nature portfolio

Peer Review File

Mechanisms of recalcitrant fucoidan breakdown in marine Planctomycetota

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript describes the isolation and identification of two fucoidan degrading Planctomycetota strains associated with marine algae. Transcriptomics and proteomics are used to identify the cazymes associated with fucoidan breakdown, and a structure of an endo-fucoidanase is presented. Finally, the authors present some interesting microscopy showing different degradation/uptake strategies for fucoidan from the two isolated strains.
General comments:
The isolation, characterisation and 'omics sections of this work are significantly stronger than the enzymology. This is interesting and thorough work but the authors need to tone down some of the more hyperbolic language, eg 'exceptionally reduced set of enzymes' in the abstract and 'exceptional recalcitrance'. From my reading of it there are very many enzymes potentially involved in the breakdown. I'm also not sure what a paradigmatic enzyme is. This part was the most confusing to me – why have the authors identified many interesting enzymes in their two strains through transcriptomics and proteomics, but then only express a single GH168 from a totally different organism? I don't expect the authors to now go and express the enzymes from 892 and 913 strains, but some more rationale for why this enzyme was selected would really help. The assays carried out are also confusing – why not show product profiles from HPAEC/TLC of the enzyme action or action of the lysate, so we can see what's actually happening. The reducing sugar assays aren't really convincing – why if this strain has its own GH168(s) does the exogenous one make a difference? Why in the fig 5b does the enzyme lower specific activity? Since its not explained well how the specific activity was calculated, I don't understand – does that mean the sample is losing reducing ends? Why is this done with only strain 892? Why are the numbers for F. vesiculous grown lysate in fig5a different to those in fig5e?
I would suggest a title change as I don't think the paper really reveals molecular mechanisms of fucoidan breakdown.
Please also change endo-fucosidase to endo fucoidanase or fucanase, the convention is that '-sidase' enzymes are considered to be exo acting apart from in very specific cases (eg GH99 endo mannosidase) but as no detailed enzymatic characterisation is carried out here I'm not sure why this name has been used.
The structural biology is nice, but is a bit overly detailed and long compared to the enzyme characterisation – with no real detail of specificity the detailed analysis doesn't tell us much, I'd suggest trimming this section down considerably, and focussing on the other parts of the work, perhaps coming back to the enzymology in future works with enzymes from the isolated strains.
The FITC labelled fucoidan data is really interesting and I hope the authors continue to work on this aspect of degradation in the future!

Specific comments/corrections
Pg 4, line 84 – clarify or remove 'paradigmatic'
Fig 1d is never referred to in the text
Fig 1e has no X-axis labels
It's not clear from the main text that the monosaccharide levels are free monos or from acid hydrolysed polysaccharide. Clarify in legend/main text
Fig 2b/e – The main text mentions lack of synteny but there's no way to compare synteny from this figure, and the supp tables are not easy to find and compare PULs to check this, is there a better way to show this (doesn't have to be in the main figures, but maybe a supplemental one)
Pg 5/6, the list of enzymes in the text is really hard to follow compared to the figure, I suggest just referring to the figure and shortening it down a lot. Similarly on pg 7 with the sulfatases, is there a more concise way of describing the data or just

pointing the reader to the figure?
Pg8 line 270 – ‘and’
Fig 5e missing axis label

Reviewer #2

(Remarks to the Author)
The manuscript aims to elucidate the metabolism mechanisms of fucoidan. Two fucoidan-utilized bacteria were screened. Genomic, transcriptomic, and proteomic analyses indicate the upregulation of PUL, particularly enzymes from GH168. The activity assay, crystal structure analysis of apo protein, and molecular docking of GH168 homolog pbFucA were conducted. Furthermore, the utilization pattern of fucoidan by 892 and 913 was observed and inferred using confocal microscopy. Fucoidan is the most structurally complex polysaccharide in the ocean. Elucidating the metabolic mechanisms of fucoidan is of significant importance in understanding the role of algae in marine carbon cycling. However, the manuscript provides limited novel information on both the metabolic pathways of fucoidan and the molecular mechanisms of endo-fucanase. The comments and recommendations are as follows. The paper should be reconsidered for publication in Nature Communication after major revisions.

1. The title "Unveiling the molecular mechanisms of recalcitrant fucoidan breakdown in marine Planctomycetota" lacks sufficient support, since the molecular mechanisms of fucoidan metabolism are not elucidated. The authors found that the enzyme from GH168 was significantly upregulated during fucoidan metabolism, but the activity assay results indicate that this enzyme cannot hydrolyze fucoidan alone. This is because exo-fucosidase, sulfatases, and other enzymes are required to pre-process the fucoidan, prior to GH168. The authors should express and characterize the key enzymes such as exo-fucosidase and sulfatases in the PULs of 892 and 913. Additionally, the biochemical properties and the cleavage sites of the GH168 enzyme should be analyzed.

2. The structure of the substrate used in the study is unclear. The structural information of the fucoidan used in this work must be added, including monosaccharide composition, content and distribution of substituent, and glycosidic linkage patterns. A clear substrate structure is essential for studying the metabolic mechanisms. Fucoidan is the most structurally complex macromolecule in the ocean. The structure of fucoidan can vary depending on the species of origin and environmental factors. Therefore, it is unconvincing to directly cite the substrate structures determined in previous studies.

3. The manuscript focuses on 892 and 913, but pbFucA belongs to another bacteria and shows only 30-40% similarity to the GH168 enzyme in 892 and 913. This deviates from the topic. The authors are asked to focus on the enzyme in 892 and 913.

4. In Fig. 5e, the hydrolytic activity of pbFucA on Ecklonia Maxima and Macrocystis pyrifera reaches -20%, which raises doubts about the activity assay results. Please explain how the control group was set up in the PAHBAH method.

5. The enzyme-substrate complexes structure of GH168 enzyme should be given. The essence of polysaccharide metabolism lies in the interaction between enzymes and substrates. Providing only an apo crystal structure is meaningless, especially considering the accurate protein structure prediction of Alphafold2.

6. The analysis of catalytic mechanism of the enzyme lacks experimental data.

7. It is inadequate to clarify the location of polysaccharide metabolism by confocal microscopy. The results of confocal microscopy can only be taken as a preliminary inference. To elucidate the primary metabolism locus of fucoidan, an in-depth analysis of fucoidan at various locations of bacteria is required.

Notes.
All of the tables should be revised to three-line table form.
Fig. 1d does not appear in the text.
The authors provided 3 crystal structures of the same enzyme without any mutation. Normally, one crystal is enough for one enzyme.
Line 272. Please delete the “)”.
Line 141. Genes within a PUL are co-expressed once the PUL is activated. The upregulation of genes does not necessarily indicate their involvement in polysaccharide degradation. The same problem exists in line 376. There is currently no evidence to demonstrate the hydrolysis activity of GH141 enzymes towards fucoidan.

Reviewer #3

(Remarks to the Author)
The manuscript by Carla Pérez-Cruz et al. entitled ‘Unveiling the molecular mechanisms of recalcitrant fucoidan breakdown in marine Planctomycetota’ describes the biochemical and structural characterization of a glycoside hydrolase active on brown algal fucan that was identified in isolated Planctomycetota by transcriptomic up-regulation when grown on fucoidans. The study also reports genomic and proteomic and physiologic data underscoring the regulation of fucan/fucoidan active enzymes. The presented results describe several divergent PULs, present in these microorganisms, that are expressed to allow the catabolism of fucoidan with a variable set of enzymes. The findings are novel and bring up an integrated

characterization of fucoidan degradation by this type of microorganisms. The experiments are well conducted, and methods are well documented. In my opinion, the study is within the scope of the journal, it contains largely original and significant results, and I only have some relatively minor comments, listed below.

Comments

1. Abstract
The authors state : that fucan/fucoidan degradation in the isolated strains “is mediated by the expression of divergent Polysaccharide Utilization Loci in these strains, using an exceptionally reduced set of enzymes”.
While the individual operon-like regions are indeed reduced with respect to the referenced verrucomicrobial one, the data presented here clearly show that, overall, a large number of enzymes are present and many if not most are up-regulated when grown on the purified substrates, adding up to roughly 70 to 80 proteins if not 100 when counting the up-regulated enzymes encoded outside the PULs; I don’t think the data allow to conclude on a single PUL being sufficient to completely degrade fucan or fucoidan? Why put the emphasis on the ‘exceptionally reduced set’?
Additionally, in the discussion section, page 14, line 513, it is unclear from where comes the estimation of up-regulation of “12 to 18 fucosidases and SAs”? If these numbers are relevant for discussion, the authors should point them out and present them more clearly in the results section pages 6 to 7 entitled “Endo-fucosidases are strongly upregulated during the degradation of different types of fucoidan”. The experiments provided by the authors do not, unambiguously, define which enzymes and how many are needed; also, the fact that the lysate of strains grown on fucoidan are needed to increase PbFucA-GH168 activity points towards a more complex situation – as the authors state themselves further down in the discussion section lines 522-524 “Interestingly, this enzyme required the action of other enzymes with complementary activities and/or specificities present in fucoidan-degrading bacteria, likely including SAs, CEs, and exo-fucosidases”. Reading these statements side by side give a bit a contradictory picture. These arguments should be more precisely supported by results of the here presented work, rendering the obvious and very interesting findings of this work much clearer.

Page 3 lines 59 and 60. Term ‘Endo-fucosidases’ ; The authors should use the generally accepted nomenclature of endo-fucanases or endo-fucoidanases, since ‘fucosidase’, in analogy to ‘glucosidase’, is the reserved term for exo-acting enzymes that specifically cleave a single monosaccharide, in general from small oligosaccharides or monosaccharide branching sugars. To avoid confusion, the authors might also take reference to the article by Deniaud-Bouët et al 2017 (reference 87) that defines the nomenclature of ‘fucan’ and ‘fucanases’ for the polysaccharide and enzymes active on homofucans, while ‘fucoidan’ and ‘fucoidanases’ design the polysaccharide and the enzymes active on the hetero-fucans. The enzyme nomenclature should be changed accordingly and homogenized throughout the manuscript (for example in Figure 1a&b the authors do use fucoidanase while they use fucosidase for the same enzymes elsewhere).

Page 5 results. The isolated Plonctomycetota strains were isolated from Fucus spiralis but growth experiments were performed on Fucus vesiculosus, is there a particular reason why changing the algal source?

Figure 2 b,e. The authors state that no evident synteny is present between the PULs of the two strains, but PULA and PULG appear to be rather similar, only that in strain 913 the PUL contains a few more genes ; isn’t this worthwhile mentioning? Also in figures 2b,e the usage of the purple color for GH168 or GH141 is not explained in the legend.

Page 9 Lines 277-278 “with the lysates of strain 892 grown in mannose and the fucoidan from U. pinnatifida and F. vesiculosus, respectively”
The formulation of this sentence is misleading, the reader gets the impression that the strain was grown in mannose and fucoidan, whereas the authors want to state that the strain was grown in mannose and the lysate of this, together with PbFucA, was used against fucoidan from the two algal strains. Please reformulate to make the situation clearer.

Supplementary Figure 11. the structural representations in a to f are tiny – they are very difficult to visually analyze and to get a realistic 3D impression of what is represented. Please provide larger panels.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have made considerable effort to address all comments from my first review, so I have no further changes to suggest, and congratulate them on an interesting manuscript

Minor typos:

Line 161 - disappearance
Line 944/947 - stined/distined instead of stained/destained

Reviewer #2

(Remarks to the Author)
The authors have done a conscientious job of answering the questions and the paper reads better. However, some key issues remain unresolved, which affects the novelty of the paper. The comments are as follows:
1. For the study on the metabolic mechanism of polysaccharides, a clear substrate structure is a prerequisite. The ambiguity of the substrate structure hinders the deduction of the cleavage site of the enzyme. The reviewer does not expect the authors to fully elucidate their structure, but the monosaccharide composition alone is not sufficient. At least some structural features should be presented, and necessary analyses (methylation analysis, FTIR, NMR) should be carried out.
2. The most attractive finding of the paper is the significant up-regulation of GH168 enzymes and their hydrolytic activity towards fucoidan from algae (albeit relatively weak), as the substrate of this family was only fucoidan from sea cucumber in the past. However, the enzymology study is insufficient, despite the addition of comparisons with other complex structure. The authors still fail to clarify how the enzyme degrades the substrate used in the paper. The authors also do not perform any analysis of the enzymatic products. The reviewer can't even be certain whether the enzyme cleaves the bond between the fucose residues, instead of galactose or mannose.
3. In the genomes, transcriptomes, and proteomes parts, the analysis is routine, although it is systematic. The conclusions in these parts lack novelty and are easy to be expected.
4. The confocal microscopy part is interesting, and some novel preliminary findings have been obtained. The reviewer hope to see further work on this part. Could the authors consider deleting the enzymology part, and combine the results of genomics, transcriptomics, proteomics, and confocal microscopy to deeply reveal the mechanism behind the different metabolic locations of these two bacteria on fucoidan.

Reviewer #3

(Remarks to the Author)
The authors have substantially amended their manuscript with additional structural and RNAseq data and experiments, rendering the outcome and conclusions stronger. They also changed the title and considered all reviewers comments to adjust to the level of outcome and subject coverage of their results. As stated before, the findings are novel and bring up an integrated view of fucoidan degradation by this type of microorganisms. The experiments are well conducted, and methods are well documented. In my opinion, the study is within the scope of the journal, it contains largely original and significant results, and I only have one additional minor comment below.

Comment

Figure 6. The figure legend of this new figure needs more precision, and perhaps contains some errors? To me, there appear to be some inconsistencies. First, the colors of the bars in **a** and **b** are not grey, blue and orange (as in Supplementary Figure 6): in Figure 6 (of main text), here, they are grey, green and red. Second, The legend describes "***c,d*** *C-PAGE analysis of the hydrolytic activity of Rho5174 and PbFucA, strain 892 lysate grown in F. vesiculosus (**L892-Fv**) and the combination of enzymes and lysate against fucoidan from F. vesiculosus (c) and against U. pinnatifida (**d**)*" while the titles above the C-Page lanes in row **d** indicate that it is **L892-up** that is combined with the enzymes.
In the results section that describes these C-page results, page 9 lines 336-337, the authors state "However, hydrolysis of fucoidan from U. pinnatifida was detected when using L892-Up (Fig. 6d)." But I don't "see" any significant bands of oligosaccharides appearing in any of the C-PAGE images of row **d**; if the authors believe to see some degradation, an arrow pointing to the band changes should help guide the readers' eye to visualize the detected degradation.

Reviewer Comments

Reviewer #1:

This manuscript describes the isolation and identification of two fucoidan degrading Planctomycetota strains associated with marine algae. Transcriptomics and proteomics are used to identify the cazymes associated with fucoidan breakdown, and a structure of an endo-fucoidanase is presented. Finally, the authors present some interesting microscopy showing different degradation/uptake strategies for fucoidan from the two isolated strains.

ANSWER: We thank the reviewer for all the constructive comments/suggestions to improve the manuscript.

General comments:

1. The isolation, characterization and 'omics sections of this work are significantly stronger than the enzymology. This is interesting and thorough work but the authors need to tone down some of the more hyperbolic language, e.g. 'exceptionally reduced set of enzymes' in the abstract and 'exceptional recalcitrance'. From my reading of it there are very many enzymes potentially involved in the breakdown.

ANSWER: We agree with the reviewer. We have toned down the language regarding the "exceptionally reduced set of enzymes" that Planctomycetota use for its degradation in the Abstract (please, see line 10 in the revised version of the manuscript). We would like to emphasize that the number of fucoidan-degrading enzymes expressed in *Lentimonas* CC4, at the proteomic level, is approximately nine times higher compared to the two Planctomycetota strains analyzed in our study, 892 and 913 (please, see Table R1 for review purposes; Sichert et al., 2020). Globally, *Lentimonas* upregulates 131 CAZymes, including exo-fucosidases, endo-fucanases and sulfatases for fucoidan degradation, while the two Planctomycetota strains upregulate only 13/15 enzymes. We have clarified this point in the Discussion section (please, see lines 494-505 and the new Supplementary Table 4 in the revised version). We have also toned down the language, replacing "exceptional recalcitrance" or "extreme recalcitrance" by "high recalcitrance" (lines 437 and 559). Please, note that fucoidan is one of the most structurally complex polysaccharides in the ocean (see comment from reviewer #2), accumulating for centuries in marine systems due to its high recalcitrance (Salmeán et al., 2022; Vidal-Melgosa et al., 2021).

	892			913			*Lentimonas*		
List of GHs and Sas potentially involved in fucoidan degradation	Nº of GH and SAs arranged in Fucoidan PULS	Nº of GH and SAs detected	Nº of GH and SAs induced	Nº of GH and SAs arranged in Fucoidan PULS	Nº of GH and SAs detected	Nº of GH and SAs induced	Nº of GH and SAs arranged in Fucoidan PULS	Nº of GH and SAs detected	Nº of GH and SAs induced
GH29	13	**9**	**6**	15	**5**	**4**	35	**23**	**22**
GH95	5	**4**	**3**	5	**1**	**1**	18	**17**	**15**
GH141	3	**1**	**0**	5	**2**	**2**	15	**15**	**12**
GH141+GH168	1	**1**	**1**	0	**0**	**0**	n.r	**0**	**n.r**
GH107	0	**0**	**0**	1	**1**	**1**	7	**3**	**1**
GH168	2	**1**	**1**	2	**1**	**1**	n.r	**0**	**n.r**
Total GH	**24**	**16**	**11**	**28**	**10**	**9**	**75**	**58**	**50**
S1_14	6	**1**	**1**	6	**0**	**0**	n.r	**0**	**n.r**
S1_15	2	**1**	**1**	8	**1**	**1**	46	**28**	**21**
S1_16	4	**1**	**0**	5	**1**	**1**	31	**18**	**14**
S1_17	3	**3**	**1**	6	**1**	**1**	22	**19**	**18**
S1_22	1	**1**	**0**	2	**2**	**1**	n.r	**22**	**22**
S1_25	2	**1**	**1**	4	**0**	**0**	14	**10**	**6**
Total SAs	**18**	**8**	**4**	**31**	**5**	**4**	**113**	**97**	**81**
Total enzymes	**42**	**24**	**15**	**59**	**15**	**13**	**188**	**155**	**131**

Table R1. Proteome investment for fucoidan degradation compared to the total of genes located in PULs or clusters in *Lentimonas* sp. CC4 and strains 892 and 913. The number of GH and SAs

detected in the proteome and induced by growth on fucoidan as carbon source is shown. Data from *Lentimonas* CC4 has been extracted from the literature (Sichert et al. 2020. Fig.1.c) (n.r, not reported data).

References

Salmeán AA, Willats WGT, Ribeiro S, Andersen TJ, Ellegaard M. Over 100-year preservation and temporal fluctuations of cell wall polysaccharides in marine sediments. *Front. Plant Sci.* 13:785902 (2022).

Sichert A, Corzett CH, Schechter MS, Unfried F, Markert S, Becher D, Fernandez-Guerra A, Liebeke M, Schweder T, Polz MF, Hehemann JH. Verrucomicrobia use hundreds of enzymes to digest the algal polysaccharide fucoidan. *Nat. Microbiol.* 5(8):1026-1039 (2020).

Vidal-Melgosa S, Sichert A, Francis TB, Bartosik D, Niggemann J, Wichels A, Willats WGT, Fuchs BM, Teeling H, Becher D, Schweder T, Amann R, Hehemann JH. Diatom fucan polysaccharide precipitates carbon during algal blooms. *Nat. Commun.* 12(1):1150 (2021).

2. I'm also not sure what a paradigmatic enzyme is.

ANSWER: We have rewritten the last part of the abstract and removed the "paradigmatic" term to improve clarity (line 12).

3. This part was the most confusing to me – why have the authors identified many interesting enzymes in their two strains through transcriptomics and proteomics, but then only express a single GH168 from a totally different organism? I don't expect the authors to now go and express the enzymes from 892 and 913 strains, but some more rationale for why this enzyme was selected would really help.

ANSWER: We thank the reviewer for this comment. We decided to concentrate on endo-fucanases as, currently, the role of these enzymes in fucoidan degradation is the least understood. By acting on internal linkages of the polysaccharide, endo-fucanases might play a crucial role in the degradation process. Specifically, the GH168 family was chosen because it was found to be the most widely distributed endo-fucanase in Planctomycetota. This enzyme family was highly upregulated during fucoidan degradation, and there was a lack of structural information. Although we initially obtained the structure of a GH168 enzyme from a member of Planctomycetota phylum (PbFucA), we acknowledge the reviewer's suggestion that it would be beneficial to study representatives of the newly isolated strains. Thus, we have expanded our efforts in this point and successfully determined the X-ray crystal structure of Rho5174 from strain 892 (Figure 7, Supplementary Figures 5 and 10, and Supplementary Table 2). In addition, we have analyzed its hydrolytic activity on various fucoidan structures from brown algae (Figure 6, Supplementary Figure 9). Consequently, we have redirected the focus of the manuscript to Rho5174, and compared the results from the structural and hydrolytic experiments with those of PbFucA, an enzyme from the same family but from a distantly related Planctomycetota organism.

4. The assays carried out are also confusing – why not show product profiles from HPAEC/TLC of the enzyme action or action of the lysate, so we can see what's actually happening. The reducing sugar assays aren't really convincing.

ANSWER: Many thanks for pointing this out to us. In the new version of the manuscript, we performed Carbohydrate Polyacrylamide Gel Electrophoresis (C-PAGE) to detect degradation products of fucoidan from various species of brown algae, after incubation with Rho5174, PbFucA and/or cellular lysates. This methodology is widely used to visualize fucoidan breakdown products (Silchenko et al. 2013, 2017, 2018; Trang et al. 2022; Vuillemin et al. 2020; Sichert et al. 2020; Silchenko et al. 2024). The PAHBAH and C-PAGE results showed that both GH168 endo-fucanases were able to increase the degradation rate of fucoidan from *F. vesiculosus* and, to a lesser extent, fucoidan from *E. maxima* and *M. pyrifera*, when combined

with the cellular lysate of strain 892. Moreover, the C-PAGE showed that Rho5174 was able to partially degrade fucoidan from *C. okamuranus* on its own. Therefore, we reorganized the presentation of our findings accordingly (lines 280-298, lines 507-515, lines 885-904, Figure 6; Supplementary Figure 9).

References

Sichert A, Corzett CH, Schechter MS, Unfried F, Markert S, Becher D, Fernandez-Guerra A, Liebeke M, Schweder T, Polz MF, Hehemann JH. Verrucomicrobia use hundreds of enzymes to digest the algal polysaccharide fucoidan. *Nat. Microbiol.* 5(8):1026-1039 (2020).

Silchenko AS, Kusaykin MI, Kurilenko VV, Zakharenko AM, Isakov VV, Zaporozhets TS, Gazha AK, Zvyagintseva TN. Hydrolysis of fucoidan by fucoidanase isolated from the marine bacterium, Formosa algae. *Mar Drugs* 11(7):2413-30 (2013).

Silchenko AS, Ustyuzhanina NE, Kusaykin MI, Krylov VB, Shashkov AS, Dmitrenok AS, Usoltseva RV, Zueva AO, Nifantiev NE, Zvyagintseva TN. Expression and biochemical characterization and substrate specificity of the fucoidanase from *Formosa* algae. *Glycobiology* 27(3):254-263 (2017).

Silchenko AS, Rasin AB, Zueva AO, Kusaykin MI, Zvyagintseva TN, Kalinovsky AI, Kurilenko VV, Ermakova SP. Fucoidan sulfatases from marine bacterium *Wenyingzhuangia fucanilytica* CZ1127T. *Biomolecules* 8(4):98 (2018).

Silchenko AS, Taran IV, Usoltseva RV, Zvyagintsev NV, Zueva AO, Rubtsov NK, Lembikova DE, Nedashkovskaya OI, Kusaykin MI, Isaeva MP, Ermakova SP. The discovery of the fucoidan-active endo-1→4-α-L-fucanase of the GH168 family, which produces fucoidan derivatives with regular sulfation and anticoagulant activity. *Int. J. Mol. Sci.* 25(1):218 (2023).

Trang VTD, Mikkelsen MD, Vuillemin M, Meier S, Cao HTT, Muschiol J, Perna V, Nguyen TT, Tran VHN, Holck J, Van TTT, Khanh HHN, Meyer AS. The endo-α(1,4) specific fucoidanase Fhf2 from *Formosa haliotis* releases highly sulfated fucoidan oligosaccharides. *Front. Plant Sci.* 13:823668 (2022).

Vuillemin M, Silchenko AS, Cao HTT, Kokoulin MS, Trang VTD, Holck J, Ermakova SP, Meyer AS, Mikkelsen MD. Functional characterization of a new GH107 endo-α-(1,4)-fucoidanase from the marine bacterium *Formosa haliotis*. Mar Drugs 18(11):562 (2020).

5. why if this strain has its own GH168(s) does the exogenous one make a difference?

ANSWER: The exogenous GH168 enzymes are used at much higher concentration (1 to 2 mg/ml) compared to endogenous GH168 enzymes from the cellular lysates.

6. Why in the fig 5b does the enzyme lower specific activity? Since its not explained well how the specific activity was calculated, I don't understand – does that mean the sample is losing reducing ends?

ANSWER: We agree with the reviewer that this was not sufficiently clear. We have substituted "specific activity" by "Absorbance 410 nm" in Figure 6 and Supplementary Figure 6. We have also normalized the data to 0. We have explained/clarified this point in the Materials and Methods Section in the revised version of the manuscript (line 873).

7. Why is this done with only strain 892?

ANSWER:
The objective of this experiment was to evaluate the activity of the enzymes from family GH168: PbFucA and, in the revised version, Rho5174. Initially, we did not detect activity for PbFucA against any fucoidan source. Thus, we used the lysates of strain 892 to assist PbFucA and Rho5174 hydrolyzing complex high-molecular fucoidan.

8. Why are the numbers for F. vesiculous grown lysate in fig5a different to those in fig5e?

ANSWER: Please, note that the experiments shown in Figures 5a and 5e in the original version of the manuscript are independent and the mean values in both experiments are within the error bars. Also, please, note that we have removed Figure 5e from this revised version because we found that the experimental data generated by the C-PAGE assay were more informative compared to those obtained following the PAHBAH method, as suggested by the reviewer. In addition, following the suggestion of reviewer #2 and based on the carbohydrate analysis of the fucoidan sources, we have removed those from *D. potatorum and F. serratus* from our hydrolytic experiments, due to the high level of impurities found in their composition.

9. I would suggest a title change as I don't think the paper really reveals molecular mechanisms of fucoidan breakdown.

ANSWER: We agree with the reviewer. We have modified the title to a more general one that now reads "Mechanisms of recalcitrant fucoidan breakdown in marine Planctomycetota".

10. Please also change endo-fucosidase to endo fucoidanase or fucanase, the convention is that '-sidase' enzymes are considered to be exo acting apart from in very specific cases (eg GH99 endo mannosidase) but as no detailed enzymatic characterisation is carried out here I'm not sure why this name has been used.

ANSWER: We agree with the reviewer. We have changed endo-fucosidase by endo-fucanase throughout the text (see e.g. lines 12, 64, 67, 163, 222, etc).

11. The structural biology is nice, but is a bit overly detailed and long compared to the enzyme characterization – with no real detail of specificity the detailed analysis doesn't tell us much, I'd suggest trimming this section down considerably, and focusing on the other parts of the work, perhaps coming back to the enzymology in future works with enzymes from the isolated strains.

ANSWER: We agree with the reviewer. We have considerably trimmed down the structural biology from the main text. Please note that during the revision of this manuscript, the X-ray crystal structure of a GH168 family member, Fun168A in complex with fucotetraose (PDB code 8YA7), was reported (Chen et al., 2024). Interestingly, this allowed us to compare our two experimentally determined structures, Rho5174 and PbFucA, with Fun168A, providing important information on the substrate specificity of the enzymes (Figures 8, Supplementary Figures 13, 15 and 17, and Supplementary Table 3).

References

1. Chen G, Dong S, Zhang Y, Shen J, Liu G, Chen F, Li X, Xue C, Cui Q, Feng Y, Chang Y. Structural investigation of Fun168A unraveling the recognition mechanism of endo-1,3-fucanase towards sulfated fucan. *Int. J. Biol. Macromol.* 271:132622 (2024).

12. The FITC labelled fucoidan data is really interesting and I hope the authors continue to work on this aspect of degradation in the future!

ANSWER: We appreciate the reviewer's positive comment on the FITC labelled fucoidan experiments. We fully agree with the suggestion to conduct future in-depth studies on the polysaccharide degradation mechanism using advanced fluorescence microscopy. These studies will undoubtedly provide additional valuable insights into how bacteria selectively degrade fucoidan and, at a later stage, other complex polysaccharides.

Specific comments/corrections:

13. Pg 4, line 84 – clarify or remove 'paradigmatic'

ANSWER: We have removed “paradigmatic” from the text.

14. Fig 1d is never referred to in the text

ANSWER: Many thanks for pointing this to us. We have now referred Figure 1d in the text (line 102).

15. Fig 1e has no X-axis labels. It’s not clear from the main text that the monosaccharide levels are free monos or from acid hydrolysed polysaccharide. Clarify in legend/main text.

ANSWER: The X-axis labels appear at the bottom of the Figure, but we agree with the reviewer that this may not have been clear. We have now fixed the X-axis labels in Figure 1e and clarified that the signal is coming from acid hydrolyzed polysaccharide.

16. Fig 2b/e – The main text mentions lack of synteny but there’s no way to compare synteny from this figure, and the supp tables are not easy to find and compare PULs to check this, is there a better way to show this (doesn’t have to be in the main figures, but maybe a supplemental one).

ANSWER: Please, note that this information can be found in Figure 3. In the latter figure, the gene configuration within each PUL is detailed, allowing the comparison of gene order between the PULs in both strains. Thus, in the revised manuscript, we have referenced Figure 3 in the main text to underscore the lack of synteny (line 135). Additionally, we have prepared a new figure to better visualize the lack of synteny in the PULs (please, see below the Figure R1, for reviewing purposes). Figure R1 displays the genome alignment of strains 892 and 913, with the PULs highlighted by colors along the genomes. The results clearly indicate the absence of synteny in the genomic regions containing the fucoidan PULs of both strains. We believe it is unnecessary to include the new Figure R1 in the manuscript. However, in the case the reviewer considers this appropriate, we can certainly incorporate the Figure into the Supplementary Information.

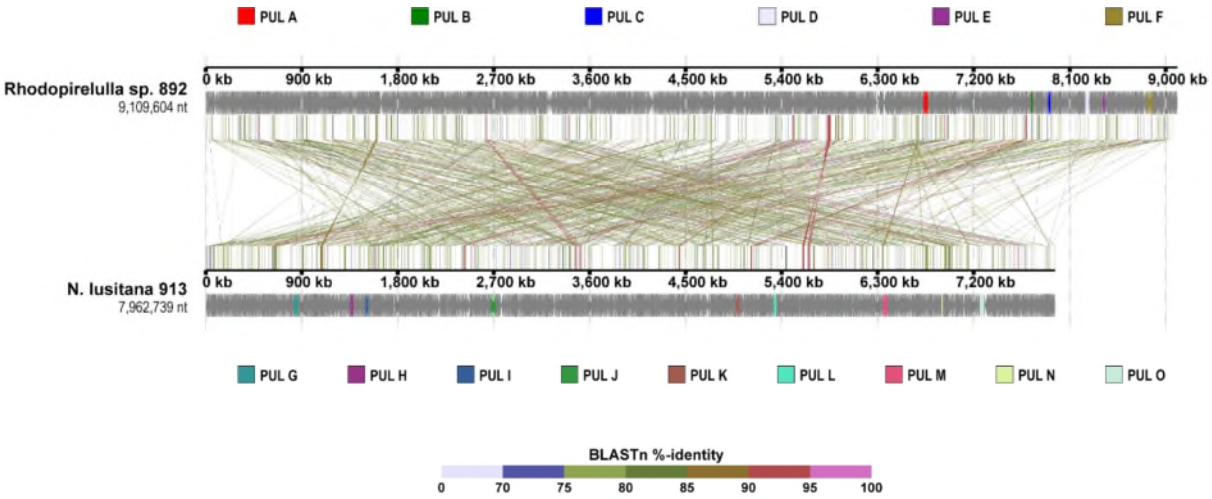

Figure R1: Genome alignment between strains 892 and 913 with BLASTn. PULs in each strain are highlighted by colours.

17. Pg 5/6, the list of enzymes in the text is really hard to follow compared to the figure, I suggest just referring to the figure and shortening it down a lot. Similarly, on pg 7 with the sulfatases, is there a more concise way of describing the data or just pointing the reader to the figure?

ANSWER: We agree with the reviewer. We have thoroughly revised the text of pages 5, 6 and 7 in the new version of the manuscript to describe the results in a more concise manner.

18. Pg8 line 270 – ‘and’

ANSWER: We have corrected this in the main text.

19. Fig 5e missing axis label.

ANSWER: We have removed Figure 5e. Please see the answer above.

Reviewer #2:

The manuscript aims to elucidate the metabolism mechanisms of fucoidan. Two fucoidan-utilized bacteria were screened. Genomic, transcriptomic, and proteomic analyses indicate the upregulation of PUL, particularly enzymes from GH168. The activity assay, crystal structure analysis of apo protein, and molecular docking of GH168 homolog pbFucA were conducted. Furthermore, the utilization pattern of fucoidan by 892 and 913 was observed and inferred using confocal microscopy. Fucoidan is the most structurally complex polysaccharide in the ocean. Elucidating the metabolic mechanisms of fucoidan is of significant importance in understanding the role of algae in marine carbon cycling. However, the manuscript provides limited novel information on both the metabolic pathways of fucoidan and the molecular mechanisms of endo-fucanase. The comments and recommendations are as follows. The paper should be reconsidered for publication in Nature Communication after major revisions.

ANSWER: We thank the reviewer for all the constructive comments/suggestions in order to improve the manuscript.

1. The title "Unveiling the molecular mechanisms of recalcitrant fucoidan breakdown in marine Planctomycetota" lacks sufficient support, since the molecular mechanisms of fucoidan metabolism are not elucidated.

ANSWER: We have modified the title to a more general one: "Mechanisms of recalcitrant fucoidan breakdown in marine Planctomycetota"

The authors found that the enzyme from GH168 was significantly upregulated during fucoidan metabolism, but the activity assay results indicate that this enzyme cannot hydrolyze fucoidan alone. This is because exo-fucosidase, sulfatases, and other enzymes are required to pre-process the fucoidan, prior to GH168.

ANSWER: The reviewer is correct. We assayed the addition of lysates from strain 892 in combination with the GH168 enzyme in the hydrolytic assays, supporting the notion that the combination of exo-fucosidases, sulfatases, and other enzymes are required to pre-process the fucoidan (please, see "Insights into fucoidan processing by GH168 enzymes from Planctomycetota" in the results section and lines 507-515).

The authors should express and characterize the key enzymes such as exo-fucosidase and sulfatases in the PULs of 892 and 913.

ANSWER: Taking into account the number of enzymes upregulated in both strains when growing on fucoidan, we believe that searching the full set of key enzymes and characterizing them is out of the scope of the current manuscript.

We kindly refer to the comment of reviewer #1 ***"I don't expect the authors to now go and express the enzymes from 892 and 913 strains".***

Additionally, the biochemical properties and the cleavage sites of the GH168 enzyme should be analyzed.

ANSWER: In the new version of the manuscript, we have made a strong effort to further advance in the analysis of the GH168 family of endo-fucanases. Specifically, we have

expressed, purified, and assayed the hydrolytic ability of Rho5174, a GH168 family endo-fucoidanase from strain 892, in processing fucoidan polymers of different sources (Figure 6, Supplementary Figures 6 and 9). In addition, we have determined the X-ray crystal structure of Rho5174 from strain 892 (Figure 7, Supplementary Figures 5 and 10, and Supplementary Table 2). Interestingly, during the review process of the current manuscript, the crystal structure of Fun168A, an endo-1,3-fucanase in complex with fucotetraose (PDB code 8YA7; Chen et al., 2024), was reported. The structural comparison of Rho5174 with Fun168A and PbFucA, an enzyme from a different member of Planctomycetota, provide important information on the substrate specificity of the enzymes (Figures 8, Supplementary Figures 13, 15 and 17, and Supplementary Table 3).

2. The structure of the substrate used in the study is unclear. The structural information of the fucoidan used in this work must be added, including monosaccharide composition, content and distribution of substituent, and glycosidic linkage patterns. A clear substrate structure is essential for studying the metabolic mechanisms. Fucoidan is the most structurally complex macromolecule in the ocean. The structure of fucoidan can vary depending on the species of origin and environmental factors. Therefore, it is unconvincing to directly cite the substrate structures determined in previous studies.

ANSWER: We agree with the reviewer that, considering the potential variation of fucoidan structure depending on the species of origin and environmental factors, obtaining information about the specific fucoidans analyzed here is very valuable. Therefore, we analyzed the relative abundance of monosaccharide building blocks and the sulfate content of the fucoidans used in this study (please, see the new Supplementary Figure 8). Based on this analysis, we have removed fucoidans from *D. potatorum* and *F. serratus* from our hydrolytic experiments due to the high level of impurities found in their composition. We agree that advancing in the elucidation of the structure of this polysaccharide (i.e. content and distribution of substituents and glycosidic linkage patterns) would be relevant for the field. However, considering its exceptional chemical complexity, this is certainly a separate several-years project. Thus, we believe that this is out of the scope of the current manuscript.

3. The manuscript focuses on 892 and 913, but pbFucA belongs to another bacteria and shows only 30-40% similarity to the GH168 enzyme in 892 and 913. This deviates from the topic. The authors are asked to focus on the enzyme in 892 and 913.

ANSWER: In the new version of the manuscript, we have modified the focus on the GH168 enzymes of 892 and 913 strains (e.g., see new Figures 7, 8)

4. In Fig. 5e, the hydrolytic activity of pbFucA on Ecklonia Maxima and Macrocystis pyrifera reaches -20%, which raises doubts about the activity assay results. Please explain how the control group was set up in the PAHBAH method.

ANSWER: We have removed Figure 5e from this revised version because we found that the experimental data generated by the C-PAGE assay were largely more informative compared to those obtained following the PAHBAH method (lines 280-298, lines 507-515, lines 885-904, Figure 6; Supplementary Figure 9).

5. The enzyme-substrate complexes structure of GH168 enzyme should be given. The essence of polysaccharide metabolism lies in the interaction between enzymes and substrates. Providing only an apo crystal structure is meaningless, especially considering the accurate protein structure prediction of Alphafold2.

ANSWER: We have made a strong effort in determining the X-ray crystal structure of Rho5174 from 892. Despite much effort, we were unable to crystallize Rho5174 or PbFucA in complex with L-fucose derivatives, by soaking and co-crystallization experiments (please, see Methods section). Please, note that the crystallization of biological macromolecules still

remains a trial and error process. We identified one molecule of 3-morpholinopropane-1-sulfonic acid (MOPS) in the central groove of the Rho5174 structure.

Interestingly, during the reviewing process of this manuscript, the crystal structure of Fun168A, an endo-1,3-fucanase in complex with fucotetraose, was reported (Fun168A-Fuc4; PDB code 8YA7; Chen et al., 2024). Specifically, Fun168A hydrolyzes the α-1,3 bonds between Fuc*p*(2OSO^{3-}) and α-L-Fuc of sulfated fucoidans from *I. badionotus* and *T. ananas* (Chen et al., 2024; Shen et al. 2020, 2023). Structural comparisons between Rho5174 and Fun168A-Fuc4 revealed that the MOPS molecule in Rho5174 occupies an equivalent position to the Fuc (-1) in the Fun168A-Fuc4 complex (Figure 7). Altogether, the structural comparison of Rho5174 with Fun168A and PbFucA, an enzyme from a different member of Planctomycetota, provide important information on the substrate specificity of the enzymes (Figures 7-8, Supplementary Figure 17 and Supplementary Table 3).

References

1. Shen J, Chang Y, Zhang Y, Mei X, Xue C. Discovery and characterization of an endo-1,3-fucanase from marine bacterium *Wenyingzhuangia fucanilytica*: a novel glycoside hydrolase family. *Front. Microbiol.* 11:1674 (2020).

2. Shen J, Chen G, Zhang Y, Mei X, Chang Y, Xue C. Characterization of a novel endo-1,3-fucanase from marine bacterium Wenyingzhuangia fucanilytica reveals the presence of diversity within glycoside hydrolase family 168. *Carbohydr. Polym.* 318:121104 (2023).

3. Chen G, Dong S, Zhang Y, Shen J, Liu G, Chen F, Li X, Xue C, Cui Q, Feng Y, Chang Y. Structural investigation of Fun168A unraveling the recognition mechanism of endo-1,3-fucanase towards sulfated fucan. *Int. J. Biol. Macromol.* 271:132622 (2024).

6. The analysis of catalytic mechanism of the enzyme lacks experimental data.

ANSWER: The classical retaining mechanism in glycoside hydrolases involves a two-step reaction facilitated by two essential residues, (i) the nucleophile and (ii) the general acid/base (McCarter et al., 1994). The nucleophile attacks the anomeric carbon and forms a covalent glycosyl-enzyme intermediate. The general acid/base residue deprotonates a water molecule, which attacks the anomeric carbon to produce the final product. In that context, the catalytic mechanism for Fun168A, a member of the GH168 family of enzymes was initially proposed (Shen et al., 2020). The study showed that D206 and E264 are critical residues of Fun168A since the mutants D206E and E264Q were inactive (Shen et al., 2020). During the review process of the current manuscript, the crystal structure of Fun168A in complex with fucotetraose (PDB code 8YA7; Chen et al., 2024), was reported. Interestingly, the distance between D206 and the atom C1 of the fucosyl residue at the 1 subsite was 3.0 Å, indicating that D206 serves as a nucleophile. In addition, the distance between E264 and the atom O1 was 2.7 Å, indicating that E264 functions as the general acid/base residue. The two residues are strictly conserved in both Rho5174 and PbFucA experimentally determined structures, and other members of the GH168 family, supporting a common catalytic mechanism (Figure 7 and Supplementary Figure 16).

References

1. Shen J, Chang Y, Zhang Y, Mei X, Xue C. Discovery and characterization of an endo-1,3-Fucanase from marine bacterium wenyingzhuangia fucanilytica: a novel glycoside hydrolase family. *Front. Microbiol.* 11 (2020).

2. McCarter JD, Withers SG. Mechanisms of enzymatic glycoside hydrolysis. *Curr. Opin. Struct. Biol.* 4:885–892 (1994).

3. Chen G, Dong S, Zhang Y, Shen J, Liu G, Chen F, Li X, Xue C, Cui Q, Feng Y, Chang Y. Structural investigation of Fun168A unraveling the recognition mechanism of endo-1,3-fucanase towards sulfated fucan. *Int. J. Biol. Macromol.* 271:132622 (2024).

7. It is inadequate to clarify the location of polysaccharide metabolism by confocal microscopy. The results of confocal microscopy can only be taken as a preliminary inference. To elucidate the primary metabolism locus of fucoidan, an in-depth analysis of fucoidan at various locations of bacteria is required.

ANSWER: In this work, we present compelling evidence that two closely related strains, 892 and 913, exhibit remarkably different behaviors in the uptake of fucoidan. This is an important contribution, since there was no previous evidence of external degradation of this polysaccharide. Describing the location of fucoidan metabolism would require combining different advanced fluorescence microscopy approaches, such as time-lapse and super-resolution microscopy, and the development of fucoidan-labeled probes compatible with these techniques, which are out of the scope of the current manuscript.

We kindly refer to the comment of reviewer #1 *"The FITC labeled fucoidan data is really interesting, and I hope the authors continue to work on this aspect of degradation in the future!".*

Notes.
All of the tables should be revised to three-line table form.

ANSWER: We thank the reviewer for the suggestion. However, we prefer to maintain the current format to preserve clarity.

Fig. 1d does not appear in the text.

ANSWER: The reviewer is correct. We have referred to Figure 1d in the main text (line 102).

The authors provided 3 crystal structures of the same enzyme without any mutation. Normally, one crystal is enough for one enzyme.

ANSWER: We provide three crystal structures of PcFucA in different space groups: $P2_1$, $P2_12_12_1$, and $P4_12_12$. Importantly, each space group exhibits a different protein packing pattern, which could certainly be of value in designing soaking experiments to study enzyme-ligand interactions and catalytic mechanisms (please, see Albesa-Jové *et al*., 2015; Albesa-Jové *et al*., 2017, 2019; Trastoy *et al*., 2020).

References

1. Albesa-Jové D, Mendoza F, Rodrigo-Unzueta A, Gomollón-Bel F, Cifuente JO, Urresti S, Comino N, Gómez H, Romero-García J, Lluch JM, Sancho-Vaello E, Biarnés X, Planas A, Merino P, Masgrau L, Guerin ME. A native ternary complex trapped in a crystal reveals the catalytic mechanism of a retaining glycosyltransferase. *Angew. Chem. Int. Ed Engl.* 54(34):9898-902 (2015).

2. Albesa-Jové D, Sainz-Polo MÁ, Marina A, Guerin ME. Structural Snapshots of α-1,3-Galactosyltransferase with Native Substrates: Insight into the Catalytic Mechanism of Retaining Glycosyltransferases. *Angew. Chem. Int. Ed Engl.* 56(47):14853-14857 (2017).

3. Albesa-Jové D, Cifuente JO, Trastoy B, Guerin ME. Quick-soaking of crystals reveals unprecedented insights into the catalytic mechanism of glycosyltransferases. *Methods Enzymol.* 621:261-279 (2019).

4. Trastoy B, Naegeli A, Anso I, Sjögren J, Guerin ME. Structural basis of mammalian mucin processing by the human gut *O*-glycopeptidase OgpA from *Akkermansia muciniphila*. *Nat. Commun.* 11(1):4844 (2020).

Line 272. Please delete the ")".

ANSWER: We have corrected this in the main text.

Line 141. Genes within a PUL are co-expressed once the PUL is activated. The upregulation of genes does not necessarily indicate their involvement in polysaccharide degradation. The same problem exists in line 376.

ANSWER: We agree with the reviewer's comment and have removed these sentences in the new version of the manuscript, accordingly.

There is currently no evidence to demonstrate the hydrolysis activity of GH141 enzymes towards fucoidan.

ANSWER: We agree with the reviewer. Please, note that this is mentioned in the main text: *"…to date, the two members of the GH141 family that have been biochemically characterized showed α-fucosidase activity on pectins and endo-xylanase activity. However, this family of enzymes has also been identified in several fucoidan processing bacteria, suggesting a key role in the hydrolysis of this polysaccharide."* (lines 375-380).

Reviewer #3 (Remarks to the Author):

The manuscript by Carla Pérez-Cruz et al. entitled 'Unveiling the molecular mechanisms of recalcitrant fucoidan breakdown in marine Planctomycetota' describes the biochemical and structural characterization of a glycoside hydrolase active on brown algal fucan that was identified in isolated Planctomycetota by transcriptomic up-regulation when grown on fucoidans. The study also reports genomic and proteomic and physiologic data underscoring the regulation of fucan/fucoidan active enzymes. The presented results describe several divergent PULs, present in these microorganisms, that are expressed to allow the catabolism of fucoidan with a variable set of enzymes. The findings are novel and bring up an integrated characterization of fucoidan degradation by this type of microorganisms. The experiments are well conducted, and methods are well documented. In my opinion, the study is within the scope of the journal, it contains largely original and significant results, and I only have some relatively minor comments, listed below.

ANSWER: We thank the reviewer for all the constructive comments/suggestions in order to improve the manuscript.

Comments

1. Abstract. The authors state: that fucan/fucoidan degradation in the isolated strains "is mediated by the expression of divergent Polysaccharide Utilization Loci in these strains, using an exceptionally reduced set of enzymes". While the individual operon-like regions are indeed reduced with respect to the referenced verrucomicrobial one, the data presented here clearly show that, overall, a large number of enzymes are present and many if not most are up-regulated when grown on the purified substrates, adding up to roughly 70 to 80 proteins if not 100 when counting the up-regulated enzymes encoded outside the PULs; I don't think the data allow to conclude on a single PUL being sufficient to completely degrade fucan or fucoidan? Why put the emphasis on the 'exceptionally reduced set'?

ANSWER: Many thanks for pointing this out to us. The emphasis on the "exceptionally reduced set of enzymes" was based in the comparison with *Lentimonas* CC4, which upregulates an important number of exo-fucosidases, endo-fucoidanases (GH29, GH95, GH141, GH107) and sulfatases (S1_14, S1_15, S1_16, S1_17, S1_22, S1_25) from the fucoidan PULs (Sichert et al. 2020) when degrading fucoidan from brown algae, up to 9-fold, as compared to 892 or 913 strains. The numbers can be compared in the new Table R1 for reviewing purposes: 131 enzymes upregulated in *Lentimonas* versus 13-15 enzymes in the Planctomycetota strains. However, we agree with the reviewer and to avoid overstatements we have toned down this sentence in the Abstract.

References

1. Sichert A, Corzett CH, Schechter MS, Unfried F, Markert S, Becher D, Fernandez-Guerra A, Liebeke M, Schweder T, Polz MF, Hehemann JH. Verrucomicrobia use hundreds of enzymes to digest the algal polysaccharide fucoidan. *Nat. Microbiol.* 5(8):1026-1039 (2020).

2. Additionally, in the discussion section, page 14, line 513, it is unclear from where comes the estimation of up-regulation of “12 to 18 fucosidases and SAs”? If these numbers are relevant for discussion, the authors should point them out and present them more clearly in the results section pages 6 to 7 entitled “Endo-fucosidases are strongly upregulated during the degradation of different types of fucoidan”.

ANSWER: We agree with the reviewer. We have now included a new Supplementary Table 5, showing the number of enzymes induced for fucoidan degradation. In addition, we have also revised/modified the text in the Results section, pages 6 to 7, accordingly. Please note that the number of enzymes upregulated in fucoidan PULs ranged from 13 to 15; it is now corrected.

3. The experiments provided by the authors do not, unambiguously, define which enzymes and how many are needed; also, the fact that the lysate of strains grown on fucoidan are needed to increase PbFucA-GH168 activity points towards a more complex situation – as the authors state themselves further down in the discussion section lines 522-524 “Interestingly, this enzyme required the action of other enzymes with complementary activities and/or specificities present in fucoidan-degrading bacteria, likely including SAs, CEs, and exo-fucosidases”. Reading these statements side by side give a bit a contradictory picture. These arguments should be more precisely supported by results of the here presented work, rendering the obvious and very interesting findings of this work much clearer.

ANSWER: We agree with the reviewer that the degradation of fucoidan is a complex process, and some aspects may not have been sufficiently clear. We have now extended our analysis to a novel GH168 enzyme from strain 892, Rho5174, and performed additional experiments using the C-PAGE assay, allowing the direct visualization of degradation products. We have incorporated, reorganized and enriched the Results and Discussion sections according to these new findings (Figure 6, Supplementary Figure 6 and 9 and Supplementary Table 2).

4. Page 3 lines 59 and 60. Term ‘Endo-fucosidases’; The authors should use the generally accepted nomenclature of endo-fucanases or endo-fucoidanases, since ‘fucosidase’, in analogy to ‘glucosidase’, is the reserved term for exo-acting enzymes that specifically cleave a single monosaccharide, in general from small oligosaccharides or monosaccharide branching sugars. To avoid confusion, the authors might also take reference to the article by Deniaud-Bouët et al 2017 (reference 87) that defines the nomenclature of ‘fucan’ and ‘fucanases’ for the polysaccharide and enzymes active on **homofucans,** while **‘fucoidan’ and ‘fucoidanases’** design the polysaccharide and the enzymes active on the hetero-fucans. The enzyme nomenclature should be changed accordingly and homogenized throughout the manuscript (for example in Figure 1a&b the authors do use fucoidanase while they use fucosidase for the same enzymes elsewhere).

ANSWER: We agree with the reviewer and appreciate this clarification. In the new version of the manuscript, we now use endofucanase for endo-acting enzymes and decided to use exofucosidase for exo-acting enzymes, to facilitate understanding and clarity for non-expert readers.

5. Page 5 results. The isolated Planctomycetota strains were isolated from *Fucus spiralis* but growth experiments were performed on Fucus vesiculosus, is there a particular reason why changing the algal source?

ANSWER: Many thanks for pointing this out to us. *F. spirales* and *F. vesiculosus* belong to the same family (*Fucaceae*) and genus (*Fucus*) of brown algae. Fucoidan from *F. spiralis* was not commercially available, and its chemical structure and composition are not well studied. Instead, we have used fucoidan from *F. vesiculosus*, which was commercially available with

high purity. In addition, its chemical structure and composition is supported by several studies.

6. Figure 2 b,e. The authors state that no evident synteny is present between the PULs of the two strains, but PULA and PULG appear to be rather similar, only that in strain 913 the PUL contains a few more genes ; isn't this worthwhile mentioning?

ANSWER: Many thanks for pointing this out to us. We have prepared a new figure to better visualize the lack of synteny in the PULs (please, see Figure R1, for reviewing purposes). Figure R1 displays the genome alignment of strains 892 and 913, with the PULs highlighted by colors along the genomes. The results clearly indicate the absence of synteny in the genomic regions containing the fucoidan PULs of both strains. This information can also be found in Figure 3. The gene configuration within each PUL is detailed, allowing the comparison of gene order between PULs in both strains. Specifically, the order of GHs and SAs between PUL A and PUL G are different, but also other complementary genes as regulators and transporters. Thus, in the revised manuscript, we have referenced Figure 3 in the main text to underscore the lack of synteny (line 135). We believe it is unnecessary to include the new Figure R1 in the Supplementary Information. However, in the case the reviewer considers this appropriate, we can certainly incorporate the Figure into the Supplementary Information.

7. Also in figures 2b,e the usage of the purple color for GH168 or GH141 is not explained in the legend.

ANSWER: We agree with the reviewer. We have fixed the figure accordingly.

8. Page 9 Lines 277-278 "with the lysates of strain 892 grown in mannose and the fucoidan from U. pinnatifida and F. vesiculosus, respectively". The formulation of this sentence is misleading, the reader gets the impression that the strain was grown in mannose and fucoidan, whereas the authors want to state that the strain was grown in mannose and the lysate of this, together with PbFucA, was used against fucoidan from the two algal strains. Please reformulate to make the situation clearer.

ANSWER: We agree with the reviewer. We have reformulated the sentence accordingly (lines 271-274).

9. Supplementary Figure 11. the structural representations in a to f are tiny – they are very difficult to visually analyze and to get a realistic 3D impression of what is represented. Please provide larger panels.

ANSWER: We agree with the reviewer. We have modified the Figure accordingly. Please, note that this is Supplementary Figure 18 in the revised version.

We wish to take this opportunity to thank again the referees for their many thoughtful suggestions that have made the manuscript so much better.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

1. The authors have made considerable effort to address all comments from my first review, so I have no further changes to suggest, and congratulate them on an interesting manuscript.

ANSWER: We very much appreciate your comments on the revised version of the manuscript.

2. Minor typos:
Line 161 – disappearance
Line 944/947 - stined/distined instead of stained/destained

ANSWER: We have modified the text accordingly.

Reviewer #2 (Remarks to the Author):
The authors have done a conscientious job of answering the questions and the paper reads better. However, some key issues remain unresolved, which affects the novelty of the paper. The comments are as follows:

3. For the study on the metabolic mechanism of polysaccharides, a clear substrate structure is a prerequisite. The ambiguity of the substrate structure hinders the deduction of the cleavage site of the enzyme. The reviewer does not expect the authors to fully elucidate their structure, but the monosaccharide composition alone is not sufficient. At least some structural features should be presented, and necessary analyses (methylation analysis, FTIR, NMR) should be carried out.

ANSWER: In our study, we use commercially available fucoidan extracts due to the lack of chemically defined fucoidan polysaccharides or oligosaccharides. These polymers are of high molecular weight and highly heterogeneous. Therefore, the characterization of fucoidan extract by methylation analysis, FTIR, NMR, or other techniques is highly challenging and largely beyond the scope of this manuscript.

4. The most attractive finding of the paper is the significant up-regulation of GH168 enzymes and their hydrolytic activity towards fucoidan from algae (albeit relatively weak), as the substrate of this family was only fucoidan from sea cucumber in the past. However, the enzymology study is insufficient, despite the addition of comparisons with other complex structure. The authors still fail to clarify how the enzyme degrades the substrate used in the paper. The authors also do not perform any analysis of the enzymatic products. The reviewer can't even be certain whether the enzyme cleaves the bond between the fucose residues, instead of galactose or mannose.

ANSWER: Our enzymatic assays are valuable for understanding how different GH168 enzymes, PbFucA and Rho5174, are capable of processing fucoidan, assisted by

other hydrolytic enzymes from fucoidan-degrading bacteria. The purification and characterization of the complex mixtures of fucoidan hydrolysis products obtained in this study are exceptionally challenging and beyond the scope of this manuscript due to the heterogeneity and unknowns in the chemical structure of this polysaccharide. We have modified the discussion section to emphasize the need for further studies on the specificity of these enzymes including the following sentence: "Future work will be needed to determine the specific linkage and cleavage sites of these enzymes."

5. In the genomes, transcriptomes, and proteomes parts, the analysis is routine, although it is systematic. The conclusions in these parts lack novelty and are easy to be expected.

ANSWER: There is only one previous study that has thoroughly addressed fucoidan degradation using omics approaches on a marine bacterium, which is affiliated with the genus *Lentimonas* from *Verrucomicrobiota*. No prior studies have centered on bacteria associated with the macroalgae microbiome, as we do here, with a focus on two *Planctomycetota* strains. We observed significant differences in the arrangement of fucoidan PULs in their genomes, and found that a relatively small number of fucosidases and sulfatases are involved in fucoidan degradation. This contrast with previous knowledge on *Lentimonas*, where the number of enzymes involved is an order of magnitude higher, as revealed by proteomics. Furthermore, we emphasize the role of endo-fucanases, which was not examined in depth in the previous study.

6. The confocal microscopy part is interesting, and some novel preliminary findings have been obtained. The reviewer hope to see further work on this part. Could the authors consider deleting the enzymology part, and combine the results of genomics, transcriptomics, proteomics, and confocal microscopy to deeply reveal the mechanism behind the different metabolic locations of these two bacteria on fucoidan.

ANSWER: We thank the reviewer for the suggestion. However, we prefer to preserve the organization of the manuscript in its current form.

Reviewer #3 (Remarks to the Author):

7. The authors have substantially amended their manuscript with additional structural and RNAseq data and experiments, rendering the outcome and conclusions stronger. They also changed the title and considered all reviewers comments to adjust to the level of outcome and subject coverage of their results. As stated before, the findings are novel and bring up an integrated view of fucoidan degradation by this type of microorganisms. The experiments are well conducted, and methods are well documented. In my opinion, the study is within the scope of the journal, it contains largely original and significant results, and I only have one additional minor comment below.

Comment

8. Figure 6. The figure legend of this new figure needs more precision, and perhaps contains some errors? To me, there appear to be some inconsistencies. First, the colors of the bars in **a** and **b** are not grey, blue and orange (as in Supplementary Figure 6): in Figure 6 (of main text), here, they are grey, green and red. Second, The legend describes "***c,d*** *C-PAGE analysis of the hydrolytic activity of Rho5174 and PbFucA, strain 892 lysate grown in F. vesiculosus (****L892-Fv****) and the combination of enzymes and lysate against fucoidan from F. vesiculosus (c) and against U. pinnatifida (****d****)*" while the titles above the C-Page lanes in row **d** indicate that it is **L892-up**that is combined with the enzymes.

ANSWER: We thank the reviewer for pointing this out to us. We have modified Figure 6 legend to correct these points.

In the results section that describes these C-page results, page 9 lines 336-337, the authors state "However, hydrolysis of fucoidan from U. pinnatifida was detected when using L892-Up (Fig. 6d)." But I don't "see" any significant bands of oligosaccharides appearing in any of the C-PAGE images of row **d**; if the authors believe to see some degradation, an arrow pointing to the band changes should help guide the readers' eye to visualize the detected degradation.

ANSWER: We agree with the reviewer that this may seem unclear. Please, note that there are no visible bands of low molecular weight appearing when using the lysates L892-Up, but there is a smear appearing after 24 hours of hydrolysis in the upper part of the C-PAGE gel, which suggests that there is a partial degradation of the fucoidan. We have modified the sentence accordingly, it now reads: "However, hydrolysis of fucoidan from *U. pinnatifida* was detected when using $L_{892\text{-}Up}$, but only at a very low level. This was indicated by a smear in the upper part of the C-PAGE gel, suggesting a partial breakdown of this fucoidan source (Fig. 6d)."