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Peer Review File

Uncovering new enzymatic tools promoting lignocellulose breakdown in the anaerobic bacterium Ruminiclostridium cellulolyticum

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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)
The two comments that I raised previously for an earlier version of this manuscript were:

1. Feruloyl esterases are well known in bacteria and fungi. There have been several reports of feruloyl esterases in anaerobic bacteria and fungi, which lessens the novelty of this work.

In their rebuttal, the authors say that they have mentioned this in the Introduction, but I can't find any mention of this in the Introduction, they have just added the references that I suggested. The authors point out that these specific enzymes are found only in anaerobic bacteria, which is a valid point. But I still think that this is a well-known class of enzyme, and what the authors have done here is to identify two enzymes that seem to be of particular importance in anaerobic bacteria vs aerobic bacteria. That seems to me to be of more specialised interest, rather than general interest.

2. The authors have not demonstrated activity on polymeric lignin substrates.

In their rebuttal, the authors point out some indirect data consistent with activity for Ccel_2017, but not for Ccel_1060. They say that it is not straightforward to demonstrate activity with polymeric substrates, but they could use a lignin prepared from a mild pretreatment method (e.g. organosolv lignin) and demonstrate release of a p-hydroxycinnamic acid by HPLC. So I don't think the point I raised has been addressed.

Overall, I think it is a good piece of work, but not transformative, and would say that it is of more specialised interest.

Reviewer #2

(Remarks to the Author)
The authors have provided a robust rebuttal to my previous review; however, the key experiment that is still missing is the incubation of purified SGNHh1060 and SGNHh2017 with wheat straw and demonstrating the release of p-coumaric acid and ferulic acid. The lyophilized supernatant would be straightforward to analyse by GC-MS, as done by the authors for the culture supernatants (Supplementary Fig. 3). As the authors point out in the rebuttal, this experiment will not demonstrate whether the products come from lignin or hemicellulose or both but it would directly confirm a potential role in opening up the lignocellulose to facilitate cell wall polysaccharide breakdown. At the moment all the evidence is indirect, although the growth experiments for SGNHh2017 are compelling.

The paper does describe a considerable of body work and provides new insight into the anaerobic lignocellulose degradation by R. cellulolyticum. The clarity of the text could be improved and there is a tendency to over-emphasis the importance of the findings to possible biorefining applications, particularly in the discussion, when the role of these hydrolases has not been unequivocally demonstrated.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have now demonstrated activity on polymeric organosolv lignin, releasing pCA and ferulic acid, so they have addressed my previous point, thank you for the new data, that does strengthen the manuscript.

Minor points: 1) it would be helpful to quote a specific activity for the release of pCA and ferulic acid 2) spelling or "organosolv" in the legend of Figure 3.

Detailed point-by-point response to reviewer comments for the manuscript Vita et al

Editor comment:

From the reports, you will see that while they find your work of some potential interest, the referees raise concerns about the advance your findings represent over earlier work and the strength of the novel conclusions that can be drawn at this stage. In particular, they agree that the stated conclusions regarding lignin degradation are not supported by the data--these concerns are sufficiently important as to preclude continued consideration at Nature Microbiology.

However, I have consulted with Tobias Goris at Communications Biology, a selective open access journal from Nature Portfolio. **They are very interested in your study and would be delighted to send a suitably revised manuscript back to the original referees, if available, should you transfer it to them.** Specifically, they ask that you either provide more solid evidence in the role of the identified enzymes in degrading ligning as outlined by R1 and R2 (specifically all their points 2) or toning down appropriately and thoroughly and throughout the whole text (including title) that the enzymes are potentially involved in anoxic lignin degradation. Negative control (as requested in point 4, R2) must be included.

First of all, many thanks for the consideration and interests for our study demonstrated by the editors and the reviewers.

In summary to respond to the reviewer comments and mainly their point 2 concerning the direct activity on lignin of the identified enzymes (see below the response point by point to reviewer comments), we performed before submission of the previous manuscript, experiments by applying them on technical polymeric lignin (Kraft and alkali lignin) that were unsuccessful. This result was expected since the preparation methods of these technical polymers induce a des-esterification of the lignin. The data were consequently not included in the previous manuscript. Moreover, to our knowledge, no current analytical technics could help to determine whether the pCA and FA extracted from lignocellulosic complex biomass like Wheat Straw (WS) comes from the lignin or the polysaccharidic fractions. Additional NMR experiments on the residual substrates after growth of the strain overproducing $SGNHh_{2017}$* were also performed following the reviewers proposed complementary experiments and the results are now included in the actual manuscript version. Combined with the previous supernatant analysis results, they are confirming the role of $SGNHh_{2017}$ that releases pCA and FA from WS but do not help us to determine from which part of the WS they are from.

Therefore, we toned down our discovery in the provided revised version of the manuscript by mentioning in the whole text an action of the SGNHs on the lignocellulosic assemblies and, with further moderation and based on the available publications, that they are potentially targeting the hydroxycinnamic acids (HCA) that are mainly esterified decorations of the WS lignin or implicated in hemicelluloses-lignin crosslinks. The same way, we changed the previous title from “How to handle lignin in plant cell walls in absence of oxygen: a bacterial lesson” to “Uncovering new enzymatic tools promoting lignocellulose breakdown in anaerobic bacteria”.

Reviewer 1 comments:

1. Feruloyl esterases are well known in fungi and bacteria. While it is interesting that they are overexpressed under anaerobic conditions in this microbe, they are not a fundamentally new class of enzyme. There are several reports of the identification of ferulate esterases from anaerobic bacteria (Donaghy et al, J. Appl. Microbiol. 2000, 88, 458-466; Goldstone et al, Proteins 2010, 78, 1457-1469) and from anaerobic fungi (Borneman et al, Appl Microbiol Biotech 1990, 33, 345-351 ; Qi et al, J Appl. Microbiol 2011, 110, 1341-1350; Wong et al, Appl Microbiol Biotech 2019, 103, 8449-8457).

Given that this class of enzyme is quite well known in the literature in anaerobic bacteria & fungi, that does lessen the novelty of this work.

We agree with the reviewer that in the original manuscript, we did use the terminology cinnamoyl ester hydrolases to qualify the $SGNHh_{1060}$ and $SGNHh_{2017}$ instead of feruloyl esterases (FAE) and that we made no mention of these well-known fungal and bacterial enzymes acting on plant biomass components. However, the novelties of our discovery are 1/ that this function was never attributed to any members of the very large SGNH superfamily encompassing very different esterases in term of sequences, homologies and substrates' specificities despite sharing a common overall structure (classification based only on the catalytic residues); and 2/ that these enzymes seem to be specific of anaerobic cellulolytic bacteria producing cellulosomes. Thus, in order to adequately discuss about the FAE, the manuscript was reorganized and rewritten to mention the FAE in the introduction where the proposed references were also added.

2. The enzyme activity of the recombinant enzymes has been shown for small molecule substrates, but hasn't been demonstrated on polymeric lignin substrates. I think it's probable that these enzymes do have activity on polymeric substrates, but that hasn't been demonstrated.

We totally agree with both reviewers' hypothesis (see responses to comments 2 and 2''' of reviewer 2) and before submission of the previous manuscript, we performed experiments where we incubated Kraft and alkali lignin with the $SGNHh_{1060}$ and $SGNHh_{2017}$ we identified and no extraction of pCA and FA could be observed. This could be explained by the effect of the treatment used to generate this type of lignins[1–3] that results in pure modified lignins depleted of their esterified HCAs, molecules that the identified enzymes target. We consequently did not perform other experiments with pure enzymes on other modified polymeric lignin because of these potential structural modifications that could impact the activity of the SGNHhs that seem to have diverse and very specific preferences of substrates ($SGNHh_{1060}$: pNPA, pNPB, MpCA; $SGNHh_{2017}$: pNPA, pNPB, pNPF, MpCA; MFA).

Moreover, we also performed after reception of the reviews and in response to comment 5 of reviewer 2, additional NMR analysis on the residual substrate after growth of the strain overexpressing $SGNHh_{2017}$* that are now included in the Fig. 1 and its legend, and further discussed in the main manuscript. It shows a similar decrease of FA content in the residual Wheat Straw (WS) compared to the one observed in the case of the wt strain. This sustains that $SGNHh_{2017}$ is at least implicated in the des-esterification of this plant biomass HCA interacting with both lignin and polysaccharides[4–11] but unfortunately it is known that NMR analysis on lignocellulosic substrate does not allow to decipher if it is esterifying lignin or polysaccharides. To our knowledge, no other biophysical technics are available to provide more solid evidence in the role of $SGNHh_{1060}$ and $SGNHh_{2017}$ in degrading lignin. We also did not perform experiments with pure enzymes on different types of natural lignified substrates (WS, wheat bran…) because, given the analysis techniques currently available, we would not be able to specify if the HCA released could originate from the lignin or the polysaccharide decorations.

Reviewer 2 comments:

1. The methods need to state that the wheat straw has been sterilised to avoid contamination of the cultures.

To correct this omission, the following sentence was added in the "Cultivation conditions" paragraph of the "Methods" section: "All insoluble substrates (C, WS or Mix) were added to the media before sterilization, subsequently sterilized with the medium, especially for WS to avoid contamination of the cultures by other microorganisms."

2. FA and pCA can originate outside of lignin e.g. ester-linked FA in hemicellulose/pectin and pCA is also ester linked in pectin / xylan. Are the authors certain the activities observed are specifically focussed on lignin modification?

We totally agree with the fact that FA and pCA are esterifying hemicellulose, pectin, xylan and lignin, thus its mention in the previous submitted manuscript[4–8,12–16]. However, to our knowledge, no analytical technics (NMR, FTIR…) are available to decipher if both released HCAs are those esterifying lignin or hemicelluloses (see response to comment 2 of reviewer 1). Since pCA is mainly decorating the WS lignin[4–6,12–15] and FA interacts with lignin and polysaccharides[4–11], we thus propose that SGNHh$_{1060}$ and SGNHh$_{2017}$ are more likely to act specifically on lignin decoration and the link between lignin and hemicelluloses. Concerning the pectin and for our knowledge, it is a minor component of WS that is mainly composed of secondary cell wall structures where this complex hetero-polysaccharide is present in trace amount or absent[17,18], reason why we did not consider that the released HCAs could come from pectin decoration removal.

2'. The authors demonstrate through a comparison of the transcriptomes of R. cellulolyticum during growth on wheat straw and non-lignified substrates that members of the SGNH hydrolase superfamily are upregulated; however, there is no direct evidence that they are present in the secretome when R. cellulolyticum is grown on wheat straw. This would be straightforward to establish by proteomics analysis of the secretome of R. cellulolyticum growing on lignin and non-lignin containing substrates. Although SGNHh1060 and SGNHh2017 were identified in a previous proteomics study, it is surprising that it was not confirmed here. Proteomics analysis would also provide evidence of the abundance and diversity of SGNH hydrolases in the secretome. This is particularly important in the light of the negative results obtained with the knockout strains.

We agree with the reviewer that this proteomic analysis demonstrating that both SGNHh$_{1060}$ and SGNHh$_{2017}$ are more abundant in the secretome of *R. cellulolyticum* grown on WS is of prime importance and that it should sustain our hypothesis of their role on lignified substrate.

Since the previous published proteomic data were acquired previously by former members of our team using hatched WS and crystalline cellulose[19], two of the substrates being used in our transcriptomic approach, we decided that these published data were robust enough and to report them in the Supplementary tables 1 and 2. Moreover, we use these previous proteomic data in the manuscript to demonstrate the presence of SGNHh$_{1060}$ only in cellulosomes prepared from WS culture and not from other plant polysaccharides, abundance that perfectly correlates with the transcriptomic data showing the induction of its encoding gene expression on lignified substrate, confirming SGNHh$_{1060}$ is one of the major enzymes implicated in the WS degradation (see table hereafter). For SGNHh$_{2017}$, in addition to its similarities with SGNHh$_{1060}$, we included this protein based on the proteomic data because they

show, as stated in the manuscript, its presence only in cellulosomes prepared from WS culture whereas it should have not been included in this study based on the transcriptomic data (see table hereafter). In the contrary for $SGNHh_{2395}$, it was included in this manuscript based on the transcriptomic data and should have not been included based on the proteomic ones (see table hereafter).

Concerning the use of the proteomics analysis to provide evidence of the abundance and diversity of SGNH hydrolases in the secretome, it is also a very interesting point discussed by reviewer 2 and it was, before the 1st submission, an option to orientate the article toward the characterization of the SGNHs in *R. cellulolyticum* and their role in the treatment/degradation on lignified substrate.

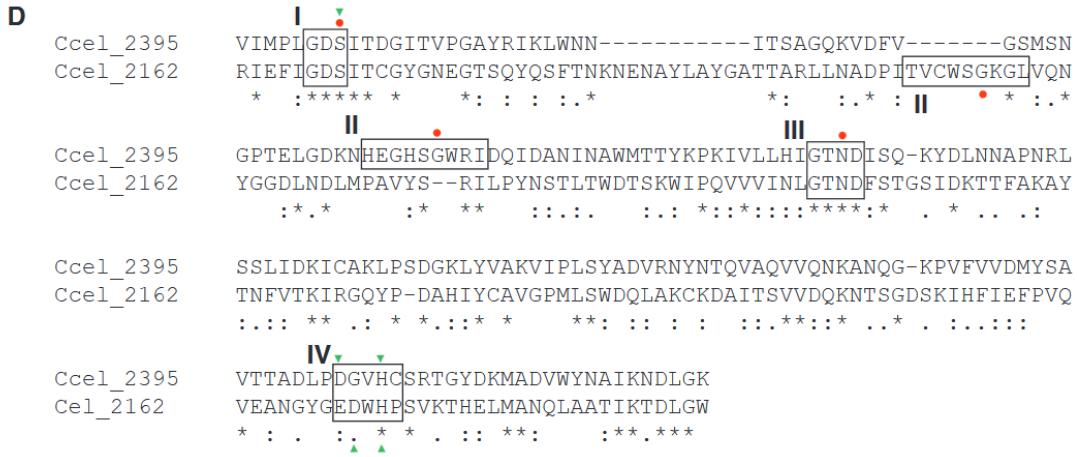
Indeed, during the comparison of the transcriptomic and proteomic data when writing the manuscript, we thought about looking into the SGNH diversity since going deeper in *R. cellulolyticum* genome, a total of six genes encoding proteins from this superfamily were detected (see table hereafter). In addition to the 3 genes encoding $SGNHh_{1060}$, $SGNHh_{2017}$ and $SGNHh_{2395}$, the two genes at loci Ccel_RS03760 and Ccel_RS16100 encode two other "SGNH/GDSL hydrolase family proteins" (Ccel_0742/$SGNHh_{0742}$ and Ccel_3210/$SGNHh_{3210}$, Supplementary table S1 and table hereafter) that are both intracellular and consequently discarded from the present study because we targeted enzymes implicated in the extracellular degradation of WS. Furthermore, the gene at locus Ccel_RS10925 also codes for a "GDSL-type esterase/lipase family protein" (Ccel_2162, Supplementary table S1 and table hereafter). Its corresponding secreted product $SGNHh_{2162}$ is composed of a N-terminal dockerin linked to "Carbohydrate esterase 2 N-terminal" (Pfam ID: PF17996) followed by a CD that has similarities with $SGNHh_{2395}$ CD (see alignment hereafter) and a function also predicted as a CE2 or an acetyl xylan esterase in CAZy database[20], and by dbCAN-[21] and ESTHER DB-[22] web servers. Although $SGNHh_{2017}$ and $SGNHh_{2162}$ encoding genes share a similar regulation pattern (Supplementary table S1 and table hereafter), the protein was detected in the cellulosomal fraction produced on all tested substrates suggesting this enzyme is active on polysaccharide(s) and It was therefore not included in our study.

We then decided to not refer to this diversity in the article for the sake of clarity in the manuscript that already contains a lot of different approaches and data.

					Detected peptide count in Blouzard et al.		
Protein IDentification based on Old gene Locus Tag (PIDOLT)	**Id protein**	**Signal Peptide (SP) prediction**	**Secretion**	**Presence of dockerin (DOC)**	**Number of peptides on cellusose NM300**	**Number of peptides on oat spelt xylan**	**Number of peptides on hatched wheat straw**
Ccel_1060	WP_015924576,1	SPI	yes	yes			8
Ccel_2017	WP_015925465,1	SPI	yes	yes			4
Ccel_2162	WP_015925599,1	SPI	yes	yes	13	3	15
Ccel_2395	WP_015925817,1	SPII	yes	yes			
Ccel_0742	WP_015924287,1						
Ccel_3210	WP_015926558,1						

			Normalized RNAseq read count				
Protein IDentification based on Old gene Locus Tag (PIDOLT)	**Locus_tag**	**Annotations**	**Cb**	**C**	**Mix**	**WSm**	**WS**
Ccel_1060	CCEL_RS05325	dockerin type I domain-containing protein	2771	2305	5032	70525	182310
Ccel_2017	CCEL_RS10195	dockerin type I domain-containing protein	3367	1103	1616	4076	3268
Ccel_2162	CCEL_RS10925	GDSL-type esterase/lipase family protein	12537	1488	2593	5960	7889
Ccel_2395	CCEL_RS12090	GDSL-type esterase/lipase family protein	412	539	634	691	2531
Ccel_0742	CCEL_RS03760	SGNH/GDSL hydrolase family protein	29	41	62	23	287
Ccel_3210	CCEL_RS16100	SGNH/GDSL hydrolase family protein	748	9193	4889	3395	2160

						Fold change			
Protein IDentification based on Old gene Locus Tag (PIDOLT)	**Locus_tag**	**WSvsCb FoldChange**	**WSvsCb padj**	**WSvsC FoldChange**	**WSvsC padj**	**WSvsMix M FoldChange**	**WSvsMix M padj**	**WSvsWSm FoldChange**	**WSvsWSm padj**
Ccel_1060	CCEL_RS05325	65,79	3,29E-53	79,101	6,88E-58	36,233	9,57E-39	2,585	1,19E-03
Ccel_2017	CCEL_RS10195	0,97	9,13E-01	2,962	1,56E-06	2,022	3,38E-03	0,801	3,77E-01
Ccel_2162	CCEL_RS10925	0,629	1,26E-01	5,3	7,20E-09	3,041	2,75E-04	1,324	3,85E-01
Ccel_2395	CCEL_RS12090	6,141	3,23E-14	4,697	1,30E-10	3,99	1,95E-08	3,657	1,31E-07
Ccel_0742	CCEL_RS03760	10,009	4,28E-08	6,903	3,04E-06	4,661	3,57E-04	12,591	3,69E-09
Ccel_3210	CCEL_RS16100	2,888	3,13E-05	0,235	7,61E-09	0,442	2,41E-03	0,636	9,28E-02



2’’’. Activity of recombinant SGNHh1060 and SGNHh2017 was demonstrated against a range of model pNP ester substrates but it is surprising that the enzymes were not incubated with lignin and/or wheat straw and the breakdown products analysed. The substrates MpCA and MFA are known to hydrolyse quite rapidly in water but the methods do not mention a negative control / blank.

Both reviewers’ comments are appropriate and as stated in the response to comment 2 of reviewer 1, we applied purified $SGNHh_{1060}$ and $SGNHh_{2017}$ on Kraft and alkali lignin but no liberation of FA and pCA was observed mainly due to these modified lignins’ preparation that des-esterify the substrates. We also did not apply them directly on WS since there is currently no analytic technics allowing us to discriminate the origin of these HCA (lignin or hemicellulose decorations). However, we demonstrated that, compared to the WS growth of the wt strain, the overexpression of $SGNHh_{2017}$* induces 1/ a larger release in the culture supernatant of both pCA, decoration esterifying mainly the lignin in WS[4–6,12–15], and FA, interacting with lignin and polysaccharides[4–11] , and 2/ using additional NMR analyses that are now included in the manuscript, a similar decrease of FA quantities in the residual substrate after growth. These data comfort the fact that $SGNHh_{2017}$ at least acts on polymeric lignified substrate by releasing pCA and FA, this second HCA undoubtedly coming from lignocellulosic substrate such as WS. By extension and based on $SGNHh_{1060}$ and $SGNHh_{2017}$ high similarities, we propose the same hypothesis for the role of $SGNHh_{1060}$.

Concerning the negative control relative to MpCA and MFA hydrolysis, the corresponding data were available in the source data file but were not included in the manuscript. To correct this omission, the following sentences “Negative controls were performed using the same experimental conditions without enzymes.” and “Negative controls were performed in the same experimental conditions without addition of enzymes in order to take in account the eventual non-enzymatic hydrolysis of the substrates in the specific activity calculation.” were added in the Fig. 3’s legend and in the “Enzymatic activity of the proteins of interest on p-nitrophenyl and p-hydroxycinnamic acid derivates” paragraph of the “Methods” section.

3. It would be relatively straightforward to demonstrate that these enzymes have a significant role in opening up the lignin structure for polysaccharide degradation by adding the enzymes to a commercial cellulase cocktail and demonstrate an increased release of reducing sugars from wheat straw.

It is an excellent comment from the reviewer and we already planned this type of experiments. Indeed, some of our collaborators are applying this approach and the tests are currently being developed. Since this manuscript is focusing on the discovery of the enzymes potentially involved in the bacterial anoxic extracellular lignin degradation, we consequently did not think to add these still ongoing experiments to this manuscript that is already rich in data.

4. SDS PAGE analysis of the purified enzymes should be included to confirm that the enzymes have been purified to homogeneity and that the activity is not due to contaminating E. coli esterases.

The Supplementary Fig. 7 was added in the Supplementary figures file. It depicts the corresponding photo of the SDS-PAGE gel analysis of the purified enzymes and we stated in its legend that "the three recombinant SGNHh heterologously produced in *E. coli* were purified to homogeneity avoiding any contamination by *E. coli*'s cytoplasmic esterases that would have interfered with the activity measurements described in Figure 3." Reference to the Supplementary Fig. 7 was adequately added in the text of the main manuscript.

5. Cultures of the overexpressing strains were analysed for fermentation products; however, only SGNHh2017 accumulated more pCA and FA. Importantly, the residual biomass was not analysed for increased lignin modification.

We agree with the reviewer that in the original manuscript, we did not carried out this important experiment to confirm that the released pCA and FA in the supernatant could be correlated with the structural WS modification and loss of these compounds upon growth of the modified strain. Consequently, we performed additional NMR analysis on the residual substrate after growth of the strain overexpressing $SGNHh_{2017}$* that are now included in in the Fig. 1 and its legend, and further discussed in the manuscript. They effectively show, compared to the WS growth of the wt strain, a similar decrease of the Wheat Straw (WS) content for FA, HCA that interacts with lignin and polysaccharides[4–11] (see responses to comments 2 of reviewer 1 and 2''' of reviewer 2).

6. Line 137 and elsewhere "tricin" not "tricine"; Line 175. The sentence starting "Are also…" needs to be rewritten.

The correction "tricine" to "tricin" was made all along the manuscript. The sentence starting by "Are also found Ccel_1243, […] Ccel_1101 (PIDOLT), which is a CotH homolog (Supplementary table 2 and Fig. 2B)." was rewritten as follow "We also identified the following proteins (Supplementary table 2 and Fig. 2B): Ccel_1243, Ccel_2622, Ccel_2735, and Ccel_3134 (Protein IDentification based on Old gene Locus Tag, PIDOLT), which are hypothetical proteins; Ccel_2642 and Ccel_3247 (PIDOLT), which have large unknown function domains; and Ccel_1101 (PIDOLT), which is a cellulosomal CotH homologue."

References

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Detailed point-by-point response to reviewer comments for the manuscript Vita et al

Editor comment:

Your manuscript entitled "Uncovering new enzymatic tools promoting lignocellulose breakdown in anaerobic bacteria" has now been seen by 2 referees, whose comments are appended below. You will see from their comments below that while they find your work of considerable interest, some important points are raised. We are interested in the possibility of publishing your study in Communications Biology, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

We therefore invite you to revise and resubmit your manuscript, taking into account the points raised. In particular,...

**Both reviewers suggest two different (although similar) and not too complicated nor time-consuming experiments with either wheat straw or pretreated lignin and subsequent product detection with either HPLC or GC-MS, which strengthen your manuscript further. I am aware that doing both might take much more time, so we do explicitly leave it up to you which one of the two routes you follow.

**As R1 points out, the novelty in describing the enzymes should not be overstated and one or two sentences should be included in the introduction.

First of all, many thanks for the willingness and the offered possibility to publish our manuscript in Communications Biology as well as for the interest and consideration for our work demonstrated by the editors and the reviewers.

Given that both reviewers rightly ask us to provide evidence of the direct activities of SGNHhs on polymeric substrate using either wheat straw or mildly pretreated lignin, and that the editor suggested that we choose one of the two proposed routes to address the reviewers' concerns, we performed the experiment on organosolv wheat straw lignin to at least investigate/demonstrate the HCA decoration removal activity of the SGNHhs. Indeed, the use of wheat straw as substrate could not, according to us, have helped to determine whether the pCA and FA extracted from this lignocellulosic complex biomass come from the lignin or the polysaccharidic fractions. The corresponding experimental data were then included (new Fig. 3B and 3C) and further discussed in the manuscript.

With regard to the reviewers' concerns about the novelty of the discovery, sentences relative to the cinnamoyl esterases (FAE) have been modified/added in the introduction-, the results- and the discussion-sections to discuss about the role of the SGNHhs, a function already performed by other well-known proteins, but nevertheless completely new for members of the SGNH superfamily. Additional measurements of SGNHhs activities on other methyl-hydroxycinnamic acid esters (not requested by the reviewers) were also performed and consequently added (modified Fig. 3A) and discussed to the article to strengthen the demonstration of their role as cinnamoyl esterases.

Concerning reviewers' points on the importance of the findings and its possible biorefining application, they have been toned down all along the manuscript with, as requested, a large rewriting and reduction of the discussion on the possible use of the SGNHS as biotechnological tools, which, as the reviewers mentioned, has not been demonstrated.

All the writing changes are in red in the revised manuscript.

Reviewer 1 comments:

1. Feruloyl esterases are well known in bacteria and fungi. There have been several reports of feruloyl esterases in anaerobic bacteria and fungi, which lessens the novelty of this work.

In their rebuttal, the authors say that they have mentioned this in the Introduction, but I can't find any mention of this in the Introduction, they have just added the references that I suggested. The authors point out that these specific enzymes are found only in anaerobic bacteria, which is a valid point. But I still think that this is a well-known class of enzyme, and what the authors have done here is to identify two enzymes that seem to be of particular importance in anaerobic bacteria vs aerobic bacteria. That seems to me to be of more specialised interest, rather than general interest.

We apologize to the reviewer 1 if the changes made in the previous revised manuscript concerning the subject of the FAE function of the SGNHhs were insufficient. To comply with this request, we have modified/added sentences in the introduction-, the results- and the discussion-sections (lines 86, 106, 294, 342, and 493) to discuss about the role of the SGNHhs, a function already performed by other well-known proteins as mentioned by reviewer 1, but nevertheless completely new for members of the SGNH superfamily. These modifications were performed in order to tone down the impact of our findings that as rightly stated by reviewer 1, seems to be important for anaerobic cellulolytic bacteria that, instead of fully delignifying the lignocellulose, have apparently selected a strategy based on the unzipping of the lignin from the parietal polysaccharides. We also decided to use the terminology cinnamoyl esterase instead of FAE since we demonstrate that SGNHs are also acting on HCA esterified on both polysaccharides and lignin (see response to comment 2 of reviewer 1) whereas for us, the terminology FAE only refers to enzymes acting on HCA esterified on the polysaccharides. Moreover, we added in the manuscript (Fig. 3A) and discussed (lines 288 to 296) additional and not requested measurements of SGNHhs activities on other methyl-hydroxycinnamic acid esters (methyl caffeic acid, methyl sinapic acid and chlorogenic acid) in order to strengthen the demonstration of SGNHs role as cinnamoyl esterases.

2. The authors have not demonstrated activity on polymeric lignin substrates.

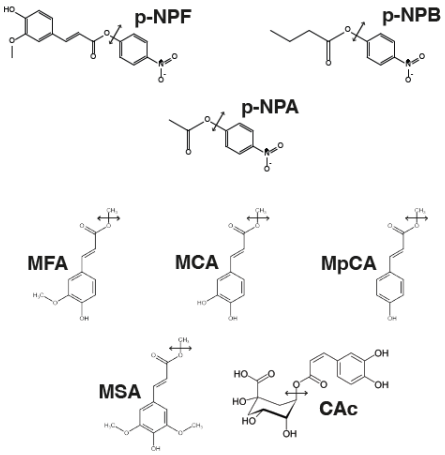
In their rebuttal, the authors point out some indirect data consistent with activity for Ccel_2017, but not for Ccel_1060. They say that it is not straightforward to demonstrate activity with polymeric substrates, but they could use a lignin prepared from a mild pretreatment method (e.g. organosolv lignin) and demonstrate release of a p-hydroxycinnamic acid by HPLC. So I don't think the point I raised has been addressed.

We totally agree with both reviewers' proposal that demonstrating the SGNHs HCA releasing activity from polymeric substrates should strengthen further our demonstration of the SGNHs function in lignocellulose degradation and both reviewers suggest two similar routes to perform the experiment (see response to reviewer 2 comment). Thus, as proposed by reviewer 2, we applied the SGNHs on wheat straw organosolv lignin and measured the quantity of FA and pCA released over time. The corresponding results were consequently added in the manuscript (new Fig. 3B and 3C, lines 297 to 306) and used to demonstrate that contrary to $SGNHh_{2395}$ that we characterized as a typical CE, $SGNHh_{1060}$ and $SGNHh_{2017}$ remove the HCA decoration from at least lignin.

Figure 3

A

Substrate	Specific activity (U.mg^{-1})		
	SGNHh$_{1060}$	SGNHh$_{2017}$	SGNHh$_{2395}$
p-NPA	25.8 ± 0.742	15.8 ± 0.552	24.3 ± 0.948
p-NPB	55.3 ± 1.61	21.5 ± 1.64	0.480 ± 0.164
p-NPF	0.219 ± 0.042	1.96 ± 0.286	0.047 ± 0.026
MFA	n.d.	3.78 ± 0.237	0.037 ± 0.024
MCA	0.507 ± 0.029	0.333 ± 0.078	n.d.
MpCA	3.96 ± 0.222	3.30 ± 0.215	n.d.
MSA	n.d.	n.d.	n.d.
CAc	0.484 ± 0.037	0.219 ± 0.043	0.055 ± 0.016



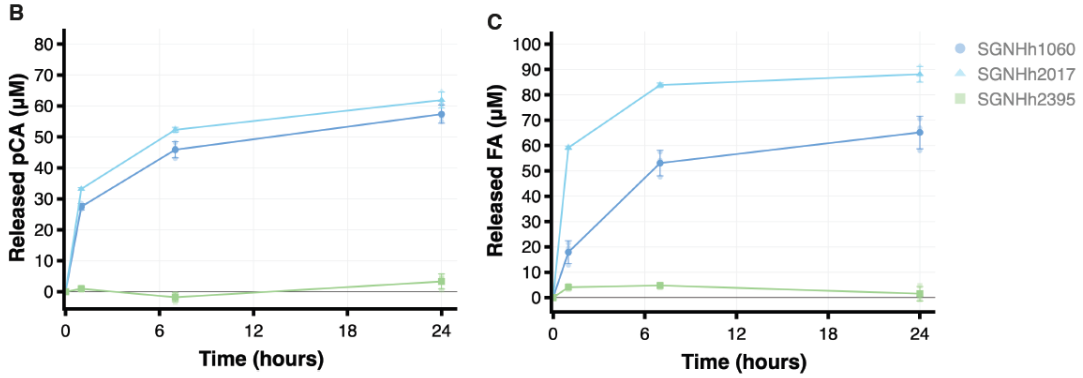


Fig. 3: Specific activities of SGNHh$_{1060}$, SGNHh$_{2017}$ and SGNHh$_{2395}$ on model esterase substrates and methyl-hydroxycinnamic acid derivates. A. The three recombinant SGNHh activities were measured spectrophotometrically following the cleavage of the ester bond from pNP-acetate (pNPA), pNP-butyrate (pNPB) and pNP-ferulate (pNPF) ([Protein] = 44-53 nM, [Substrate] = 1 mM), or from methyl ferulic acid (MFA), methyl caffeic acid (MCA), methyl coumaric acid (M*p*CA), methyl sinapic acid (MSA) and chlorogenic acid (CAc) ([Protein] = 35-48 nM, [Substrate] = 100 µM). The experiments were performed in triplicates at 37°C in 50 mM potassium phosphate pH 7 with addition of 1 % Tween 80 in the buffer for pNPF assays. Negative controls were performed using the same experimental conditions without enzymes. Calculated specific activities are reported in mg of total protein. 1U = µmol pNP/min. n.d. : not detected. The structure of the substrates is represented on the right and double arrows indicate the position of the cleaved bond. **B.** and **C.** The quantities (µM) of *p*CA (**B.**) and FA (**C.**) released according to time (hours) by the three recombinant SGNHh when individually applied on WS organoslov lignin are depicted ([Protein] = 1 µM, [Substrate] = 5 g.L^{-1}). The experiments were performed in triplicates at 37°C in 50 mM potassium phosphate pH 7. Negative controls were performed using the same experimental conditions without enzymes and the presented data were corrected by substraction of the quantities measured in the negative control to the ones measured for each enzyme.

Reviewer 2 comments:

The authors have provided a robust rebuttal to my previous review; however, the key experiment that is still missing is the incubation of purified SGNHh1060 and SGNHh2017 with wheat straw and demonstrating the release of p-coumaric acid and ferulic acid. The lyophilized supernatant would be straightforward to analyse by GC-MS, as done by the authors for the culture supernatants (Supplementary Fig. 3). As the authors point out in the rebuttal, this experiment will not demonstrate

whether the products come from lignin or hemicellulose or both but it would directly confirm a potential role in opening up the lignocellulose to facilitate cell wall polysaccharide breakdown. At the moment all the evidence is indirect, although the growth experiments for SGNHh2017 are compelling.

As mentioned in the response to reviewer 1 comment 2, we totally agree with both reviewers' proposal that demonstrating the SGNHs HCA releasing activity from polymeric substrates would strengthen our manuscript further. We considered to perform this experiment on wheat straw as requested by reviewer 2 but reviewer 1 proposed to use instead lignin prepared from a mild pretreatment method (e.g. organosolv lignin). Since the editor let us the choice to explore one or the other proposed routes and that according to us, the use of wheat straw could not have helped to determine whether the pCA and FA extracted come from the lignin or the polysaccharidic fractions, we chose to perform and discuss the experiments carried on wheat straw organosolv lignin and at least demonstrate that $SGNHh_{1060}$ and $SGNHh_{2017}$ remove the HCA decoration from lignin (see response to reviewer 2 comment 1).

The paper does describe a considerable of body work and provides new insight into the anaerobic lignocellulose degradation by R. cellulolyticum. The clarity of the text could be improved and there is a tendency to over-emphasis the importance of the findings to possible biorefining applications, particularly in the discussion, when the role of these hydrolases has not been unequivocally demonstrated.

We apologize to reviewer 2 (and also reviewer 1) if it appeared to him/her in the previous revised version of the manuscript that we tend to over-emphasis the importance of the findings to possible biorefining applications. Then, we toned down our comments on these subjects throughout the manuscript and also largely rewrote and reduce the discussion on the possible use of the SGNHS as biotechnological tools.

Detailed point-by-point response to reviewer comments for the manuscript Vita et al

Editor comment:

Your manuscript entitled "Uncovering new enzymatic tools promoting lignocellulose breakdown in anaerobic bacteria" has now been seen again by one of our referees, whose comments appear below. In light of their advice I am delighted to say that we are happy, in principle, to publish a suitably revised version in Communications Biology.

Include any additional editorial comments about the process or referee reports

We therefore invite you to revise your paper one last time to address the remaining concerns of our reviewers. At the same time we ask that you edit your manuscript to comply with our format requirements and to maximise the accessibility and therefore the impact of your work.

While we do not have a strict limit on citations, the amount of about 150 seems a bit inflationary. Check and delete those which are not really necessary, especially in bulk citations in lines 80, 87, 121 and the last paragraph of the discussion.

First of all, we are really grateful to the editor and the reviewers for their help and comments during the reviewing process that allowed us to strengthen our work and manuscript. We also greatly thank them and are really happy for the acceptance of our paper to be published in Communications Biology.

Given the reviewer 1's remaining concerns, we added as requested the SGNHh specific activities for the release of pCA and FA from WS organosolv lignin in the manuscript (lines 262 to 267) and we corrected the misspelling "organoslov" to "organosolv" in the legend of Figure 3.

With regard to the editor's points and the edition of the manuscript to comply with Communications Biology format requirements, we modified the manuscript following the Final Revision Instructions. Most particularly, we edited the title that now includes the name of the bacterium studied in our work, we added a section "Statistics and Reproducibility" in the Methods, we made the RNAseq data discussed in this publication publicly accessible and we deflated the amount of references from 154 to 133.

Reviewer 1 comments:

The authors have now demonstrated activity on polymeric organosolv lignin, releasing pCA and ferulic acid, so they have addressed my previous point, thank you for the new data, that does strengthen the manuscript.

Minor points: 1) it would be helpful to quote a specific activity for the release of pCA and ferulic acid 2) spelling or "organosolv" in the legend of Figure 3.

First of all, many thanks to reviewer 1 for his/her reactivity during the full revision process and also to both reviewers for their helpful comments that greatly strengthen our manuscript. To comply with the two minor points requested by reviewer 1, we added/edited a paragraph including the SGNHh specific activities for the release of pCA and FA from WS organosolv lignin in the manuscript (lines 262 to 267) and we corrected the misspelling "organoslov" to "organosolv" in the legend of Figure 3.