster (Merck, Darmstadt, Germany), whole cell protein (W) samples were obtained prior to removal of the cell debris by centrifugation (13,000 x g, 4 °C, 10 min). Samples of the soluble protein fraction (S) were taken from the respective supernatant.

Enzyme purification

Cell pellets were thawed and resuspended in 50 mM NaPi with 300 mM NaCl (pH 8.0) and lysed by three cycles of sonication (2.0 min, 30% pulse, 50% power). After centrifugation (10,000 x g, 4 °C, 20 min), the supernatant was filtered (0.45 µm) and loaded onto a 5 mL HisTrap FF crude column (GE Healthcare, Freiburg, Germany) equilibrated with lysis buffer. Alternatively, Rotigarose-His/Ni beads (Karl Roth, Karlsruhe, Germany) were used in gravity flow columns. After washing, the protein was eluted with 50 mM NaPi and 300 mM NaCl containing 300 mM imidazole (pH 8.0). Fractions containing the protein of interest were pooled and desalted using PD-10 columns (GE Healthcare, Freiburg, Germany) equilibrated with 50 mM NaPi pH 7.4. Proteins were analyzed by SDS-PAGE on 12.5% acrylamide gels. 1% (v/v) 2,2,2-trichloroethanol was used for the visualization of proteins under UV light⁵¹. Alternatively, proteins were stained with Coomassie Blue (PhastGel® Blue R). All enzymes were used undiluted, or in dilutions of 1:5, 1:10 or 1:20 with enzyme storage buffer (Supplementary Table 11).

Alternatively, cells were subjected to mechanical lysis and cytoplasmic extracts were loaded onto an Histrap column (5ml, GE Healthcare) equilibrated with 50 mM Tris, 0.2 M NaCl, 20 mM imidazol, 1 mM CaCl₂ at pH 8.0. Recombinant proteins were eluted with around 250 mM imidazole and then loaded onto a Hiprep Desalting column (26/10, 53ml, GE Healthcare) in order to eliminate the imidazole, which notably interfered with sulfatase activity. Purified enzymes were concentrated (Amicon® Ultra Centrifugal Filter, 30 kDa) to a concentration of 1 mg mL⁻¹ (Nanodrop).

750 **Purification of ulvan**

751 Green tide *Ulva* sp. was collected near Roscoff (France) and dried. Alternatively, dried *Ulva*
752 biomass from the Atlantic coast in Spain was purchased as organic sea lettuce (Kulau,
753 Berlin, Germany). Ulvan was extracted according to the literature⁵². The dialysis step was
754 exchanged by precipitation with acetone (80% (v/v) final concentration). After washing,
755 acetone-precipitated ulvan was dissolved in deionized water and freeze-dried. Alternatively,
756 ulvan was obtained from Agrival (Plouenan, France) or Elicityl (Grenoble, France).

757 **Enzyme assays**

758 Generally, reactions were performed in 50 mM HEPES pH 7.4 with 100 mM NaCl or 35 mM
759 Tris pH 8.0 with 50 mM NaCl. Initial degradation of ulvan into larger oligomers was monitored
760 by C-PAGE, while smaller degradation products and the conversion of purified oligomers
761 was analyzed by FACE. For lyases, the increase in absorbance at 235 nm was recorded
762 over time. For unsaturated uronyl hydrolases (GH88 and GH105), the decrease in
763 absorbance at 235 nm of ulvan lyase products was monitored. For screening reactions, 10%
764 (v/v) clarified lysate as used for the SDS-PAGE was added. Untreated ulvan was generally
765 used at a concentration of 1 g L⁻¹ while purified oligomers were used at 0.25 mg mL⁻¹.
766 Incubation was performed overnight at room temperature.

767 **Sulfatase activity assay on ulvan polymers**

768 Activity assays were conducted on three different ulvan polymers from *Ulva* species: a
769 commercial ulvan from Elicityl (Grenoble, France), an ulvan which was a gift from the
770 company Agrival (Plouenan, France), and an ulvan extracted from *Ulva* sp. harvested on
771 Brittany north coast (Roscoff, France). 10 µL of each ulvan solution (1% w/v in H₂O) was
772 incubated with 10 µL of purified sulfatase (1 mg mL⁻¹) in a final volume of 80 µL of 25 mM
773 Tris-HCl, 0.1 M NaCl, 0.5 mM CaCl₂, pH 8.0 buffer mix, for 18 h at 37 °C. For each reaction,
774 a control sample was prepared using similar conditions but with an inactivated enzyme

(100 °C, 10 min). Reaction mixtures and blanks were then filtered (10 kDa, Amicon® Ultra, Millipore) to measure the amount of free sulfate in the filtrates.

Ulvan-specific sulfatase activity was measured by high-performance anion-exchange chromatography (HPAEC). Using an ICS5000 system (*Thermo Scientific Dionex*), anions from reaction mixture filtrates were injected (AS-AP Autosampler) and separated using an AG11-HC guard column (4x50 mm) mounted in series with an AS11-HC anion-exchange column (4x250 mm). Elutions were performed with isocratic 12 mM NaOH at a flow rate of 1 mL min⁻¹ (Single Pump-5), and the detection of anions was leaded by an Analytical CD Conductivity Detector associated to a suppressor (ASRS 500, 4 mm) running at 50 mA. Using a standard curve of sulfate, concentration of sulfate released by the enzymatic reaction was calculated from the difference of the amount of sulfate between samples and the associated blanks.

Sulfatase activity assay on characterized ulvan oligosaccharides

10 µL of ulvan oligosaccharides (0.5-1% w/v in H₂O) were incubated with 15 µL of purified sulfatase (0.5 mg mL⁻¹) in a final volume of 75 µL of 5 mM Tris-HCl, 10 mM NaCl, 0.5 mM CaCl₂, pH 8.0 buffer, for 18 h at 37 °C. The recombinant enzymes P33_GH105 or P36_GH78 were added (2 µL – 3 mg mL⁻¹). Each reaction mixture was centrifuged (14,000 x g for 10 min) before injection. Oligosaccharide detection was realized by HPAEC analyzes on the same ICS 5000 system described for the sulfate quantification. Elutions were performed at a flow rate of 0.5 mL min⁻¹ using a NaOH multistep gradient from 8 to 280 mM (45 min). Oligosaccharides were detected by conductivity mode under a current suppression of 50-300 mA.

Carbohydrate polyacrylamide gel electrophoresis

Fluorophore-assisted carbohydrate electrophoresis (FACE) was performed with 2-aminoacridone (AMAC) as fluorophore⁵³.

For carbohydrate polyacrylamide gel electrophoresis (C-PAGE), samples were mixed with an equal volume of FACE loading buffer⁵³. Gels and running conditions were identical to FACE. Carbohydrates were visualized by staining with Stains-All solution (0.25 g L⁻¹ in 1.7 mM Tris-HCl pH 7.5 with 25% (v/v) isopropanol). The gels were destained with 25% (v/v) isopropanol in deionized water.

Purification of oligomers and structure determination

Ulvan was digested with purified enzymes in Tris-HCl pH 8.5 at room temperature. Oligomers were separated on two XK 26/100 (GE Healthcare, Freiburg, Germany) in series filled with Bio-Gel P-2 (Rio-Rad, Munich, Germany) using 100 mM (NH₄)₂CO₃ as mobile phase at a flow rate of 1 mL min⁻¹. After lyophilization of the fractions containing the products, oligomers were dissolved in D₂O and lyophilized three times before NMR spectra were recorded on a Bruker Avance III HD 600 (600 MHz) spectrometer (Bruker, Billerica, USA) in D₂O solutions. The structures were independently elucidated based on 1D and 2D (COSY, HSQC, HMBC, TOCSY) methods and the assigned ¹H and ¹³C-NMR signals were then compared with literature data, showing excellent consistency^{8,9}. For samples containing uronic acid structures, it was required to neutralize the otherwise acidic NMR samples with Na₂HPO₄ to pH 7-8 (pH-electrode calibrated to H⁺) in order to achieve fully resolved signals for the carboxylic acid and neighboring positions (¹³C). HPLC-ELS-MS analysis was performed by injection of ~0.1% solutions (1–5 µL) on a Nexera UHPLC system from Shimadzu (equipped with two binary LC-30AD pumps plus degassers, a CTO-20 column oven) and a LCMS-2200 EV MS-detector and an additional ELS-detector (JASCO ELS-2041). Analysis was performed with mobile phase A = H₂O (0.1% HCOOH) and mobile phase B = CH₃CN on a C18 column (XSelect CSH XP C18 2.5 µm 3 x 50 mm) at 40 °C. Flow rate was 1.3 mL min⁻¹ (0–3 min) with 5% B from 0–0.15 min, 5–98% B from 0.15–2.2 min and 98%–5% B from 2.2–2.5 min.

Crystallization of proteins and structure determination

Crystallization trials of P18_S1_7 (pFA13 construct) and of the family S1_25 sulfatase module of the bimodular GH78 L-rhamnosidase P36 (pET28 construct, referred to as P36_S1_25) were undertaken at room temperature using the vapor-diffusion method in sitting drops containing a 2:1 ratio of pure protein (12.9 and 13.0 mg mL⁻¹, respectively) and of precipitant solution. P18_S1_7 and P36_S1_25 were mixed with reservoir solution containing 100 mM MIB pH 5.0 and 25 % PEG 1,500 and 100 mM MES pH 6.5 and 25 % PEG 2,000 MME, respectively. Crystals of the L-rhamnose mutarotase P21_mutarotase (pFA16 construct, concentration: 14.9 mg mL⁻¹) were obtained by the hanging-drop vapor-diffusion method at room temperature and also at a 2:1 protein/precipitant ratio with a reservoir solution containing 100 mM sodium acetate pH 4.6 and 4.3 M sodium formate. Crystals of P18_S1_7, P21_mutarotase and P36_S1_25 were cryoprotected with 10%, 14% and 14% glycerol, respectively, and flash-frozen in liquid nitrogen. X-ray diffraction experiments were carried out at 100 K at beamlines PROXIMA-1 (PX1) for P18_S1_7 and P21_mutarotase and PROXIMA-2 for P36_S1_25 (SOLEIL Synchrotron, GIF-sur-YVETTE, France). Diffraction data of P18_S1_7, P21_mutarotase and P36_S1_25 were obtained at 1.23, 1.47 and 2.91 Å, respectively, and were processed using XDS⁵⁴. Scaling and merging were performed using the program Aimless from the CCP4 package⁵⁵. The structure of P21_mutarotase (a dimer of 2 x 115 residues), P18_S1_7 (475 residues), and P36_S1_25 (467 residues) were solved by molecular replacement with the CCP4 suite program MolRep⁵⁶ using the structures of the rhamnose mutarotase RhaU from *Rhizobium leguminosarum* (PDB entry: 2QLX)³¹, of the human iduronate-2-sulfatase (5FQL)²³ and of the putative sulfatase YidJ from *Bacteroides fragilis* (2QZU) as starting models, respectively. Refinement and model building of P18_S1_7 and P21_mutarotase were undertaken using the PHENIX program suite⁵⁷ and the Coot software⁵⁸. Initial refinement of the P36_S1_25 structure was performed with BUSTER⁵⁹ and PHENIX⁵⁷, and then manual examination and rebuilding of the refined coordinates were carried out in Coot⁵⁸. Structural validation was undertaken using MOLPROBITY⁶⁰.

SEC-purified P17_GH2 crystallized in 1:1 ratio of 7 mg mL⁻¹ protein in 20 mM Tris pH 8.0 and mother liquor in the JBScreen PACT ++ HTS and JBScreen Classic HTS I (Jena Bioscience). A single crystal from the screen grown in 20 % PEG 3350, 0.1 M Bis-Tris pH 7.5, 0.2 M sodium bromide was cryo-protected in 30 % glycerol prior X-ray crystallography. The diffraction data were collected at DESY P11 automatically integrated in XDS and scaled and merged in Aimless^{54,61}.

The structure of P17_GH2 was solved by molecular replacement using 5dmy as search model in phaser⁶². The structure was built automatically using buccaneer and manually in Coot building directly into the 2Fo-Fc maps^{58,63}. Structural validation was carried out using MOLPROBITY⁶⁰.

Data availability

All data that support the findings of this study are available from the corresponding authors upon reasonable request. The protein structures are deposited in the PDB under 6HHM, 6HHN, 6HPD and 6HR5. Mass spectrometry data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository⁶⁴ with the dataset identifier PXD009299. The sequences of the newly characterized ulvan-degrading enzymes can be found in the GenBank sequence database under the respective locus tags mentioned in Table 1.

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Table 1 | List of PUL H-encoded and relevant non-PUL H-encoded proteins with abbreviations used in the text, corresponding locus tags and functional annotation as well as their relative abundance (mean log₂ ratio) with the respective carbon source. Empty/white squares refer to non-quantified proteins while grey squares indicate OFF-proteins that could not be quantified due to a lack of ¹⁴N signals (see Online Methods)

PUL H-encoded proteins (for ulvan and ulvan-derived monosaccharide utilization)			log ₂ ratio		
Abbreviation	Locus tag	Functional annotation	fru	rha	ulv
P1_GH88	*21900	unsaturated glucuronyl hydrolase (GH88)			
P2_SusD	*21910	SusD-like protein			
P3_TBDR	*21920	TonB-dependent receptor			
P4_HK	*21930	histidine kinase			
P5_isomerase	*21940	4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase			
P6_dehydrogenase	*21950	2-deoxy-D-gluconate 3-dehydrogenase			
P7	*21960	conserved hypothetical protein			
P8_GH2	*21970	beta-galactosidase (GH2)			
P9_lactonase	*21980	6-phosphogluconolactonase			
P10_PLnc	*21990	ulvan lyase (PLnc)			
P11_S1_7	*22000	iduronate-2-sulfatase (S1_7)			
P12_S1_8	*22010	arylsulfatase (S1_8)			
P13_S1_16	*22020	arylsulfatase (S1_16)			
P14_S1_7	*22030	arylsulfatase (S1_7)			
P15_GH2	*22040	glycoside hydrolase (GH2)			
P16_GH2	*22050	beta-galactosidase (GH2)			
P17_GH2	*22060	beta-galactosidase (GH2)			
P18_S1_7	*22070	arylsulfatase (S1_7)			
P19_S1_27	*22080	sulfatase (S1_27)			
P20_GH78	*22090	alpha-L-rhamnosidase (GH78)			
P21_mutarotase	*22100	L-rhamnose mutarotase			
P22	*22110	conserved hypothetical protein			
P23	*22120	conserved hypothetical protein			
P24_GH3	*22130	beta-glucosidase (GH3)			
P25_SusD	*22140	SusD-like protein			
P26_TBDR	*22150	TonB-dependent receptor			
P27_GH43	*22160	beta-xylosidase (GH43)			
P28_GH78	*22170	alpha-L-rhamnosidase (GH78)			
P29	*22180	conserved hypothetical protein			
P30_PL28	*22190	ulvan lyase (PL28)			
P31_GH39	*22200	glycoside hydrolase (GH39)			
P32_S1_8	*22210	arylsulfatase (S1_8)			
P33_GH105	*22220	glycoside hydrolase (GH105)			
P34_GH3	*22230	beta-glucosidase (GH3)			
P35_oxidoreductase	*22240	oxidoreductase			
P36_GH78/S1_25	*22250	alpha-L-rhamnosidase/sulfatase (GH78/S1_25)			
P37	*22260	hypothetical protein			
P38_SusD	*22270	SusD-like protein			
P39_TBDR	*22280	TonB-dependent receptor			
Non-PUL H-encoded proteins (for ulvan-derived monosaccharide utilization)			log ₂ ratio		
Abbreviation	Locus tag	Functional annotation	fru	rha	ulv
NP1_dehydrogenase	*21840	aldehyde dehydrogenase A			
NP2_dehydrogenase	*21850	L-lactate dehydrogenase			
NP3_aldolase	*21860	class II aldolase/adducin family protein			
NP4_kinase	*21870	pentulose/hexulose kinase			
NP5_isomerase	*21880	rhamnose isomerase ^a			
NP6_aldolase	*21890	rhamnulose-1-phosphate aldolase			
NP7_kinase	*160	xylulose kinase			
NP8_isomerase	*170	xylose isomerase			
NP9_oxidoreductase	*9410	D-mannonate oxidoreductase			
NP10_dehydratase	*9420	mannonate dehydratase			
NP11_isomerase	*9430	uronate isomerase			
NP12_kinase	*9800	2-dehydro-3-deoxygluconate kinase			
NP13_aldolase	*9820	aldolase ^b			
NP14_kinase	*11640	2-dehydro-3-deoxygluconate kinase			
NP15_kinase	*16400	2-dehydro-3-deoxygluconate kinase			
NP16_XylE	*180	D-xylose transporter XylE ^c			
NP17_ABC	*11090	ABC transporter, ATP-binding protein			
NP18_ABC	*25150	ABC transporter, ATP-binding protein			
NP19_ABC	*7480	ABC transporter, ATP-binding protein			
NP20_ABC	*12820	ABC transporter, ATP-binding protein			
Proteins were numbered (P1 - P39: PUL H-encoded proteins, NP1 - NP20: non-PUL H-encoded proteins) and protein function was indicated. In the case of glycoside hydrolases (GH) and sulfatases (S), families and subfamilies were specified ^{21,22} , e.g. GH2 (family) or S1_7 (family and subfamily). *BN863_, e.g. “*21900” refers to locus tag BN863_21800; ^a identified by BLAST against the Uniprot database, previously annotated as xylose isomerase-like TIM barrel domain protein, ^b 4-hydroxy-2-oxoglutarate aldolase / 2-dehydro-3-deoxyphosphogluconate aldolase, ^c only captured by subproteome analysis of ulvan-grown cells (Supplementary Data Set 2), fru: fructose, rha: rhamnose, ulv: ulvan			log ₂ ratio 1.5 -7		

Ulvan

Rha

DKI

GlcA

Xyl

e