



HAL
open science

Characterization of three bacterial glycoside hydrolase family 9 endoglucanases with different modular architectures isolated from a compost metagenome

Laure Aymé, Agnès Hébert, Bernard Henrissat, Vincent Lombard, Nathalie Franche, Stéphanie Perret, Etienne Jourdir, Senta Heiss-Blanquet

► To cite this version:

Laure Aymé, Agnès Hébert, Bernard Henrissat, Vincent Lombard, Nathalie Franche, et al.. Characterization of three bacterial glycoside hydrolase family 9 endoglucanases with different modular architectures isolated from a compost metagenome. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 2021, 1865 (5), pp.129848. 10.1016/j.bbagen.2021.129848 . hal-03150854

HAL Id: hal-03150854

<https://ifp.hal.science/hal-03150854>

Submitted on 5 Mar 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Characterization of three bacterial glycoside hydrolase family 9**
2 **endoglucanases with different modular architectures isolated**
3 **from a compost metagenome**

4 Laure Aymé^a, Agnès Hébert^a, Bernard Henrissat^{b,c,d}, Vincent Lombard^{b,c}, Nathalie Franche^e, Stéphanie
5 Perret^e, Etienne Jourdiar^a and Senta Heiss-Blanquet^{a*}

^aIFP Energies Nouvelles, 1 - 4 avenue du Bois-Préau, 92852 Rueil-Malmaison, France

^bArchitecture et Fonction des Macromolécules Biologiques (AFMB), CNRS, 163 avenue de Luminy,
13288 Aix Marseille Université, Marseille, France

^cINRAE, USC1408 Architecture et Fonction des Macromolécules Biologiques (AFMB), 163 avenue
de Luminy, 13288 Marseille, France

^dDepartment of Biological Sciences, King Abdulaziz University, Jeddah, Saudi Arabia

6 ^eAix Marseille Université, CNRS, LCB, 31 Chemin Joseph Aiguier, 13009 Marseille, France

7

8

*Corresponding author:

Senta Heiss-Blanquet, IFP Energies Nouvelles, 1 et 4, avenue de Bois-Préau, 92852 Rueil-Malmaison
Cedex, France

+33 1 47 52 72 56, senta.blanquet@ifpen.fr, ORCID 0000-0001-8533-3274

9

10 **Abstract**

11 **Background:** Environmental bacteria express a wide diversity of glycoside hydrolases (GH). Screening
12 and characterization of GH from metagenomic sources provides an insight into biomass degradation
13 strategies of non-cultivated prokaryotes.

14 **Methods:** In the present report, we screened a compost metagenome for lignocellulolytic activities and
15 identified six genes encoding enzymes belonging to family GH9 (GH9a-f). Three of these enzymes
16 (GH9b, GH9d and GH9e) were successfully expressed and characterized.

17 **Results:** A phylogenetic analysis of the catalytic domain of pro- and eukaryotic GH9 enzymes
18 suggested the existence of two major subgroups. Bacterial GH9s displayed a wide variety of modular
19 architectures and those harboring an N-terminal Ig-like domain, such as GH9b and GH9d, segregated
20 from the remainder. We purified and characterized GH9 endoglucanases from both subgroups and
21 examined their stabilities, substrate specificities and product profiles. GH9e exhibited an original
22 hydrolysis pattern, liberating an elevated proportion of oligosaccharides longer than cellobiose. All of
23 the enzymes exhibited processive behavior and a synergistic action on crystalline cellulose. Synergy
24 was also evidenced between GH9d and a GH48 enzyme identified from the same metagenome.

25 **Conclusions:** The characterized GH9 enzymes displayed different modular architectures and distinct
26 substrate and product profiles. The presence of a cellulose binding domain was shown to be necessary
27 for binding and digestion of insoluble cellulosic substrates, but not for processivity.

28 **General significance:** The identification of six GH9 enzymes from a compost metagenome and the
29 functional variety of three characterized members highlight the importance of this enzyme family in
30 bacterial biomass deconstruction.

31

32 **Keywords:** metagenome, cellulose hydrolysis, endoglucanase, glycoside hydrolase

33

34 **1. Introduction**

35 Carbohydrates are the building blocks of the most structurally diverse class of biopolymers, such as α -
36 and β -glucans, chitosan, xanthan, agar or pectins [1]. Nature has thus evolved a huge variety of
37 enzymes able to synthesize, modify or degrade these polymers. Among them, glycoside hydrolases
38 (GHs) (EC 3.2.1.x) catalyzing the hydrolysis of glycosidic bonds are of special interest as their activity
39 is of major importance for the deconstruction of plant- and animal-derived materials such as starch,
40 cellulose or chitin [2]. GHs are one of the five enzyme classes which are classified further in the
41 comprehensive Carbohydrate-Active enZymes (CAZy) database [3,4] with updated information
42 regarding their substrate specificities, catalytic mechanisms and three-dimensional structures [5]. Each
43 sequence-based family groups together enzymes sharing common structural and mechanistic features.
44 Some families are monospecific with their members acting on only one substrate, but many families
45 group together enzymes with different substrate specificities [3]. Currently, over 165 GH families are
46 listed in the CAZy database and this number keeps on growing [6], reflecting the huge diversity of the
47 enzymatic arsenal devoted to carbohydrate degradation.

48 Cellulose, the most abundant organic compound on earth, represents an important feedstock for the
49 synthesis of biofuels and platform chemicals [7]. This linear biopolymer composed of β -1,4-linked
50 glucose molecules forms partly crystalline microfibrils, making enzymatic attack difficult [8]. Its
51 enzymatic degradation requires the concerted action of several types of activities [8,9].

52 Endoglucanases (EC 3.2.1.4) randomly cleave internal β -1,4 glycosidic linkages and increase the
53 number of chain ends; cellobiohydrolases (EC 3.2.1.91) attack the cellulose chain at the reducing or
54 the non-reducing end, processively cleaving every second glycosidic bond to form cellobiose, the
55 smallest structural repeating unit of cellulose; finally, β -glucosidases (EC 3.2.1.21) hydrolyze
56 cellobiose into glucose units. Recently, oxidative cleavage of polysaccharides by Lytic Polysaccharide
57 Monooxygenases (LPMOs) has been demonstrated and shown to enhance the hydrolytic activity of
58 cellulase cocktails [10,11].

59 GHs and LPMOs often display a modular structure where the catalytic domain (CD) can be appended
60 to one or more carbohydrate-binding modules (CBMs). These auxiliary domains allow binding to
61 insoluble polysaccharides. They are functionally and structurally independent modules, able to target a
62 specific polysaccharide, bringing the CD into close proximity to the substrate [12,13]. CBMs are
63 currently divided into 86 families in the CAZy database. Other accessory modules, such as N-terminal
64 immunoglobulin (Ig)-like domains [14] or fibronectin type III domains [15], can also be associated
65 with CDs. N-terminal Ig-like domains have been shown to interact directly with the CD and to
66 stabilize it in the *Alicyclobacillus acidocaldarius* Cel9A and the *Clostridium thermocellum*
67 cellobiohydrolase CbhA [16,17].

68 Cellulases are widely distributed in nature and produced by many microorganisms, mainly bacteria
69 and fungi [18], but they are also found in plants [19], some protozoa [20] and some animals [21–23].
70 Microorganisms have elaborated different strategies for cell wall deconstruction [18]. Aerobic
71 cellulose-degrading bacteria and fungi secrete free cellulases, while anaerobic microbes sometimes
72 express multienzyme complexes termed cellulosomes [8]. In these multienzymatic complexes, a
73 scaffold protein binds various catalytic subunits via cohesion-dockerin interactions [24]. Non-
74 cellulosomal cellulases can also be found in anaerobic microorganisms [25,26], where they are present
75 either in a free form or attached to the cell wall. The advantage of the cellulosomal organization is the
76 proximity of different enzyme activities which generates synergies between enzymes [27]. A similar
77 strategy is applied by the recently characterized thermophilic anaerobic bacterium *Caldicellulosiruptor*
78 *bescii* which produces multimodular enzymes within a single gene product presenting important
79 synergistic interactions [28].

80 Cellulolytic bacterial species that have been characterized include the anaerobic *Ruminiclostridium*
81 *cellulolyticum* and *C. thermocellum* or the aerobic species *Thermobifida fusca* [24,29–33]. Their
82 genomes encode cellulases mainly from families GH5, GH9 and GH48. Many enzymes of the latter
83 family are described as processive GH and bacteria generally contain only a few GH48-encoding
84 genes. In contrast, GH5 and GH9 cellulases which display endoglucanase activities are often more
85 abundant. Thus, the *C. cellulolyticum* genome encodes 13 GH9s and each of the enzymes has a

86 particular specificity in terms of substrate preference, processivity or binding affinity [34]. GH9
87 endocellulases often act synergistically with GH48 glucanases, wherein the synergistic factor varies
88 as a function of the GH9 partner enzyme [35,27]. In contrast, the genome of *Clostridium*
89 *phytofermentans* encodes only one GH9 enzyme that has been shown to be a major contributor to
90 cellulose degradation [36].

91 In nature, microorganisms often act in a synergistic way to efficiently degrade plant biomass. Previous
92 studies have shown that metagenomes of relevant environments such as soils or compost contain a
93 large number of hemicellulose and cellulose encoding genes, especially if they have been enriched on
94 lignocellulosic substrates [37–39]. Major bacterial phyla present in these aerobic enrichments are
95 *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* [40–42]. These communities thus constitute an
96 interesting reservoir for new enzymes which can be of biotechnological relevance. Concerning the
97 identification of genes involved in lignocellulose degradation, shotgun DNA sequencing delivers a
98 global picture of the functional potential of the microbial community, but suffers from the
99 inconvenience of yielding only very few full-length clones. In contrast, activity-based screening has
100 the advantage of retrieving full coding sequences and identifying genes with low sequence homology
101 to genes of known function [43].

102 In the present study, a metagenomic library was constructed from compost which had been enriched
103 on lignocellulosic biomass relevant to industrial ethanol production. Screening on model substrates
104 yielded a large number of hemicellulase and cellulose encoding genes, in particular six novel genes
105 encoding GH9 cellulases. The high diversity of still uncharacterized enzymes within the GH9 family,
106 combined with their likely importance in cellulolytic cocktails of aerobic and anaerobic bacteria, led
107 us to characterize and compare three representatives of this family. They originate from two different
108 phyla and are representative of several modular architectures of GH9 enzymes. The results revealed
109 that each of them has distinct biochemical characteristics, including substrate preferences, product
110 profiles and ability to synergize with other enzymes.

111 **2. Materials and methods**

112 **2.1 Cloning of metagenome-derived bacterial GH9s for expression in *Escherichia coli***

113 Compost samples from a municipal compost platform were incubated for seven months with a
114 lignocellulosic substrate consisting of pretreated *Miscanthus giganteus*, wheat straw and poplar and
115 DNA was extracted as previously described [38]. The composition of the lignocellulosic material is
116 detailed in [38]. High molecular weight DNA was separated on a 0.5% low melt agarose gel and the
117 size range of around 40 kb was isolated. After end-repair and a supplementary purification step using
118 Phase Lock Gel (Quantabio, Beverly, MA, USA), the fragments were cloned into pCC2FOS vector
119 and packaged (Epicentre, Madison, WI, USA). The resulting library of about 48,000 clones was
120 screened on AZCL-xyloglucan and AZCL-hydroxycellulose. Clones were grown overnight in
121 microplates with the addition of 0.02 % arabinose to induce a high-copy number of fosmids. After
122 transfer onto LB/chloramphenicol agar plates and overnight growth, an overlay containing lysozyme
123 and AZCL-substrates was applied. Plates were incubated at 55°C for five days to allow enzymatic
124 hydrolysis of the chromogenic substrates [44]. Positive clones were retrieved by observation of clones
125 using a low-power stereo microscope. After a second, identical screening round, positive fosmid
126 clones were sequenced using Illumina sequencing, assembled using the Velvet algorithm and
127 submitted to CAZy family assignment using FASTY (V35) [3,45]. Protein sequences of CAzymes that
128 were relevant for biomass degradation were BLASTed against the non-redundant Protein database for
129 taxonomic assignment using the closest homolog [46]. Full length genes encoding putative GH9
130 cellulases were subcloned into the pET300/CT-DEST vector using Gateway technology (Thermo
131 Fisher Scientific/Invitrogen, Waltham, MA, USA) and transformed into *E. coli* BL21(DE3) cells
132 (MerckMilliporeSigma, Burlington, MA, USA) for protein production. The full-length gene encoding
133 the unique GH48 identified in the positive fosmid clones was subcloned in a pET22
134 (MerckMilliporeSigma, Burlington, MA, USA). The primers NdeI-GH48
135 (5'AAAAAACATATGCGGGTCGCCTGTGACGTGACCTAC3') and XhoI-GH48
136 (5'AAAAAACTCGAGGGGGAAGAGCAGGCCGTAC3') were designed to amplify the entire gene

137 by PCR. Using the restriction sites *NdeI* and *XhoI* (underscored in the primers) in a pET22b(+)
138 digested with the same enzymes, the gene was cloned in frame with the sequence encoding a 6xHis-
139 tag at the 3' terminus. The recombinant vector pET-GH48 was used to transform the *E. coli*
140 BL21(DE3) strain for protein production.

141 **2.2 Bioinformatics analysis of the GH9 family**

142 GH9 sequences extracted from the compost metagenome underwent a protein BLAST analysis [46] to
143 determine the taxonomic order of each metagenome-derived GH9 using their respective closest
144 homologs. To construct a phylogenetic tree, the sequences of the 172 characterized GH9s available on
145 the CAZy database on April 17, 2019 were analyzed along with the 6 metagenome-derived GH9s
146 using Geneious software version 9.1.8 (Biomatters, Auckland, New Zealand). Sequences were aligned
147 using Clustal Omega [47] operating with 10 iterations. Only the aligned protein CDs were conserved.
148 Signal peptides and N- and C-terminal extensions that are not fully conserved across the alignment,
149 were removed. Proteins containing partial CDs were excluded from the analysis. The resulting amino
150 acid sequences (168 sequences + 6 metagenome-derived GH9s) were realigned using Clustal Omega.
151 The Neighbor-Joining (NJ) algorithm was used for distance tree building with a bootstrap resampling
152 method using 1000 replicates and a support threshold of 75%. The resulting phylogenetic tree was
153 displayed and annotated using the iTOL online tool [48]. For each sequence, information regarding
154 domain nature and organization, taxonomy and available 3D-structure of the CD was retrieved from
155 the Uniprot [49], Pfam [50] and CDD [51] databases.

156 **2.3 Heterologous expression of the recombinant metagenome-derived GH9s**

157 Recombinant 6xHis-tagged proteins were expressed in *E. coli* BL21 (DE3) cells grown in
158 autoinduction MagicMedia medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented
159 with 100 µg/ml ampicillin. 500 ml cell cultures were grown in 2000 ml flasks at 250 rpm, incubated at
160 37 °C for 24 h. Cells were harvested by centrifugation at 5000 g for 10 min, washed with 0.9% (w/v)
161 NaCl and stored at -80°C. GH48 enzyme was produced in the recombinant *E. coli* BL21(DE3) strain
162 grown at 37°C to an optical density at 600 nm of 1.5, followed by the addition of 200 µM isopropyl-β-

163 D-thiogalactopyranoside (IPTG) overnight at 20°C, with shaking. Cells were harvested by
164 centrifuging for 15 min at 6000 g and processed directly for protein purification.

165 **2.4 Protein purification**

166 All purification steps were carried out at 4°C. Bacterial cell pellets expressing recombinant proteins
167 (GH9b, GH9d and GH9e) were thawed and resuspended in the lysis buffer (25 mM triethanolamine
168 pH 7.0, 150 mM NaCl, 5% (v/v) glycerol, 15 mM imidazole) supplemented with SigmaFAST Protease
169 Inhibitor Cocktail (1 tablet/50 ml, (MerckMilliporeSigma, Burlington, MA, USA), 1 mg/ml lysozyme
170 (MerckMilliporeSigma, Burlington, MA, USA) and 15 µg/ml DNase I (MerckMilliporeSigma,
171 Burlington, MA, USA). Cells were disrupted by sonication using a Bioblock Scientific Vibra-Cell
172 Ultrasonic Processor (Sonics & Materials Inc). Extracts were spun down at 12 000 g for 15 min.
173 Supernatants were loaded onto HisTrap FF crude columns (GE Healthcare, Chicago, IL, USA) pre-
174 equilibrated with the lysis buffer. Washing and elution were carried out in lysis buffer supplemented
175 with imidazole using a two-step gradient (washing at 40 mM imidazole and elution at 112, 88 and 185
176 mM imidazole for GH9b, GH9d and GH9e, respectively). Purified proteins were concentrated using
177 Vivaspin 20 centrifugal concentrator (30 kDa molecular weight cut-off, Sartorius) and loaded onto a
178 Superdex 200 10/300 GL size-exclusion column pre-equilibrated with gel filtration buffer (25 mM
179 triethanolamine pH 7.0, 150 mM NaCl, 5% (v/v) glycerol). Elution was carried out using an isocratic
180 flux of buffer. The column was calibrated with Bio-Rad's gel filtration standard (Biorad, Hercules,
181 CA, USA). When size-exclusion chromatography was omitted (GH9e only), imidazole elution buffer
182 was exchanged with gel filtration buffer by diafiltration using the Vivaspin centrifugal concentrator.
183 For GH48 purification, cells were resuspended in 30 mM Tris-HCl (pH 8) and disrupted in a French
184 Press. The crude extract was spun down (10 min, 10000 g) and the supernatant containing the His-
185 tagged proteins was loaded onto a column of Ni-nitrilotriacetic acid superflow resin (Qiagen, Hilden,
186 Germany) equilibrated with 30 mM Tris-HCl (pH 8) and eluted using the same buffer supplemented
187 with 60 mM imidazole. The fractions containing the purified protein were pooled and concentrated by
188 ultrafiltration (Vivaspin 10 kDa cutoff, Sartorius, Göttingen, Germany) and loaded onto an anion
189 exchange chromatography column (HiTrap Q-sepharose, GE Healthcare, Chicago, IL, USA)

190 equilibrated with 30 mM Tris-HCl (pH 8) then eluted with a linear NaCl gradient (0- 0.5 M). Fractions
191 of interest were pooled, dialyzed and concentrated by ultrafiltration (Vivaspin 20, 30 kDa cutoff,
192 Sartorius, Göttingen, Germany) in 30 mM Tris-HCl (pH 8).

193 Protein concentrations were determined using a Nanodrop spectrophotometer with extinction
194 coefficients calculated using the ExPASy ProtParam tool [52]. Purified proteins were snap-frozen in
195 liquid nitrogen and stored at -80°C for over a year without any significant loss of activity.

196 **2.5 SDS-PAGE**

197 Proteins were separated by SDS-PAGE using 10% polyacrylamide Mini-PROTEAN TGX precast gels
198 (BioRad, Hercules, CA, USA) with Tris-glycine running buffer (BioRad, Hercules, CA, USA) and
199 lithium dodecyl sulfate (LDS) sample buffer (Thermo Fisher Scientific, Waltham, MA, USA)
200 supplemented with 50 mM dithiothreitol (DTT). Protein samples were denatured at 70°C for 10 min
201 before loading. The gels were stained with BioSafe Coomassie (Biorad, Hercules, CA, USA).

202 **2.6 Substrates**

203 Carboxymethyl cellulose (CMC) (degree of substitution 0.7) was obtained from SERVA. Low
204 viscosity barley β -glucan, low viscosity Konjac glucomannan and tamarind seed amyloid xyloglucan
205 were purchased from Megazyme. 2-hydroxyethyl-cellulose (HEC) and laminarin were obtained from
206 Merck. Phosphoric acid-swollen cellulose (PASC) was prepared from microcrystalline cellulose
207 Avicel PH-101 (MerckMilliporeSigma, Burlington, MA, USA) as follows: 5 g of Avicel PH-101 was
208 dissolved in 100 ml of 85% phosphoric acid at room temperature and precipitated with cold distilled
209 water. Fibers were collected and washed 6 times in cold distilled water. The pH of the suspension was
210 adjusted to 6.5 with 1 M sodium carbonate. Cellulose was washed again 4 times in cold distilled water.
211 Resulting PASC was collected, drained and stored at - 20°C until use.

212 **2.7 Activity measurements on soluble substrates**

213 Reducing sugar concentration was determined using the 3,5-dinitrosalicylic acid (DNS) assay [53] and
214 a D-glucose standard curve. The effect of pH and temperature on endoglucanase activity was assayed
215 with CMC. Respective enzymes (20 nM to 300 nM) were added to the assay mixture containing 1%

216 (w/v) CMC diluted in McIlvaine's citrate-phosphate buffer [54]. The assay mixture was incubated for
217 10 min. For each enzyme, optimal pH and temperature were respectively assayed in the ranges of 2.7
218 to 7.4 and 34°C to 85°C. Reactions were stopped by adding 2 volumes of DNS solution (300 g/l
219 potassium sodium tartrate and 10 g/l DNS in 0.4 M sodium hydroxide) and were then heated for 5 min
220 at 95°C. The absorbance was measured at 540 nm using a microplate spectrophotometer (Multiskan
221 ascent, Thermo Fisher Scientific, Waltham, MA, USA).

222 For stability assays, enzymes were incubated for 24h at different pHs (2.7-7.4) or temperatures (0°C-
223 84°C) and residual activities were measured under optimal conditions for each enzyme.

224 Specific activities of each enzyme were evaluated on several substrates (low viscosity barley β -glucan,
225 low viscosity Konjac glucomannan, tamarind seed amyloid xyloglucan, 2-hydroxyethyl-cellulose and
226 laminarin) diluted to 1% (w/v) in McIlvaine's citrate-phosphate buffer. Respective enzymes (20 nM to
227 300 nM) were assayed at their corresponding optimal temperatures and pHs.

228 To determine kinetic parameters on CMC, hydrolysis reactions were carried out at the optimal pH and
229 temperature with a concentration of CMC varying from 0.25% to 4% (w/v). The experimental data
230 were fitted to the Michaelis-Menten equation using the maximum likelihood method described in
231 [55]. The Michaelis constant (K_M) and the turn-over number (k_{cat}) were determined from the model.

232 To measure the effect of different metal ions on the activity, hydrolysis reactions were carried out for
233 10 min on 1% (w/v) CMC supplemented or not supplemented with 1 mM $MnCl_2$, $MgCl_2$, $CaCl_2$ or
234 $ZnCl_2$. Respective enzymes (80 nM to 240 nM) were assayed at their corresponding optimal
235 temperatures and pHs.

236 **2.8 Product profile on crystalline and amorphous cellulose**

237 50 nM of each enzyme was incubated at 50°C in the presence of 10 g/l PASC or Avicel PH-101
238 diluted in McIlvaine's citrate-phosphate buffer, pH 5.9. After 1h, 6h or 24h, the reactions were
239 stopped by adding two volumes of 200 mM NaOH. The mixtures were then filtered at 0.22 μ m to
240 remove insoluble substrates. The glucose and corresponding oligosaccharide concentrations were
241 determined using high-performance anion-exchange chromatography (HPAEC) coupled with pulsed

242 amperometric detection (PAD) (Dionex, Thermo Fisher Scientific, Waltham, MA, USA) and using a
243 CarboPac PA1 column (Thermo Fisher Scientific, Waltham, MA, USA). Data acquisition from the
244 detector and determination of retention times and peak areas were carried out using Chromeleon
245 software version 6.8 (Thermo Fisher Scientific, Waltham, MA, USA). The column was operated at a
246 constant flow rate of 0.3 ml/min at 30 °C. Before injection, the column was equilibrated with 100% of
247 eluent A (0.1 M NaOH) over 5 min. Elution was carried out using a linear gradient of 0–15% of eluent
248 B (1 M sodium acetate in 0.1 M NaOH) over 11 min, followed by a step at 15% of eluent B for 4 min.
249 Glucose and oligosaccharide solutions in the concentration range of 0.1-20 mg/L were used as external
250 standards. Specific activities on Avicel and PASC were inferred from total glucose and
251 oligosaccharide contents.

252 **2.9 Substrate binding assay**

253 Binding of the enzymes to insoluble substrates, Avicel PH-101 and PASC, was assayed using the
254 method carried out by [34]. Briefly, 1.5 µM of each enzyme was incubated at 4°C for 1 h at 400 rpm
255 in 200 µl of cellulose (PASC or Avicel) at 7 g/l in McIlvaine's citrate-phosphate buffer pH 5.9. The
256 samples were subsequently centrifuged at 10 000 g for 10 min, and the supernatants were collected
257 and mixed with 3X LDS sample buffer supplemented with 150 mM dithiothreitol. The cellulose-
258 containing pellets were washed twice with McIlvaine's citrate-phosphate buffer, pH 5.9 and
259 resuspended in 200 µl of diluted LDS sample buffer supplemented with 50 mM dithiothreitol. The
260 protein samples were boiled for 10 min and the samples were centrifuged for 10 min at 10 000 g. The
261 supernatants were collected and 15 µl was separated by SDS-PAGE as described above.

262 **2.10 Synergy assay**

263 Assays were carried out on Avicel PH-101 (20g/l) with 50 nM of enzyme mixtures at different molar
264 ratios. Avicel PH-101 was hydrolyzed with shaking (850 rpm) for 4h at 50°C and pH 5.9 in
265 McIlvaine's citrate-phosphate buffer. The reactions were stopped by boiling for 10 min. The reducing
266 ends released in the supernatant were assayed by the modified 2,2'-bicinchoninate (BCA) method
267 [56]. Synergistic factors were calculated by dividing the activity of the mixture by the sum of the
268 individual activities.

269 **2.11 Processivity assay**

270 3.5 g/L PASC was hydrolyzed by 1 μ M of each enzyme (GH9b, GH9d or GH9e) with shaking (850
271 rpm) for 6h at 50°C and pH 5.9 in McIlvaine's citrate-phosphate buffer. The reactions were stopped by
272 boiling for 10 min. The supernatant was extracted and the cellulose pellet was washed three times with
273 McIlvaine's buffer. After centrifuging, the reducing ends released in the supernatant and pellet were
274 assayed by the modified BCA method established by [56]. The processivity was determined from the
275 distribution of the reducing ends released in the cellulose pellet (insoluble) and the supernatant
276 (soluble) according to [57].

277 **2.12 Sequence data**

278 All of the sequences in this article were submitted to the GenBank database and have the following
279 accession numbers: MT186814 (GH9a), MT186815 (GH9b), MT186816 (GH9c), MT186817 (GH9d),
280 MT186818 (GH9e), MT186819 (GH9f), MT186820 (GH48).

281

282 **3. Results**

283 **3.1 Sequence and phylogenetic analyses of novel metagenome-derived bacterial GH9 enzymes**

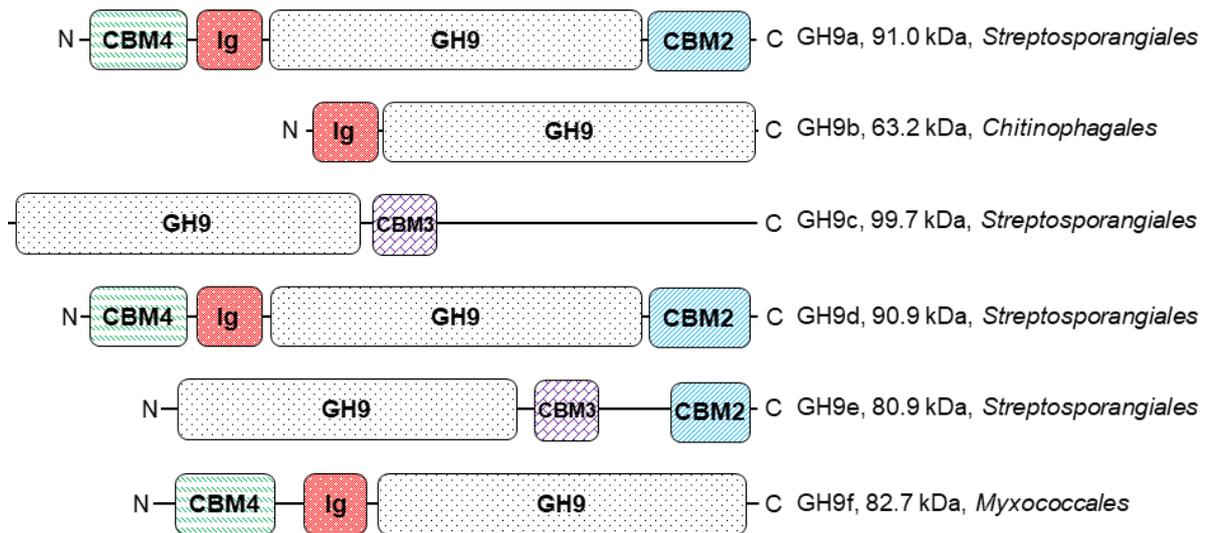
284 Compost samples were enriched on lignocellulosic biomass under aerobic conditions for seven
285 months, after which metagenomic DNA was extracted. A library of 48,000 clones was screened on
286 AZCL-cellulose and AZCL-xyloglucan. After two screening rounds, 74 positive clones were
287 sequenced and ORFs were subjected to CAZy family assignment using the same procedures as those
288 applied for the updates of the CAZy database [4,2]. 31 genes encoding CAZymes potentially involved
289 in plant biomass deconstruction and possessing a signal peptide for secretion could be identified
290 (Supplementary Table S1). Most of them encode glycoside hydrolases, but five carbohydrate esterases
291 from families CE1 and CE4, one AA10 LPMO and two gene fragments containing a carbohydrate
292 binding domain CBM2 were also retrieved. The overall identities with sequences from the non-
293 redundant protein database ranged from 32% (a CE1 carbohydrate esterase) to 92% (for GH74a and

294 GH51 enzymes). Concerning the probable phylogenetic origin of these genes, two thirds were most
295 closely related to sequences from the *Actinobacteria* phylum, 16% displayed highest identity scores to
296 sequences from the *Bacteroidetes* phylum and two genes were closest to sequences from
297 *Proteobacteria*. Only three genes were related to other phyla. This result is consistent with the
298 observed phylogenetic distribution of the shotgun metagenome from the same enriched compost [38],
299 suggesting that the metagenomic sequences identified here by functional screening might indeed
300 originate from species involved in biomass deconstruction in the present compost community.

301 For most GH families, only one or two representatives were retrieved from the fosmid library, but
302 notable exceptions are the GH74 and GH9 families, where respectively five and six genes were
303 identified. As GH9 have mostly endo- or exoglucanase activity and are important players in plant
304 biomass deconstruction, these enzymes were investigated further. The six enzymes (denoted GH9a-
305 GH9f) harbored a putative signal peptide suggesting extracellular localization, and they display
306 different modular organizations (Fig. 1). Four of them displayed the highest identity scores (66-83%)
307 with sequences from the *Streptosporangiales* order, whereas GH9b and GH9f showed the most
308 similarity to sequences from a *Thermoflavifilum* species (belonging to the *Chitinophagales* order, 78%
309 identity) and from the genus *Sorangium* (*Myxococcales* order, 69% identity), respectively. All but the
310 GH9b enzyme possess CBMs belonging to families 2, 3 or 4 attached to their catalytic domains. Four
311 proteins harbor a N-terminal Ig-like module located upstream of the CD. GH9a and GH9d display a
312 similar domain architecture as well as a very high sequence identity (94.5% over the full-length
313 sequence and reaching 96% over the CD) (Fig. 1).

314

315

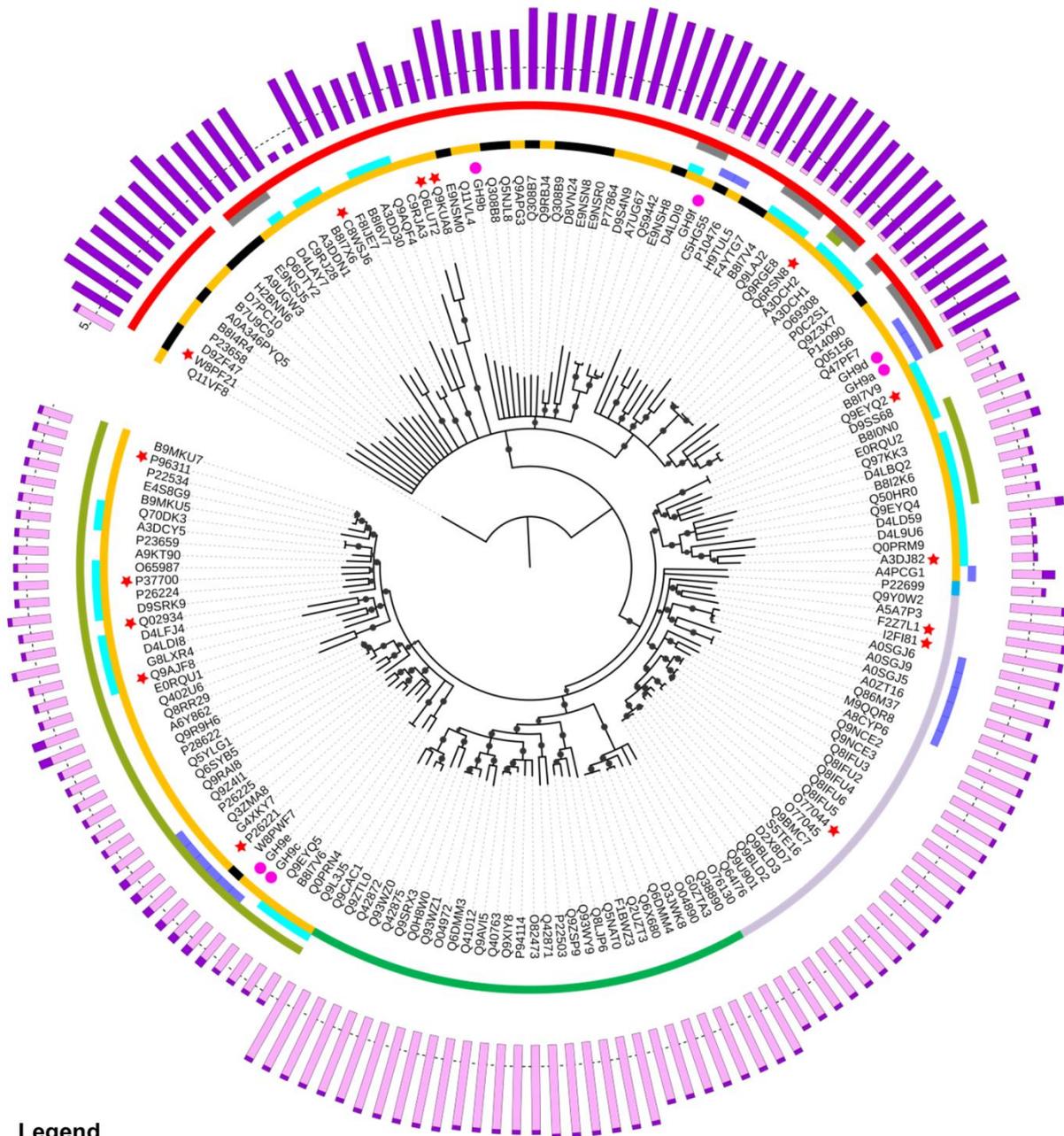


316

317 **Fig. 1** Domain architecture of the metagenomic-derived bacterial GH9s. The CD (GH9) and accessory
 318 modules (N-terminal immunoglobulin-like domain (Ig), CBM2, CBM3 and CBM4) are represented
 319 with their relative sequence lengths. The molecular weights of mature proteins were calculated using
 320 the ExPASy ProtParam tool [52]. Taxonomic orders were predicted by BLAST [46] using the
 321 phylogenetic origin of the closest homolog. N, N-terminal; C, C-terminal

322

323 The CDs of our metagenome-derived GH9s were aligned with the 168 characterized GH9s that
 324 contain a full-length CD and which were listed in the CAZy database on April 17, 2019. The resulting
 325 alignment was used to build a phylogenetic tree (Supplementary Fig. S1 and Fig. 2). Two sub-families
 326 appeared upon phylogenetic analysis: GH9 enzymes devoid of an Ig-like module (Pfam accession
 327 number PF02927) formed a clade distinct from that containing Ig-like module-containing GH9s. GH9
 328 sequences without an Ig-like module are divided into several taxonomical nodes that separate plant,
 329 animal and bacterial proteins with bootstrap values larger than 0.8. Bacterial proteins devoid of an Ig-
 330 like module segregate in at least two different clades. Among our metagenome-derived enzymes, Ig-
 331 like module-containing GH9a and GH9d, with a 96% sequence identity between their CDs, showed
 332 the smallest evolutionary distance. GH9b and GH9f, also harboring an Ig-like module and with,
 333 respectively, 48% and 29% sequence identity to GH9d, were clustered in two distinct nodes.



Legend

Tree scale: 0.1 —			
Annotations	Taxonomic Kingdoms	Domain composition	C-terminal loop length
● metagenome-derived GH9	■ Bacteria	■ N-terminal Ig-like domain	■ Loop A
★ 3D structure available	■ Viridiplantae	■ CBM4	■ Loop B
	■ Metazoa	■ CBM3	
	■ Amoebozoa	■ CBM2	
	■ Unclassified or uncultured	■ Dockerin	

334

335

336 **Fig. 2** Phylogeny of the GH9 family. Circular phylogram based on the alignment of the CDs of the six
337 metagenome-derived GH9s and the 168 characterized GH9 sequences extracted from the CAZy
338 database. The multiple sequence alignment was built using Clustal Omega and the Neighbor-Joining
339 (NJ) algorithm was used for distance tree building. Bootstrap values higher than 80 are represented
340 with a dark dot. Metagenome-derived GH9s are highlighted with a fuchsia dot. Crystallized CDs with
341 a known three-dimensional (3D) structure are highlighted by a red star. The inner ring is colored
342 according to the taxonomic kingdom of the corresponding organism. The middle rings are colored as a
343 function of the presence of one of the five major accessory modules (N-terminal Ig-like domain,
344 CBM2, CBM3, CBM4 or dockerin). The respective lengths of the two possible C-terminal loops are
345 represented in the outer ring as a stacked column chart with a dashed line corresponding to a length of
346 5 residues. C-terminal loops associated with Ig-like domain-containing GH9s (loop A) or with GH9s
347 exhibiting no Ig-like module (loop B) are respectively represented in violet and in pink. The
348 phylogenetic tree annotation was carried out with iTOL [48]

349

350 The tree distance matrix highlighted a low sequence identity between GH9s containing or not
351 containing an Ig-like module (Supplementary Table S2). However, it is likely that they share a
352 common ancestor, as evidenced by residual sequence identity and their similar three-dimensional
353 structures (Supplementary Fig. S2). The CD is a conserved $(\alpha/\alpha)_6$ barrel fold with similar secondary
354 structure orientations. Moreover, our phylogenetic analysis highlighted the presence of a GH9 from
355 *Cytophaga hutchinsonii* (Uniprot accession number Q11VF8), devoid of an Ig-like module and
356 distantly related to both sub-families. Taken together, these results are in line with a common origin
357 for all GH9s and an early loss of the Ig-like domain during evolution.

358 We could identify 19 different modules in the 174 aligned GH9s highlighting the wide variety of
359 modular architectures in this family (Supplementary Table S3). Among them, only 5 modules are
360 shared by at least 9% of the proteins. All bacterial GH9s, except two, harbored at least one accessory
361 module (i.e. CBM or Ig-like module). CBM4 and CBM9 are closely related and identified under the
362 Pfam accession number PF02018. All of the CBM4/9-containing GH9 enzymes also harbored an Ig-

363 like domain. In contrast to bacteria, eukaryotic GH9 enzymes, which are all devoid of an Ig-like
364 domain, present much less variability in their modular architecture. Strikingly, they also all lack helper
365 modules, with the exception of some metazoan enzymes comprising a CBM2 module. Two bacterial
366 subgroups can be found among the group of enzymes without Ig-like domain: in one of them,
367 corresponding to the enzymes formerly termed “Theme B” enzymes [58], all enzymes contain a
368 CBM3, which is not true for the second subgroup, where only part of the enzymes contain a CBM3.
369 The second major branch grouping together Ig-like domain-containing enzymes, also displays two
370 subgroups: one of them includes a majority of enzymes with CBM4 (corresponding to the previously
371 termed “Theme D” enzymes). The second one, corresponding to “Theme C” enzymes, comprises a
372 majority of enzymes without a CBM, but also some enzymes with a CBM4 module. Consequently, the
373 presence or absence of several modules, such as CBM2, CBM3 or dockerin, appeared to be
374 independent of the phylogenetic clustering, suggesting that only the upstream Ig-like module has co-
375 evolved with the CD.

376 Multiple sequence alignment of the CD highlighted the presence of two distinct C-terminal loops,
377 respectively present in the Ig-like module-containing GH9s (loop A) and in the GH9s from the second
378 branch (loop B) (Fig. 2). Taking a closer look at the structure of two crystallized GH9 members, each
379 belonging to one of the two subfamilies, loop A and B are both bordering the substrate binding cleft
380 (Supplementary Fig. S3). Wu and Davies have already pointed out this difference and explained the
381 different modes of action of the exo-acting GH9 of *Vibrio cholerae* (UniProt: Q9KUA8) and endo-
382 acting GH9s by the presence of a longer loop restricting the active-site binding pocket in the exo-
383 acting enzyme. [59] Our analysis suggests that the longer loop A is present in most Ig-like domain
384 containing enzymes, even if they are endo-acting. Two GH9s without any Ig-like module (UniprotKB
385 accession number A9UGW3 and O69308) appeared among the Ig-like module-containing GH9s and
386 displayed the corresponding loop A. However, these proteins are annotated as partial or fragmentary,
387 according to the CAZy and UniprotKB databases, respectively. Therefore, it is possible that the full-
388 length sequences of these proteins harbor an Ig-like domain.

389 **3.2 Purification of three metagenome-derived bacterial GH9s**

390 In order to characterize the newly identified GH9 enzymes, we intended to subclone them for
391 expression in *E. coli*. For unknown reasons, GH9a and GH9f clones could not be obtained. The strain
392 containing the GH9c-encoding gene did not yield any protein. However, two GH9s harboring an Ig-
393 like domain (GH9b and GH9d) and one GH9 belonging to the branch without an Ig-like domain
394 (GH9e) were successfully cloned and expressed in *E. coli*. Proteins were purified to homogeneity
395 (Supplementary Fig. S4) with estimated purities of 99.5% (GH9b), 91.6% (GH9d), 96.8% (GH9e), as
396 determined by densitometry. Upon separation by size-exclusion chromatography, the proteins
397 displayed apparent molecular weights (MW) that were different from expected (Supplementary Fig.
398 S4). GH9d exhibited the apparent MW of a trimer, assuming it displays a globular shape. GH9b and
399 GH9e MWs appeared respectively 2.4 and 2.1 times smaller, suggesting a non-globular shape. In order
400 to increase yields, GH9e which was poorly expressed but eluted with a high purity rate upon affinity
401 chromatography, was not separated by gel filtration for further experiments.

402 **3.3 Functional characterization on soluble substrates**

403 In order to investigate substrate specificities of metagenome-derived GH9s, specific activities of
404 GH9b, GH9d and GH9e were measured on various soluble substrates (Table 1). The most prominent
405 activities were obtained on β -glucan for the three enzymes. GH9b exhibited the highest activity on all
406 soluble substrates, while GH9e displayed specific activities that were at least 4.2 times lower than for
407 GH9b. All three enzymes showed notable activities on CMC and glucomannan, a linear copolymer of
408 glucose and mannose, joined by β -(1 \rightarrow 4)-linkages. In order to evaluate the ability of each enzyme to
409 cleave β -(1 \rightarrow 3) linkages, the enzymes were incubated with laminarin, a (1,3)- β -D-glucan with β -
410 (1 \rightarrow 6) branching. Compared to β -glucan, a mixed linkage glucose polysaccharide containing β -(1 \rightarrow 3)
411 and β -(1 \rightarrow 4) bonds, no significant activity was detected on laminarin, indicating that the enzymes are
412 specific for β -(1 \rightarrow 4) glycosidic bonds. Overall, specific activities were lower on substrates containing
413 substitutions (CMC, hydroxyethyl cellulose, xyloglucan) compared to non-substituted substrates like
414 β -glucan and glucomannan, suggesting that the functional groups (respectively carboxymethyl,
415 hydroxyethyl and xylose) might cause steric hindrance, thereby rendering access for hydrolases

416 difficult. This might be especially true for xyloglucan due to its bulkier substitution compared to
 417 carboxymethyl or hydroxyethyl side chains. Taken together, these results demonstrate that the purified
 418 enzymes exhibit endo- β -1,4-glucanase activity.

419

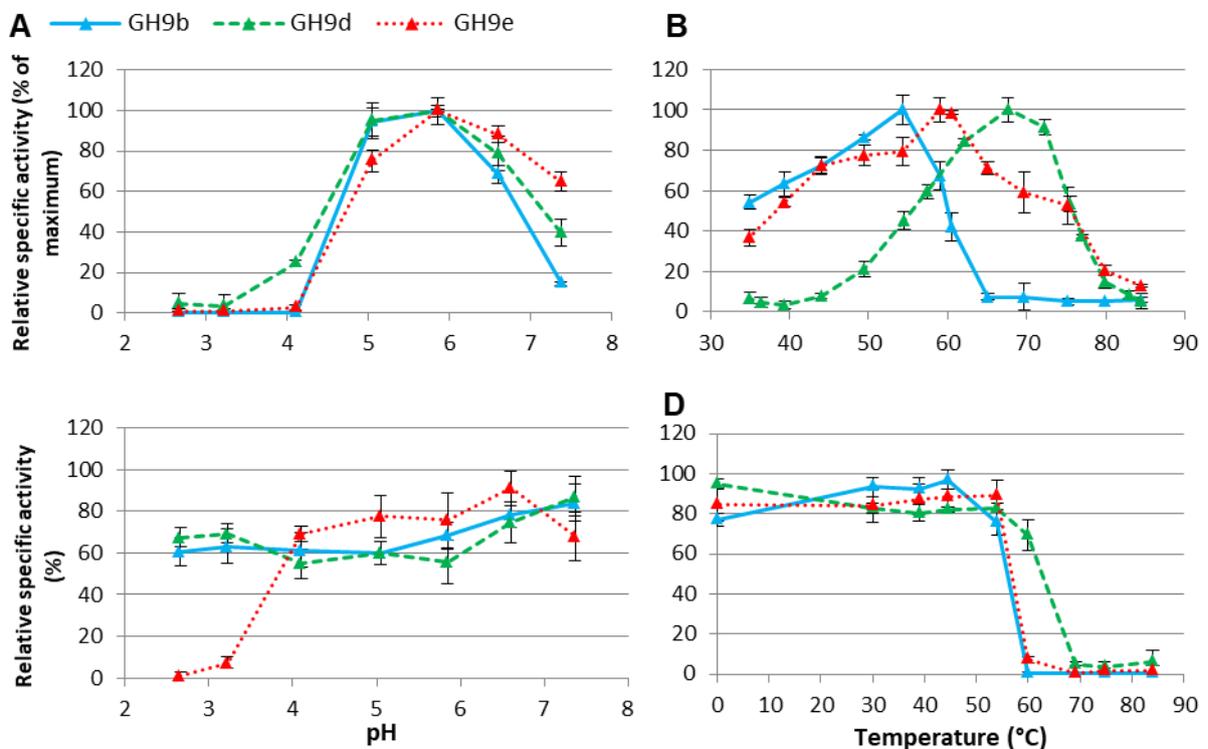
	Specific activities ($\mu\text{mol min}^{-1} \text{nmol}^{-1}$)		
	GH9b	GH9d	GH9e
xyloglucan (Tamarind seed)	0.49 ± 0.12	0.15 ± 0.07	0.00 ± 0.00
HEC	0.81 ± 0.01	0.01 ± 0.03	0.09 ± 0.01
CMC	3.94 ± 0.03	1.46 ± 0.07	0.93 ± 0.01
glucomannan (Konjac)	9.94 ± 0.36	3.25 ± 0.47	2.04 ± 0.06
β -glucan (Barley)	26.86 ± 0.74	18.62 ± 0.45	6.13 ± 0.34
Laminarin (<i>Laminaria digitata</i>)	0.14 ± 0.12	0.04 ± 0.20	0.03 ± 0.03

420 **Table 1** Specific activities of bacterial GH9s on 1% (*w/v*) soluble substrates. Hydrolysis reactions
 421 were carried out on various soluble substrates for 10 min under optimal conditions (pH 5.9 and
 422 respective temperatures of 54°C (GH9b), 68°C (GH9d) and 60°C (GH9e) (Fig. 4)). Each value is the
 423 mean \pm standard deviation of three replicates and is expressed in $\mu\text{mol}/\text{min}/\text{nmol}$.

424

425 The effect of pH and temperature on the three endo- β -1,4-glucanases as well as the kinetic parameters
 426 were determined on CMC, a model substrate used to characterize cellulolytic activity. A common
 427 optimal pH of 5.9 was obtained with rather narrow pH ranges (pH 5 - 6.6) where at least 60% relative
 428 activity was observed (Fig. 3A). Regarding pH stability (Fig. 3C), GH9b and GH9d retained at least
 429 50% residual activity after 24h of incubation at pHs in the range of 2.7-7.4, whereas GH9e lost its
 430 activity below pH 4.1. Variation of the incubation temperature affected the metagenome-derived GH9s
 431 differently (Fig. 3B). GH9b displayed an optimal temperature of 54°C, the optimum for GH9e was
 432 60°C. GH9d proved to be the most thermophilic enzyme with an optimal temperature of 68°C. GH9e
 433 showed more than 50% relative activity over a wide range of temperatures (39-75°C), in contrast to
 434 GH9b (35-59°C) and GH9d (58-76°C). Temperature stability profiles (Fig. 3D) highlighted that GH9b

435 and GH9e both lost activity after a 24h incubation above 54°C, while GH9d appeared more stable with
 436 a residual activity of 69% at 60°C.



437
 438 **Fig. 3** Effect of temperature and pH on the activity of metagenome-derived GH9s on 1% (*w/v*) CMC.
 439 (A) Optimal pH. Hydrolysis was carried out at the given pHs and at 68°C (GH9d), 54°C (GH9b) or
 440 60°C (GH9e) for 10-20 min. (B) Optimal temperature. Activity was measured after incubation at the
 441 indicated temperature and at pH 5.9. (C) pH stability. Activity was measured under optimal conditions
 442 (temperature, pH) after a 24h incubation of the enzymes at the designated pH. (D) Temperature
 443 stability. Activity was measured under optimal conditions after a 24h incubation at the indicated
 444 temperatures. Each value is the mean \pm standard deviation of three replicates.

445
 446 Kinetic parameters were estimated on CMC (Table 2). Saturation curves fitted the Michaelis-Menten
 447 hyperbola. Equation parameters (v_{max} and K_M) were estimated using the maximum likelihood method.
 448 Results revealed a lower affinity of GH9e for CMC with a Michaelis constant (K_M) at least 10 times
 449 higher than GH9b or GH9d. The turnover numbers (k_{cat}) were close, with a value that was 2 times

450 higher for GH9e. Overall, the catalytic efficiency of GH9e on this soluble substrate is 4.7 to 7 times
451 lower than GH9d and GH9b, respectively.

452

	GH9b	GH9d	GH9e
K_M (g l ⁻¹)	42.0 ± 2,0	57.1 ± 3.1	574 ± 19
v_{max} (μM min ⁻¹)	1204 ± 29	310 ± 8.7	7788 ± 177
k_{cat} (s ⁻¹)	263.9 ± 6.3	241.1 ± 6.8	541.9 ± 12
k_{cat}/K_M (l g ⁻¹ s ⁻¹)	6.3 ± 0.5	4.2 ± 0.4	0.9 ± 0.05

453 **Table 2** Kinetic parameters of bacterial GH9s on CMC. Activity was measured under optimal
454 conditions (temperature, pH) for 10 min in a CMC concentration range of 0.25-4% (w/v). Each value
455 represents the mean ± standard deviation of two independent experiments, each carried out in
456 triplicate.

457

458 3.4 Hydrolysis activities on cellulosic substrates

459 We employed lignin-free model biomass substrates, crystalline (Avicel) and amorphous (PASC)
460 cellulose, to compare the product profiles of our metagenome-derived bacterial GH9s. All analyses
461 were carried out at a temperature of 50°C in order to maintain a residual activity of more than 80%
462 over 24h for each enzyme. Results highlighted different propensities to hydrolyze these insoluble
463 substrates (Table 3). GH9e, with the lowest activities measured on soluble substrates, demonstrated
464 the highest activity on Avicel and PASC after 6h and 24 h of incubation. GH9b, with higher specific
465 activities on soluble substrates, exhibited the lowest activities on crystalline cellulose with values 6
466 times lower than GH9e after 24 h of incubation. Such contrasting activities on soluble and insoluble
467 substrates are typical for cellulases and have been already reported [60,61,34]. This discrepancy
468 between the activities of GH9b and GH9e on insoluble substrates (4.4 and 6-fold higher activities of
469 GH9e on PASC and Avicel at 24h, respectively) could be related to the presence of two CBMs in the
470 GH9e enzyme, in contrast to GH9b which does not contain a cellulose binding domain. The GH9d
471 activity appeared only moderate on both insoluble substrates under the present conditions. However,

472 previous assays on CMC demonstrated that at 50°C, GH9d has only 20% of its maximum activity
473 (Fig. 3B).

474

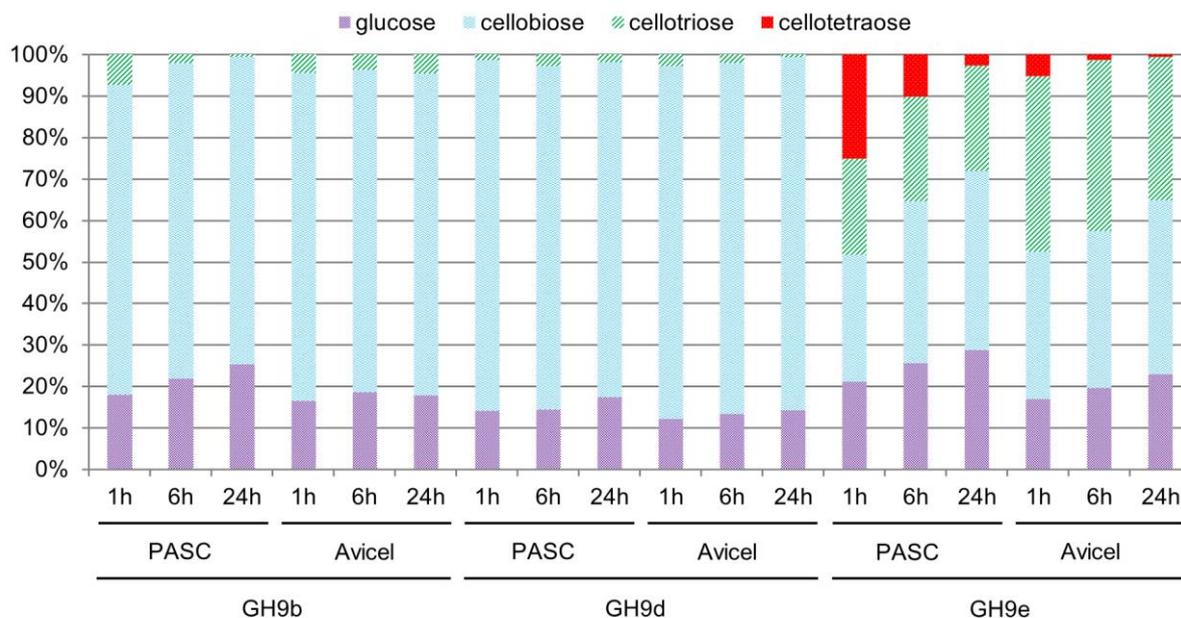
Substrate	Incubation time	Hydrolysis activities ($\mu\text{mol nmol}^{-1}$)		
		GH9b	GH9d	GH9e
PASC	1 h	3.38 \pm 0.08	0.64 \pm 0.04	3.07 \pm 0.12
	6 h	3.72 \pm 0.08	1.80 \pm 0.11	9.15 \pm 0.10
	24 h	4.21 \pm 0.10	3.09 \pm 0.34	18.58 \pm 0.35
Avicel	1 h	0.98 \pm 0.07	1.05 \pm 0.02	2.53 \pm 0.07
	6 h	1.40 \pm 0.08	1.78 \pm 0.03	5.73 \pm 0.20
	24 h	1.68 \pm 0.09	2.74 \pm 0.12	10.38 \pm 0.27

475 **Table 3** Hydrolysis activities of bacterial GH9s on 2% (*w/v*) PASC or Avicel are expressed in μmol of
476 reducing sugars liberated per nmol of enzyme after 1 h, 6 h or 24 h at 50°C. The products were
477 quantified by HPAEC-PAD using corresponding external standards. Each value represents the mean \pm
478 standard deviation of three replicates.

479

480 The product profiles revealed interesting differences (Fig. 4). For GH9b and GH9d, the major product
481 was cellobiose, whereas GH9e displayed a distinct substrate hydrolysis pattern with an elevated
482 proportion of oligosaccharides. Cellotriose and cellotetraose reached 48% of the total sugar content
483 after a one-hour incubation on PASC or Avicel, while cellobiose only reached 31% and 35% of total
484 sugar, respectively. The relative proportions of cellotriose and cellotetraose decreased over time and
485 were respectively reduced to 28% and 35% after a 24h incubation time. These results point to
486 differences in the mode of action of GH9e on the one hand, and GH9b and GH9d on the other hand.

487



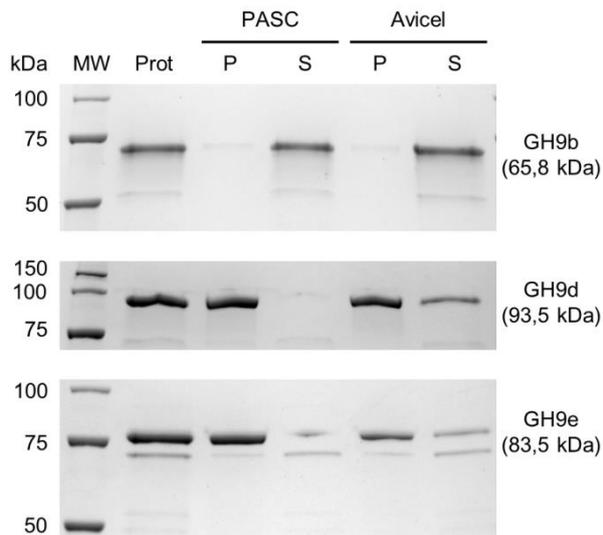
488

489 **Fig. 4** Product profiles on crystalline and amorphous cellulose. 2% (w/v) PASC or Avicel were
 490 hydrolyzed for 1h, 6h or 24h at pH 5.9 and at 50°C. Products were quantified by HPAEC-PAD using
 491 corresponding external standards. The relative content of each compound is shown.

492

493 3.5 Substrate binding, processivity and synergy on cellulosic substrates

494 The binding of each GH9 to amorphous and crystalline cellulose was investigated (Fig. 5). No
 495 detectable binding of GH9b, which is devoid of CBM, was observed on either substrate. Both CBM-
 496 containing GH9s (GH9d and GH9e) bound extensively to amorphous cellulose, especially for GH9d
 497 for which no soluble fraction was detectable. The affinity of GH9d and GH9e for crystalline cellulose
 498 seemed lower than for amorphous cellulose.



499

500 **Fig. 5** Binding of GH9 enzymes to cellulosic substrates. Proteins (1.5 μM) were incubated with
 501 amorphous (PASC) or crystalline (Avicel) cellulose (7 g/L) for 1 h at 4 $^{\circ}\text{C}$. The resulting pellets (P,
 502 bound proteins) and supernatants (S, unbound protein) were collected and the proteins were denatured
 503 in sample buffer. The samples underwent SDS-PAGE along with protein solutions incubated without
 504 any substrate (Prot).

505

506 We evaluated the processivity of each enzyme using the modified BCA assay, which allows for a
 507 quantification of reducing extremities in the micromolar range [56]. We analyzed the distribution of
 508 reducing sugars among the insoluble and soluble fractions resulting from the activity of each enzyme
 509 on PASC. The vast majority of reducing extremities (>96%) was detected in the soluble fraction of all
 510 samples (Table 4). This result suggests that all three enzymes act processively and that a CBM is not
 511 necessarily a prerequisite for this mode of action.

512

Enzyme	Fraction	Reducing ends concentration, μM (%)
GH9b	Soluble	322.6 ± 21.1 (96.4%)
	Insoluble	12.1 ± 23.2 (3.6%)
GH9d	Soluble	222.0 ± 2.4 (97.5%)

	Insoluble	5.7 ± 12.2 (2.5%)
GH9e	Soluble	1554.0 ± 40.0 (99,1%)
	Insoluble	14.1 ± 6.1 (0.9%)

513 **Table 4** Distribution of reducing sugar ends released from PASC. Hydrolysis reactions were carried
514 out with 1 µM of each enzyme for 4 hours at pH 5.9 and 50°C.

515

516 The synergistic properties of the three bacterial GH9s were studied between themselves and with a
517 GH48 family member retrieved from the same metagenomic screening (Table 5). Like the GH9
518 enzymes, this modular protein containing a CBM2 and a GH48 catalytic domain was recombinantly
519 expressed in *E. coli* (Supplementary Fig. S5). Binary and ternary mixtures of the three endoglucanases
520 exhibited moderate synergy between the bacterial GH9s. All enzyme combinations enhanced the
521 activity by a factor of less than 1.5. The highest synergy could be observed by combining GH9b and
522 GH9d. Binary mixtures of GH48 with each of the three GH9s highlighted a significant synergy
523 between GH48 and GH9d. This enzyme combination yielded the highest synergism factor obtained
524 out of all the assayed enzyme mixtures.

525

Enzyme mixture	Molar ratio in mixture	Synergism factor
GH9b + GH9d	1:1	1.4
GH9b + GH9e	1:1	1.2
GH9d + GH9e	1:1	1.3
GH9b + GH9d + GH9e	1:1:1	1.1
GH9b + GH48	1:1	0.9
GH9d + GH48	1:1	1.6
GH9e + GH48	1:1	0.8

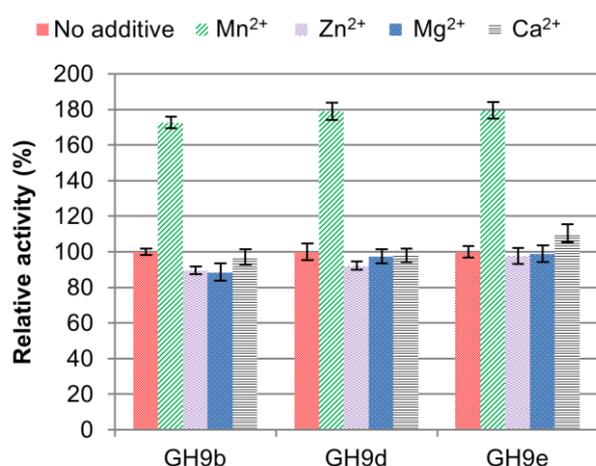
526 **Table 5** Synergy effect of binary or ternary mixtures of bacterial GH9s and in binary mixtures with a
527 GH48 enzyme. Hydrolysis reactions were carried out on Avicel PH-101 for 6 hours at pH 5.9 and
528 50°C with 50 nM enzymes at different molar ratios. The synergism factor represents the activity of
529 each mixture divided by the sum of the activities of the mixture components.

530

531 3.6 Effect of metal ions

532 It has been shown that cellulase activity can be enhanced or inhibited in the presence of divalent
533 cations [62–65]. We therefore determined the effect of various metal ions (manganese, zinc,
534 magnesium and calcium) on the activity of the three bacterial GH9s (Fig. 6). Endoglucanase activity
535 increased by 70% to 80% in the presence of manganese ions. Calcium ions appeared to be a moderate
536 stimulator of GH9e, with a 10% activity increase, while they had no impact on GH9b and GH9d. Zinc
537 and magnesium ions either slightly inhibited the activity or had no impact.

538



539

540 **Fig. 6** Effect of 1 mM metal ions on the activity of metagenome-derived GH9s. Hydrolysis reactions
541 were carried out on 1% (w/v) CMC for 10 min under optimal conditions (temperature, pH). Each
542 value is the mean \pm standard deviation of three replicates and is expressed as a percentage of the
543 activity of the corresponding enzyme without additive.

544

545 **4. Discussion**

546 Due to their diversity, environmental bacteria constitute a useful source of promising enzyme
547 candidates for biomass conversion [66]. Compost has been shown to contain a wide diversity of
548 lignocellulose-degrading enzymes which could be enriched by long-term incubation on lignocellulosic
549 biomass [38]. This ecosystem was therefore chosen as a source for the construction of a metagenomic
550 library in order to mine the uncultivable part of cellulose-degrading microflora and to identify novel
551 efficient cellulases. Genes encoding putative cellulases from families GH5, GH6, GH12, GH9 and
552 GH48 were retrieved by functional screening. Compared to other GH families, a surprisingly large
553 number of GH9 encoding genes were identified. This highlights the important role of this class of
554 enzymes in the degradation of lignocellulosic materials by bacteria. GH9 enzymes display various
555 modes of action on cellulosic substrates: some are exoglucanases, such as *C. thermocellum*
556 cellobiohydrolase CbhA [16], while others are processive endocellulases, that remain attached to the
557 polysaccharide chain after the initial hydrolysis step [58,67], or more classical dissociative
558 endoglucanases [68,69]. More rarely, exo- β -D-glucosaminidase, mannanase and xyloglucanase
559 activities have been evidenced for GH9 enzymes [70,71]. In order to understand the role of the GH9
560 enzymes in our compost enrichment better, a thorough characterization was undertaken.

561 The sequence comparison of characterized GH9 enzymes carried out in the present study highlighted a
562 wide variety of modular architectures within this enzyme class. In contrast to previous classifications
563 of GH9s [58,72–74], the phylogenetic groups of our analysis, including the newly isolated GH9s, do
564 not reflect the modular architecture of the enzymes. For instance, several domains, like CBM2, CBM3
565 or dockerin, are each found in several distant clades. The only feature that seems to have co-evolved
566 with the catalytic domain is the N-terminal Ig-like domain, and the two distinct subfamilies are
567 characterized by the absence or presence of this domain, respectively. Among the six metagenomic
568 GH9s identified from our metagenome, three enzymes belonging to different clades were
569 characterized.

570 GH9e is part of a subgroup of enzymes which all contain a CBM3 module. However, some of them,
571 including GH9e, also possess a CBM2. Of these, two enzymes have previously been extensively
572 characterized: the *Cellulomonas fimi* CenB (UniProt: P26225) [60] and the *Thermobifida fusca* Cel9A
573 (UniProt: P26221) endoglucanases [75,61,76]. The specific activities of GH9e on CMC and PASC are
574 similar to those of both former enzymes [60,61]. All three enzymes seem to be more efficient on
575 PASC than on crystalline cellulose [60,61]. Compared to the other two metagenomic GH9 enzymes
576 characterized in the present study, GH9e is the one with the highest activity on crystalline and
577 amorphous cellulose after a 6h or a 24h hydrolysis.

578 Within the Ig-like domain containing subgroup which includes GH9d, only very few enzymes possess
579 a CBM2 and a CBM4 module (Fig.2). One of them is GH9d; the other enzymes are GH9a (this study)
580 and UniProt Q47PF7 from *T. fusca* for which no kinetic data have been published. To our knowledge,
581 GH9d thus represents the first GH9 enzyme containing these two modules to be characterized in more
582 detail. *H. thermocellum* CenC is part of the same subgroup as GH9d, but harbors a CBM3 instead of a
583 CBM2. Compared to this enzyme, GH9d displays a higher turnover number on CMC [77]. An
584 interesting feature of GH9d is the similar activity on both PASC and Avicel. This contrasts with the
585 substrate preferences of GH9e and GH9b as well as of other characterized enzymes of their respective
586 subgroups, suggesting that the modular organization of GH9d might contribute to the efficient
587 deconstruction of crystalline cellulose as well as the amorphous substrate by this enzyme.

588 The representatives of the second subgroup of Ig-like domain-containing enzymes which have been
589 described in most detail are *R. cellulolyticum* Cel9U and Cel9W [34]. GH9b has very similar specific
590 activities to those of the formerly characterized enzymes. GH9b has a higher affinity constant than the
591 *R. cellulolyticum* enzymes, but a comparable k_{cat}/K_m to Cel9W. The major reaction product of GH9b
592 on Avicel and PASC was cellobiose, suggesting a G2-processive endoglucanase type of action, similar
593 to the closely related *H. thermocellum* Cel9D [78].

594 GH9 enzymes have previously also been isolated from other metagenomes, such as soil or compost
595 metagenomes [62,79]. Most of them are Ig-like domain containing enzymes devoid of a CBM.

596 Similarly to GH9b, they displayed high activity on soluble substrates, such as CMC or β -glucan
597 [80,62,79]. The Cel9 endoglucanase from a bagasse pile metagenome with the same modular
598 structure as GH9e showed high activity on β -glucan, but not on the other cellulosic substrates tested
599 [81].

600 All three GH9 enzymes had optimal temperatures above 50°C and a pronounced thermostability below
601 60°C, with GH9d being the most thermophilic and thermostable endoglucanase. These results are in
602 line with activities measured for secreted or cellulosomal GH9 family counterparts presenting
603 heterogeneous modular organizations [77,82,83,63]. The reported optimal temperatures ranged from
604 40°C for EngZ from *C. cellulovorans* to 70°C for *H. thermocellum* CenC, with thermostability up to at
605 least 50°C [83,77]. Optimal temperatures for previously identified metagenomic GH9 enzymes were
606 shown to differ from 30°C to 75°C, although the enzymes have been retrieved from mesophilic
607 environments [81,62,80].

608 All of the three GH9 enzymes displayed Michaelis-Menten kinetics on CMC, despite the processive
609 activity observed. CMC is a substituted substrate that might hinder any processive action, as
610 demonstrated by the absence of activity of cellobiohydrolase on this soluble substrate. Therefore,
611 CMC could force endoglucanases to detach from the substrate in order to bind and liberate a new
612 product. This condition might be necessary in order to obey the Michaelis-Menten model.

613 The GH9e enzyme displayed a distinct product profile on cellulose with a high relative content of
614 cellotetraose (G4) and cellotriose (G3) oligosaccharides. A similar cellulose hydrolysis pattern was
615 previously obtained with *T. fusca* Cel9A [84,85]. Our phylogenetic analysis highlighted that GH9e CD
616 is closely related to *T. fusca* Cel9A (76% identity). Both proteins show a similar modular organization
617 and an ability to processively hydrolyze cellulosic substrates and produce oligosaccharides. Cellulases
618 with this particular hydrolysis pattern are found in both cellulosomal [78,86] and non-cellulosomal
619 GH9s [87,84,88]. In their comparative study of the cellulosomal cellulases from *C. thermocellum*, Leis
620 *et al.* [78] identified G4-type processive endoglucanases only within the GH9 family. These enzymes

621 catalyze the cleavage and release of defined cello-oligosaccharides with G4 as an intermediate product
622 at the beginning of the hydrolysis reaction.

623 In our phylogenetic tree, characterized G4-producing GH9s with a processive activity
624 [87,84,88,78,58,67] clustered in the same clade as GH9e, which also displayed processivity. These
625 G4-producing GH9s and the metagenome-derived GH9c and GH9e harbor a CBM3 found C-terminal
626 to the CD. In the vast majority of G4-producing GH9s, including GH9e, this CBM3 belongs to type c
627 (CBM3c) based on sequence identity [74]. CBM3cs do not exhibit all of the conserved residues found
628 in type a and b CBM3s, including residues involved in cellulose-binding [58]. Furthermore, a
629 truncated form of *H. thermocellum* Cel9I composed of only the catalytic domain and a CBM3c
630 module failed to bind to cellulose [58]. Rather, this CBM appears to play a helper role in activity, as
631 cellulose degradation is markedly reduced and processivity is lost in *T. fusca* Cel9A upon deletion of
632 its CBM3c [85]. CBM3cs are therefore thought to be involved in modulating the activity and
633 processivity of processive GH9 endoglucanases.

634 The role of other CBMs on processivity is less clear and might depend on other structural features of
635 the enzymes. The processivity of *T. fusca* Cel9A was reduced upon deletion of its CBM2 and a role of
636 the CBMX2s of *C. cellulosi* Cel9a in processivity has also been evidenced [85,89]. The mechanisms
637 involved in processivity for GH9 enzymes without CBM3c are still less well understood [90]. In the
638 present study, the processive mode of action observed on amorphous cellulose for all three GH9
639 enzymes, including GH9b devoid of CBM, suggests that the presence of a CBM is not a necessary
640 prerequisite for processivity. However, deletion of CBM2 and/or CBM4 should be carried out to
641 determine the role of these CBMs in processivity for GH9d and GH9e.

642 Manganese ion is an activator of all three bacterial GH9s assayed on CMC. As the CD is the only
643 common module of our three bacterial endoglucanases, this result suggests a potential stabilizing role
644 for manganese ions on the CDs of GH9 enzymes. However, supplementing with manganese has
645 already been assayed on CMC for several GH9 family members, with contrasting results. Mn^{2+}
646 significantly enhanced the activity of a termite GH9 [64] and of another GH9 cloned from a compost

647 metagenome [62]. It had no positive impact on *C. thermocellum* Cel9W activity [65] while it
648 significantly inhibited the activity of Cel9K from *Paenibacillus* sp. X4 [63]. Altogether, this suggests
649 that discrete factors, not fully conserved, might influence manganese binding. Further experiments
650 such as differential scanning fluorimetry would be necessary in order to detect ligand interactions that
651 might promote protein stability [91].

652 Of our three GH9s, the highest synergy on Avicel cellulose was obtained between GH9b and GH9d.
653 Each of these enzymes had limited activity on crystalline cellulose, but they displayed contrasting
654 binding abilities. It is possible that, due to the presence of both a CBM2 and a CBM4, GH9d binds to
655 specific sites of the substrates and renders it more accessible for GH9b. GH9e, which harbors a CBM2
656 and a CBM3 at its C-terminus, might not be as efficient as GH9d in creating accessible sites for GH9b,
657 thus leading to lower synergy with GH9b. Alternatively, the high activity of GH9e compared with the
658 other two enzymes (about 2.5 to 6 times higher, depending on hydrolysis time) might explain the more
659 limited synergistic properties observed with GH9e. Similar synergy factors than with GH9b were
660 found for GH9d and GH9e in combination with the GH48 family member retrieved from our compost
661 metagenome. The best synergy was observed by mixing GH9d and GH48, which is in good agreement
662 with the previously demonstrated synergy between GH9 and GH48 enzymes [35,27].

663

664 **5. Conclusions**

665 A wide diversity of GH9 enzymes was retrieved in a compost metagenome and three new bacterial
666 members of this family were characterized as thermophilic and processive endo-1,4- β -glucanases
667 activated by manganese. The highest activity on insoluble substrates was achieved by GH9d and
668 GH9e containing a CBM. GH9e displayed a peculiar processive activity, rapidly producing
669 cellotetraose and cellotriose from amorphous cellulose. GH9d, one of the first GH9 enzymes with
670 CBM4 and a CBM2 to be characterized, has various interesting features: high stability in a wide range
671 of pH and temperatures, similar activity on soluble and insoluble substrates, and the highest synergy

672 with GH9b and GH48 enzymes. These properties could make it suitable for biomass saccharification
673 for bioethanol production. From a more fundamental point of view, it will be interesting to determine
674 the relative contribution of the two binding modules to affinity, activity and processivity on diverse
675 substrates by means of deletion mutants and to characterize more representatives of the same modular
676 structure in further studies in order to reveal any common features.

677

678 **Acknowledgments**

679 The authors thank Nicolas Lopes Ferreira for fruitful discussions and for providing PASC, as well as
680 Simon Arragain for critical review of the manuscript. This work was supported by Agence Nationale
681 de Recherche (Grant number: ANR-12-BIO-ME-006-01)

682

683 **Authors' contributions**

684 LA and NF purified and characterized the enzymes, and LA drafted the manuscript. AH constructed
685 the metagenomic library and isolated positive clones. BH and VL carried out the CAZyme annotation
686 and BH proofread the manuscript. EJ and LA carried out the phylogenetic analysis. SHB designed the
687 study and SP and SHB supervised the work and participated in manuscript drafting and editing.

688

689 **6. References**

690 [1]N. Karaki, A. Aljawish, C. Humeau, L. Muniglia, J. Jasniewski, Enzymatic modification of
691 polysaccharides: Mechanisms, properties, and potential applications: A review, *Enzyme and Microbial*
692 *Technology* 90 (2016) 1–18.

693 [2]B.L. Cantarel, P.M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, B. Henrissat, The
694 Carbohydrate-Active EnZymes database (CAZy): An expert resource for Glycogenomics, *Nucleic*
695 *acids research* 37 (2009) D233-D238.

696 [3]B. Henrissat, G. Davies, Structural and sequence-based classification of glycoside hydrolases,
697 *Current opinion in structural biology* 7 (1997) 637–644.

698 [4]V. Lombard, H. Golaconda Ramulu, E. Drula, P.M. Coutinho, B. Henrissat, The carbohydrate-
699 active enzymes database (CAZy) in 2013, *Nucleic acids research* 42 (2014) D490-5.

700 [5]G. Davies, B. Henrissat, Structures and mechanisms of glycosyl hydrolases, *Structure* (London,
701 England : 1993) 3 (1995) 853–859.

702 [6]M.-L. Garron, B. Henrissat, The continuing expansion of CAZymes and their families, *Current*
703 *opinion in chemical biology* 53 (2019) 82–87.

704 [7]L.R. Lynd, C.E. Wyman, T.U. Gerngross, *Biocommodity Engineering*, *Biotechnol Progress* 15
705 (1999) 777–793.

706 [8]L.R. Lynd, P.J. Weimer, W.H. van Zyl, I.S. Pretorius, *Microbial cellulose utilization: Fundamentals*
707 *and biotechnology*, *Microbiology and molecular biology reviews* : MMBR 66 (2002) 506-77, table of
708 contents.

709 [9]Y.-H.P. Zhang, L.R. Lynd, Toward an aggregated understanding of enzymatic hydrolysis of
710 cellulose: Noncomplexed cellulase systems, *Biotechnology and bioengineering* 88 (2004) 797–824.

711 [10]P.V. Harris, D. Welner, K.C. McFarland, E. Re, J.-C. Navarro Poulsen, K. Brown, R. Salbo, H.
712 Ding, E. Vlasenko, S. Merino, F. Xu, J. Cherry, S. Larsen, L. Lo Leggio, Stimulation of
713 lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: Structure and
714 function of a large, enigmatic family, *Biochemistry* 49 (2010) 3305–3316.

715 [11]S.J. Horn, G. Vaaje-Kolstad, B. Westereng, V.G. Eijsink, Novel enzymes for the degradation of
716 cellulose, *Biotechnology for biofuels* 5 (2012) 45.

- 717 [12]A.B. Boraston, D.N. Bolam, H.J. Gilbert, G.J. Davies, Carbohydrate-binding modules: Fine-
718 tuning polysaccharide recognition, *The Biochemical journal* 382 (2004) 769–781.
- 719 [13]D. Guillén, S. Sánchez, R. Rodríguez-Sanoja, Carbohydrate-binding domains: Multiplicity of
720 biological roles, *Applied microbiology and biotechnology* 85 (2010) 1241–1249.
- 721 [14]M. Juy, A.G. Amrt, P.M. Alzari, R.J. Poljak, M. Claeysens, P. Béguin, J.-P. Aubert, Three-
722 dimensional structure of a thermostable bacterial cellulase, *Nature* 357 (1992) 89–91.
- 723 [15]I.A. Kataeva, R.D. Seidel, A. Shah, L.T. West, X.-L. Li, L.G. Ljungdahl, The fibronectin type 3-
724 like repeat from the *Clostridium thermocellum* cellobiohydrolase CbhA promotes hydrolysis of
725 cellulose by modifying its surface, *Applied and environmental microbiology* 68 (2002) 4292–4300.
- 726 [16]I.A. Kataeva, V.N. Uversky, J.M. Brewer, F. Schubot, J.P. Rose, B.-C. Wang, L.G. Ljungdahl,
727 Interactions between immunoglobulin-like and catalytic modules in *Clostridium thermocellum*
728 cellulosomal cellobiohydrolase CbhA, *Protein engineering, design & selection : PEDS* 17 (2004) 759–
729 769.
- 730 [17]H. Liu, J.H. Pereira, P.D. Adams, R. Sapro, B.A. Simmons, K.L. Sale, Molecular simulations
731 provide new insights into the role of the accessory immunoglobulin-like domain of Cel9A, *FEBS*
732 *letters* 584 (2010) 3431–3435.
- 733 [18]Y.J. Bomble, C.-Y. Lin, A. Amore, H. Wei, E.K. Holwerda, P.N. Ciesielski, B.S. Donohoe, S.R.
734 Decker, L.R. Lynd, M.E. Himmel, Lignocellulose deconstruction in the biosphere, *Current opinion in*
735 *chemical biology* 41 (2017) 61–70.
- 736 [19]B.R. Urbanowicz, A.B. Bennett, E. Del Campillo, C. Catalá, T. Hayashi, B. Henrissat, H. Höfte,
737 S.J. McQueen-Mason, S.E. Patterson, O. Shoseyov, T.T. Teeri, J.K.C. Rose, Structural organization
738 and a standardized nomenclature for plant endo-1,4-beta-glucanases (cellulases) of glycosyl hydrolase
739 family 9, *Plant physiology* 144 (2007) 1693–1696.
- 740 [20]R. Ramalingam, J.E. Blume, H.L. Ennis, The *Dictyostelium discoideum* spore germination-
741 specific cellulase is organized into functional domains, *Journal of bacteriology* 174 (1992) 7834–7837.

742 [21]K. Suzuki, T. Ojima, K. Nishita, Purification and cDNA cloning of a cellulase from abalone
743 *Haliotis discus hannai*, *European journal of biochemistry* 270 (2003) 771–778.

744 [22]T. Arimori, A. Ito, M. Nakazawa, M. Ueda, T. Tamada, Crystal structure of endo-1,4- β -glucanase
745 from *Eisenia fetida*, *Journal of synchrotron radiation* 20 (2013) 884–889.

746 [23]S. Khademi, L.A. Guarino, H. Watanabe, G. Tokuda, E.F. Meyer, Structure of an endoglucanase
747 from termite, *Nasutitermes takasagoensis*, *Acta crystallographica. Section D, Biological*
748 *crystallography* 58 (2002) 653–659.

749 [24]W. Schwarz, The cellulosome and cellulose degradation by anaerobic bacteria, *Applied*
750 *microbiology and biotechnology* 56 (2001) 634–649.

751 [25]E. Berger, D. Zhang, V.V. Zverlov, W.H. Schwarz, Two noncellulosomal cellulases of
752 *Clostridium thermocellum*, Cel9I and Cel48Y, hydrolyse crystalline cellulose synergistically, *FEMS*
753 *microbiology letters* 268 (2007) 194–201.

754 [26]N. Franche, C. Tardif, J. Ravachol, S. Harchouni, P.-H. Ferdinand, R. Borne, H.-P. Fierobe, S.
755 Perret, Cel5I, a SLH-Containing Glycoside Hydrolase: Characterization and Investigation on Its Role
756 in *Ruminiclostridium cellulolyticum*, *Plos One* 11 (2016) e0160812.

757 [27]H.-P. Fierobe, E.A. Bayer, C. Tardif, M. Czjzek, A. Mechaly, A. Bélaïch, R. Lamed, Y. Shoham,
758 J.-P. Bélaïch, Degradation of Cellulose Substrates by Cellulosome Chimeras: SUBSTRATE
759 TARGETING VERSUS PROXIMITY OF ENZYME COMPONENTS, *Journal of Biological*
760 *Chemistry* 277 (2002) 49621–49630.

761 [28]R. Brunecky, M. Alahuhta, Q. Xu, B.S. Donohoe, M.F. Crowley, I.A. Kataeva, S.-J. Yang, M.G.
762 Resch, M.W.W. Adams, V.V. Lunin, M.E. Himmel, Y.J. Bomble, Revealing nature's cellulase
763 diversity: The digestion mechanism of *Caldicellulosiruptor bescii* CelA, *Science (New York, N.Y.)*
764 342 (2013) 1513–1516.

765 [29]M.K. Bhat, T.M. Wood, The cellulase of the anaerobic bacterium *Clostridium thermocellum*:
766 Isolation, dissociation, and reassociation of the cellulosome, *Carbohydrate Research* 227 (1992) 293–
767 300.

768 [30]I. Fendri, C. Tardif, H.-P. Fierobe, S. Lignon, O. Valette, S. Pagès, S. Perret, The cellulosomes
769 from *Clostridium cellulolyticum* : Identification of new components and synergies between
770 complexes, *The FEBS Journal* 276 (2009) 3076–3086.

771 [31]E.M. Gomez del Pulgar, A. Saadeddin, The cellulolytic system of *Thermobifida fusca*, *Critical*
772 *Reviews in Microbiology* 40 (2014) 236–247.

773 [32]J.-C. Blouzard, P.M. Coutinho, H.-P. Fierobe, B. Henrissat, S. Lignon, C. Tardif, S. Pagès, P. de
774 Philip, Modulation of cellulosome composition in *Clostridium cellulolyticum*: Adaptation to the
775 polysaccharide environment revealed by proteomic and carbohydrate-active enzyme analyses,
776 *Proteomics* 10 (2010) 541–554.

777 [33]J.L.A. Brás, A. Cartmell, A.L.M. Carvalho, G. Verzé, E.A. Bayer, Y. Vazana, M.A.S. Correia,
778 J.A.M. Prates, S. Ratnaparkhe, A.B. Boraston, M.J. Romão, C.M.G.A. Fontes, H.J. Gilbert, Structural
779 insights into a unique cellulase fold and mechanism of cellulose hydrolysis, *Proceedings of the*
780 *National Academy of Sciences of the United States of America* 108 (2011) 5237–5242.

781 [34]J. Ravachol, R. Borne, C. Tardif, P. de Philip, H.-P. Fierobe, Characterization of all family-9
782 glycoside hydrolases synthesized by the cellulosome-producing bacterium *Clostridium cellulolyticum*,
783 *The Journal of biological chemistry* 289 (2014) 7335–7348.

784 [35]M. Kostylev, D.B. Wilson, Synergistic interactions in cellulose hydrolysis, *Biofuels* 3 (2012) 61–
785 70.

786 [36]A.C. Tolonen, A.C. Chilaka, G.M. Church, Targeted gene inactivation in *Clostridium*
787 *phytofermentans* shows that cellulose degradation requires the family 9 hydrolase Cphy3367,
788 *Molecular microbiology* 74 (2009) 1300–1313.

789 [37]T. Mori, I. Kamei, H. Hirai, R. Kondo, Identification of novel glycosyl hydrolases with
790 cellulolytic activity against crystalline cellulose from metagenomic libraries constructed from bacterial
791 enrichment cultures, *Springerplus* 3 (2014) 365.

792 [38]S. Heiss-Blanquet, F. Fayolle-Guichard, V. Lombard, A. Hébert, P.M. Coutinho, A. Groppi, A.
793 Barre, B. Henrissat, Composting-Like Conditions Are More Efficient for Enrichment and Diversity of

794 Organisms Containing Cellulase-Encoding Genes than Submerged Cultures, Plos One 11 (2016)
795 e0167216-e0167216.

796 [39]K.M. DeAngelis, J.M. Gladden, M. Allgaier, P. D'haeseleer, J.L. Fortney, A. Reddy, P.
797 Hugenholtz, S.W. Singer, J.S. Vander Gheynst, W.L. Silver, B.A. Simmons, T.C. Hazen, Strategies
798 for Enhancing the Effectiveness of Metagenomic-based Enzyme Discovery in Lignocellulolytic
799 Microbial Communities, BioEnergy Research 3 (2010) 146–158.

800 [40]W. Mhuantong, V. Charoensawan, P. Kanokratana, S. Tangphatsornruang, V. Champreda,
801 Comparative analysis of sugarcane bagasse metagenome reveals unique and conserved biomass-
802 degrading enzymes among lignocellulolytic microbial communities, Biotechnology for biofuels 8
803 (2015) 16.

804 [41]M. de Vries, A. Schöler, J. Ertl, Z. Xu, M. Schloter, Metagenomic analyses reveal no differences
805 in genes involved in cellulose degradation under different tillage treatments, FEMS microbiology
806 ecology 91 (2015).

807 [42]A.P. Reddy, C.W. Simmons, P. D'haeseleer, J. Khudyakov, H. Burd, M. Hadi, B.A. Simmons,
808 S.W. Singer, M.P. Thelen, J.S. Vanderghenst, Discovery of microorganisms and enzymes involved in
809 high-solids decomposition of rice straw using metagenomic analyses, Plos One 8 (2013) e77985-
810 e77985.

811 [43]C. Simon, R. Daniel, Achievements and new knowledge unraveled by metagenomic approaches,
812 Applied microbiology and biotechnology 85 (2009) 265–276.

813 [44]M. Nyssönen, H.M. Tran, U. Karaoz, C. Weihe, M.Z. Hadi, J.B.H. Martiny, A.C. Martiny, E.L.
814 Brodie, Coupled high-throughput functional screening and next generation sequencing for
815 identification of plant polymer decomposing enzymes in metagenomic libraries, Front Microbiol 4
816 (2013) 282.

817 [45]W.R. Pearson, T. Wood, Z. Zhang, W. Miller, Comparison of DNA Sequences with Protein
818 Sequences, Genomics 46 (1997) 24–36.

819 [46]S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped
820 BLAST and PSI-BLAST: A new generation of protein database search programs, *Nucleic acids*
821 *research* 25 (1997) 3389–3402.

822 [47]F. Madeira, Y.M. Park, J. Lee, N. Buso, T. Gur, N. Madhusoodanan, P. Basutkar, A.R.N. Tivey,
823 S.C. Potter, R.D. Finn, R. Lopez, The EMBL-EBI search and sequence analysis tools APIs in 2019,
824 *Nucleic acids research* 47 (2019) W636-W641.

825 [48]I. Letunic, P. Bork, Interactive Tree Of Life (iTOL) v4: Recent updates and new developments,
826 *Nucleic acids research* 47 (2019) W256-W259.

827 [49]UniProt Consortium, UniProt: A worldwide hub of protein knowledge, *Nucleic acids research* 47
828 (2019) D506-D515.

829 [50]R.D. Finn, J. Mistry, B. Schuster-Böckler, S. Griffiths-Jones, V. Hollich, T. Lassmann, S. Moxon,
830 M. Marshall, A. Khanna, R. Durbin, S.R. Eddy, E.L.L. Sonnhammer, A. Bateman, Pfam: Clans, web
831 tools and services, *Nucleic acids research* 34 (2006) D247-51.

832 [51]A. Marchler-Bauer, Y. Bo, L. Han, J. He, C.J. Lanczycki, S. Lu, F. Chitsaz, M.K. Derbyshire,
833 R.C. Geer, N.R. Gonzales, M. Gwadz, D.I. Hurwitz, F. Lu, G.H. Marchler, J.S. Song, N. Thanki, Z.
834 Wang, R.A. Yamashita, D. Zhang, C. Zheng, L.Y. Geer, S.H. Bryant, CDD/SPARCLE: Functional
835 classification of proteins via subfamily domain architectures, *Nucleic acids research* 45 (2017) D200-
836 D203.

837 [52]E. Gasteiger, C. Hoogland, A. Gattiker, S. Duvaud, M.R. Wilkins, R.D. Appel, A. Bairoch,
838 Protein Identification and Analysis Tools on the ExPASy Server, in: J.M. Walker (Ed.), *The*
839 *Proteomics Protocols Handbook*, Humana Press; Springer e-books, Totowa, NJ, 2005.

840 [53]G.L. Miller, Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar, *Anal.*
841 *Chem.* 31 (1959) 426–428.

842 [54]T.C. McIlvaine, A buffer solution for colorimetric comparison, *J. Biol. Chem.* 49 (1921) 183–186.

843 [55]J.G. Raaijmakers, Statistical analysis of the Michaelis-Menten equation, *Biometrics* 43 (1987)
844 793–803.

845 [56]Y.-H.P. Zhang, L.R. Lynd, Determination of the number-average degree of polymerization of
846 cellodextrins and cellulose with application to enzymatic hydrolysis, *Biomacromolecules* 6 (2005)
847 1510–1515.

848 [57]D.C. Irwin, M. Spezio, L.P. Walker, D.B. Wilson, Activity studies of eight purified cellulases:
849 Specificity, synergism, and binding domain effects, *Biotechnology and bioengineering* 42 (1993)
850 1002–1013.

851 [58]R. Gilad, L. Rabinovich, S. Yaron, E.A. Bayer, R. Lamed, H.J. Gilbert, Y. Shoham, Cell, a
852 noncellulosomal family 9 enzyme from *Clostridium thermocellum*, is a processive endoglucanase that
853 degrades crystalline cellulose, *Journal of bacteriology* 185 (2003) 391–398.

854 [59]L. Wu, G.J. Davies, Structure of the GH9 glucosidase/glucosaminidase from *Vibrio cholerae*, *Acta*
855 *Crystallogr F Struct Biol Commun* 74 (2018) 512–523.

856 [60]P. Tomme, A.L. Creagh, D.G. Kilburn, C.A. Haynes, Interaction of Polysaccharides with the N-
857 Terminal Cellulose-Binding Domain of *Cellulomonas fimi* CenC. 1. Binding Specificity and
858 Calorimetric Analysis, *Biochemistry* 35 (1996) 13885–13894.

859 [61]W. Zhou, D.C. Irwin, J. Escovar-Kousen, D.B. Wilson, Kinetic Studies of *Thermobifida fusca*
860 Cel9A Active Site Mutant Enzymes, *Biochemistry* 43 (2004) 9655–9663.

861 [62]C.-J. Duan, M.-Y. Huang, H. Pang, J. Zhao, C.-X. Wu, J.-X. Feng, Characterization of a novel
862 theme C glycoside hydrolase family 9 cellulase and its CBM-chimeric enzymes, *Applied*
863 *microbiology and biotechnology* 101 (2017) 5723–5737.

864 [63]J.P. Lee, Y.A. Kim, S.K. Kim, H. Kim, Characterization of a Multimodular Endo- β -1,4-Glucanase
865 (Cel9K) from *Paenibacillus* sp. X4 with a Potential Additive for Saccharification, *Journal of*
866 *microbiology and biotechnology* 28 (2018) 588–596.

867 [64]P. Zhang, X. Yuan, Y. Du, J.-J. Li, Heterologous expression and biochemical characterization of a
868 GHF9 endoglucanase from the termite *Reticulitermes speratus* in *Pichia pastoris*, *BMC biotechnology*
869 18 (2018) 35.

870 [65]K. Kumar, S. Singal, A. Goyal, Role of carbohydrate binding module (CBM3c) of GH9 β -1,4
871 endoglucanase (Cel9W) from *Hungateiclostridium thermocellum* ATCC 27405 in catalysis,
872 Carbohydrate Research 484 (2019) 107782.

873 [66]R. López-Mondéjar, C. Algora, P. Baldrian, Lignocellulolytic systems of soil bacteria: A vast and
874 diverse toolbox for biotechnological conversion processes, Biotechnology advances (2019).

875 [67]X.-Z. Zhang, N. Sathitsuksanoh, Y.-H.P. Zhang, Glycoside hydrolase family 9 processive
876 endoglucanase from *Clostridium phytofermentans*: Heterologous expression, characterization, and
877 synergy with family 48 cellobiohydrolase, Bioresource technology 101 (2010) 5534–5538.

878 [68]M.M. Kesavulu, J.Y. Tsai, H.L. Lee, P.H. Liang, C.D. Hsiao, Structure of the catalytic domain of
879 the *Clostridium thermocellum* cellulase CelT, Acta crystallographica. Section D, Biological
880 crystallography 68 (2012) 310–320.

881 [69]G. Parsiegla, A. Belaïch, J.P. Belaïch, R. Haser, Crystal structure of the cellulase Cel9M
882 enlightens structure/function relationships of the variable catalytic modules in glycoside hydrolases,
883 Biochemistry 41 (2002) 11134–11142.

884 [70]Y. Honda, N. Shimaya, K. Ishisaki, M. Ebihara, H. Taniguchi, Elucidation of exo-beta-D-
885 glucosaminidase activity of a family 9 glycoside hydrolase (PBPR0520) from *Photobacterium*
886 *profundum* SS9, Glycobiology 21 (2011) 503–511.

887 [71]V. Phakeenuya, K. Ratanakhanokchai, A. Kosugi, C. Tachaapaikoon, A novel multifunctional
888 GH9 enzyme from *Paenibacillus curdlanolyticus* B-6 exhibiting endo/exo functions of cellulase,
889 mannanase and xylanase activities, Applied microbiology and biotechnology 104 (2020) 2079–2096.

890 [72]A. Belaïch, G. Parsiegla, L. Gal, C. Villard, R. Haser, J.-P. Belaïch, Cel9M, a new family 9
891 cellulase of the *Clostridium cellulolyticum* cellulosome, Journal of bacteriology 184 (2002) 1378–
892 1384.

893 [73]S.Y. Ding, E.A. Bayer, D. Steiner, Y. Shoham, R. Lamed, A novel cellulosomal scaffoldin from
894 *Acetivibrio cellulolyticus* that contains a family 9 glycosyl hydrolase, Journal of bacteriology 181
895 (1999) 6720–6729.

896 [74]S. Jindou, Q. Xu, R. Kenig, M. Shulman, Y. Shoham, E.A. Bayer, R. Lamed, Novel architecture
897 of family-9 glycoside hydrolases identified in cellulosomal enzymes of *Acetivibrio cellulolyticus* and
898 *Clostridium thermocellum*, *FEMS microbiology letters* 254 (2006) 308–316.

899 [75]F. Mingardon, J.D. Bagert, C. Maisonnier, D.L. Trudeau, F.H. Arnold, Comparison of family 9
900 cellulases from mesophilic and thermophilic bacteria, *Appl Environ Microbiol* 77 (2011) 1436–1442.

901 [76]D.B. Wilson, Studies of *Thermobifida fusca* plant cell wall degrading enzymes, *Chem Record* 4
902 (2004) 72–82.

903 [77]I.u. Haq, F. Akram, M.A. Khan, Z. Hussain, A. Nawaz, K. Iqbal, A.J. Shah, CenC, a multidomain
904 thermostable GH9 processive endoglucanase from *Clostridium thermocellum*: Cloning,
905 characterization and saccharification studies, *World journal of microbiology & biotechnology* 31
906 (2015) 1699–1710.

907 [78]B. Leis, C. Held, F. Bergkemper, K. Dennemarck, R. Steinbauer, A. Reiter, M. Mechelke, M.
908 Moerch, S. Graubner, W. Liebl, W.H. Schwarz, V.V. Zverlov, Comparative characterization of all
909 cellulosomal cellulases from *Clostridium thermocellum* reveals high diversity in endoglucanase
910 product formation essential for complex activity, *Biotechnology for biofuels* 10 (2017) 240.

911 [79]Y. Zhou, X. Wang, W. Wei, J. Xu, W. Wang, Z. Xie, Z. Zhang, H. Jiang, Q. Wang, C. Wei, A
912 novel efficient β -glucanase from a paddy soil microbial metagenome with versatile activities,
913 *Biotechnology for biofuels* 9 (2016) 36.

914 [80]H. Nacke, M. Engelhaupt, S. Brady, C. Fischer, J. Tautzt, R. Daniel, Identification and
915 characterization of novel cellulolytic and hemicellulolytic genes and enzymes derived from German
916 grassland soil metagenomes, *Biotechnology letters* 34 (2012) 663–675.

917 [81]P. Kanokratana, L. Eurwilaichitr, K. Pootanakit, V. Champreda, Identification of glycosyl
918 hydrolases from a metagenomic library of microflora in sugarcane bagasse collection site and their
919 cooperative action on cellulose degradation, *Journal of Bioscience and Bioengineering* 119 (2015)
920 384–391.

921 [82]A.O.S. Lima, M.C. Quecine, M.H.P. Fungaro, F.D. Andreote, W. Maccheroni, W.L. Araújo, M.C.
922 Silva-Filho, A.A. Pizzirani-Kleiner, J.L. Azevedo, Molecular characterization of a beta-1,4-
923 endoglucanase from an endophytic *Bacillus pumilus* strain, *Applied microbiology and biotechnology*
924 68 (2005) 57–65.

925 [83]S.J. Kim, J.E. Joo, S.D. Jeon, J.E. Hyeon, S.W. Kim, Y.S. Um, S.O. Han, Enhanced
926 thermostability of mesophilic endoglucanase Z with a high catalytic activity at active temperatures,
927 *International journal of biological macromolecules* 86 (2016) 269–276.

928 [84]J. Sakon, D. Irwin, D.B. Wilson, P.A. Karplus, Structure and mechanism of endo/exocellulase E4
929 from *Thermomonospora fusca*, *Nature structural biology* 4 (1997) 810–818.

930 [85]D. Irwin, D.H. Shin, S. Zhang, B.K. Barr, J. Sakon, P.A. Karplus, D.B. Wilson, Roles of the
931 catalytic domain and two cellulose binding domains of *Thermomonospora fusca* E4 in cellulose
932 hydrolysis, *Journal of bacteriology* 180 (1998) 1709–1714.

933 [86]V.V. Zverlov, N. Schantz, W.H. Schwarz, A major new component in the cellulosome of
934 *Clostridium thermocellum* is a processive endo-beta-1,4-glucanase producing cellotetraose, *FEMS*
935 *microbiology letters* 249 (2005) 353–358.

936 [87]C. Reverbel-Leroy, S. Pages, A. Belaich, J.P. Belaich, C. Tardif, The processive endocellulase
937 CelF, a major component of the *Clostridium cellulolyticum* cellulosome: Purification and
938 characterization of the recombinant form, *Journal of bacteriology* 179 (1997) 46–52.

939 [88]L. Gal, C. Gaudin, A. Belaich, S. Pages, C. Tardif, J.P. Belaich, CelG from *Clostridium*
940 *cellulolyticum*: A multidomain endoglucanase acting efficiently on crystalline cellulose, *Journal of*
941 *bacteriology* 179 (1997) 6595–6601.

942 [89]K.-D. Zhang, W. Li, Y.-F. Wang, Y.-L. Zheng, F.-C. Tan, X.-Q. Ma, L.-S. Yao, E.A. Bayer, L.-S.
943 Wang, F.-L. Li, Processive Degradation of Crystalline Cellulose by a Multimodular Endoglucanase
944 via a Wirewalking Mode, *Biomacromolecules* 19 (2018) 1686–1696.

945 [90]S. Wu, S. Wu, Processivity and the Mechanisms of Processive Endoglucanases, *Applied*
946 *Biochemistry and Biotechnology* 190 (2020) 448–463.

947 [91]F.H. Niesen, H. Berglund, M. Vedadi, The use of differential scanning fluorimetry to detect ligand
948 interactions that promote protein stability, *Nature protocols* 2 (2007) 2212–2221.

Supplementary Tables

Table S1 CAZymes identified by metagenomic screening on AZCL-HEC or AZCL-xyloglucan

The taxon of the closest homolog, on the order and genus level, was determined by BLASTP similarity searches against the non-redundant protein database.

n.d. the genus of the closest homolog could not be determined.

CAZy family	ID	CBM	presumed activity	% identity	Taxon of closest homolog	
					genus	order
GH3	GH3a	-	beta-glucosidase, beta-xylosidase	66	<i>Nitrospirillum</i>	<i>Rhodospirillales</i>
GH5	GH5a	-	endoglucanase	60	<i>Calditrix</i>	<i>Calditrichales</i>
	GH5b	CBM2		68	<i>Catellatospora</i>	<i>Micromonosporales</i>
GH6	GH6a	CBM2	endoglucanase, exoglucanase	66	<i>Actinomadura</i>	<i>Streptosporangiales</i>
GH9	GH9a	CBM2 CBM4	endoglucanase, exoglucanase	66	<i>Sphaerisporangium</i>	<i>Streptosporangiales</i>
	GH9b	-		78	<i>Thermo flavifilum</i>	<i>Chitinophagales</i>
	GH9c	CBM3		67	<i>Streptosporangium</i>	<i>Streptosporangiales</i>
	GH9d	CBM2 CBM4		66	<i>Sphaerisporangium</i>	<i>Streptosporangiales</i>
	GH9e	CBM2 CBM3		83	<i>Microbispora</i>	<i>Streptosporangiales</i>
	GH9f	CBM4		69	<i>Sorangium</i>	<i>Myxococcales</i>
GH10	GH10	CBM22 CBM22 CBM22 CBM 9	xylanase	60	<i>Verrucosispora</i>	<i>Micromonosporales</i>
GH11	GH11a	CBM2	xylanase	62	<i>Micromonospora</i>	<i>Micromonosporales</i>
	GH11b	-		77	<i>Thermobifida</i>	<i>Streptosporangiales</i>
GH12	GH12a	-	endoglucanase	70	<i>Catellatospora</i>	<i>Micromonosporales</i>
GH43	GH43a	-	arabinanase, xylanase	87	<i>Thermo flavifilum</i>	<i>Chitinophagales</i>
GH48	GH48a	CBM2	endoglucanase, exoglucanase	81	<i>Microbispora</i>	<i>Streptosporangiales</i>
GH51	GH51a	-	endoglucanase, xylanase	92	<i>Thermo flavifilum</i>	<i>Chitinophagales</i>
GH53	GH53a	-	galactanase	89	<i>Thermo flavifilum</i>	<i>Chitinophagales</i>
GH74	GH74a	CBM2	xyloglucanase	92	<i>Microbispora</i>	<i>Streptosporangiales</i>
	GH74b	CBM2		85	<i>Microbispora</i>	<i>Streptosporangiales</i>
	GH74c	CBM2		75	<i>Microbispora</i>	<i>Streptosporangiales</i>
	GH74d	CBM2		81	<i>Microbispora</i>	<i>Streptosporangiales</i>
	GH74e	-		83	<i>Microbispora</i>	<i>Streptosporangiales</i>
GH115	GH115a	-	xylane-glucuronidase	64	<i>Opitutus</i>	<i>Opitales</i>
CE1	CE1a	-	acetyl xylan esterase	55	<i>Actinophytocola</i>	<i>Pseudonocardiales</i>
	CE1b	-		57	<i>Catenulispora</i>	<i>Catenulisporales</i>
	CE1c	-		32	<i>Lewinella</i>	<i>Sphingobacteriales</i>
CE4	CE4a	-	acetyl xylan esterase	59	n.d.	<i>Thermomicrobiales</i>
AA10	AA10a	CBM2	LPMO	64	<i>Micromonospora</i>	<i>Micromonosporales</i>
CBM2	CBM2a	-	unknown	51	<i>Dactylosporangium</i>	<i>Micromonosporales</i>
	CBM2b	-	unknown	45	<i>Micromonospora</i>	<i>Micromonosporales</i>

Table S2 Distance matrix of the multiple sequence alignment of 174 characterized GH9s

(see Excel file)

Table S3 Protein domains attached to at least one of the 174 characterized GH9s and their occurrence

Domain	Pfam accession number	Occurrence	
		Protein number	%
Glycoside hydrolase family 9	PF00759	174	100,0
Cellulase N-terminal Ig-like domain	PF02927	55*	33,3
CBM3	PF00942	47	27,0
Dockerin	PF00404	41	23,6
CBM2	PF00553	18	10,3
CBM4/9	PF02018	16	9,2
Fibronectin type III domain	PF00041	6	3,4
CBM49	PF09478	4	2,3
Glycoside hydrolase family 48	PF02011	3	1,7
PKD domain	PF00801	2	1,1
CBM10	PF02013	2	1,1
CBMX2	PF03442	2	1,1
CBM64	PF18666	2	1,1
Glycoside hydrolase family 5	PF00150	1	0,6
Glycoside hydrolase family 16	PF00722	1	0,6
F5/8 type C domain	PF00754	1	0,6
Carbohydrate family 9 binding domain-like	PF06452	1	0,6
Immunoglobulin I-set domain	PF07679	1	0,6
Glycoside hydrolase family 44	PF12891	1	0,6

* 55 Ig-like domain-containing proteins according to Pfam database; 3 supplementary proteins are identified respectively with CDD database

(UniProt References C9RJA3 and Q6LUT2) and InterPro (Q9APG3)

Supplementary figures

Figure S1: Alignment of 168 characterized GH9 enzymes and the six identified metagenomic GH9 enzymes.

The multiple sequence alignment was built using Clustal Omega, and the aligned fasta file was submitted to ESPript 3.0 (<http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi>). Identical or similar amino acids are colored in red, and boxed in blue if the global similarity score is > 0.7. 100% conserved amino acids are highlighted in red. Loop A of Ig-like-domain-containing enzymes is located between positions 467 and 483. Residues composing loop B are situated between positions 453 and 454.

(see corresponding pdf file)

Figure S2: Superposition of the three-dimensional structures of three bacterial GH9s belonging to distinct clades

X-ray crystal structures of *T. fusca* Cel9A (Sakon et al. 1997) (UniProt Reference P26221, PDB ID: 1TF4, green), *C. thermocellum* CbhA (Schubot et al. 2004) (Q6RSN8, PDB ID: 1UT9, blue) and *R. cellulolyticum* Cel9M (Parsiegla et al. 2002) (Q9EYQ2, PDB ID: 1IA6, red) were superposed using PyMol (DeLano 2002). Protein three-dimensional structures are displayed using the cartoon representation.

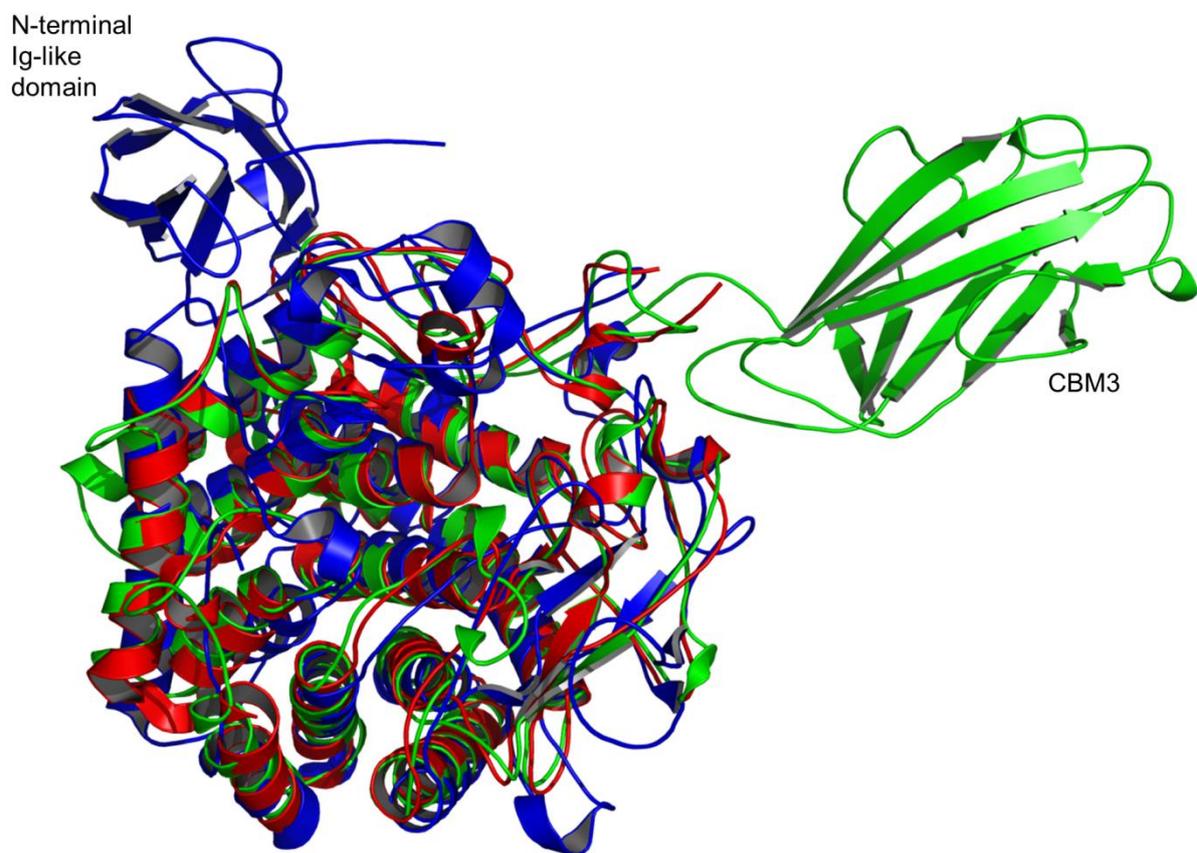


Figure S3: Superposition of the three-dimensional structures of two bacterial GH9s belonging to distinct clades with co-crystallized substrate

X-ray crystal structures of *T. fusca* Cel9A (Sakon et al. 1997) (UniProt Reference P26221, PDB ID: 1JS4, green), *C. thermocellum* CbhA (Schubot et al. 2004) (Q6RSN8, PDB ID: 1RQ5, red) were superposed using PyMol (DeLano 2002). Protein three-dimensional structures are displayed using the cartoon representation. Co-crystallized substrates (cellotriose for TfCel9A and cellotetraose for CtCbhA) are displayed as ball-and-stick. Loops A (Arg558-Gln575, CtCbhA) and B (Trp430-Ser436, TfCel9A) are respectively colored in magenta and red.

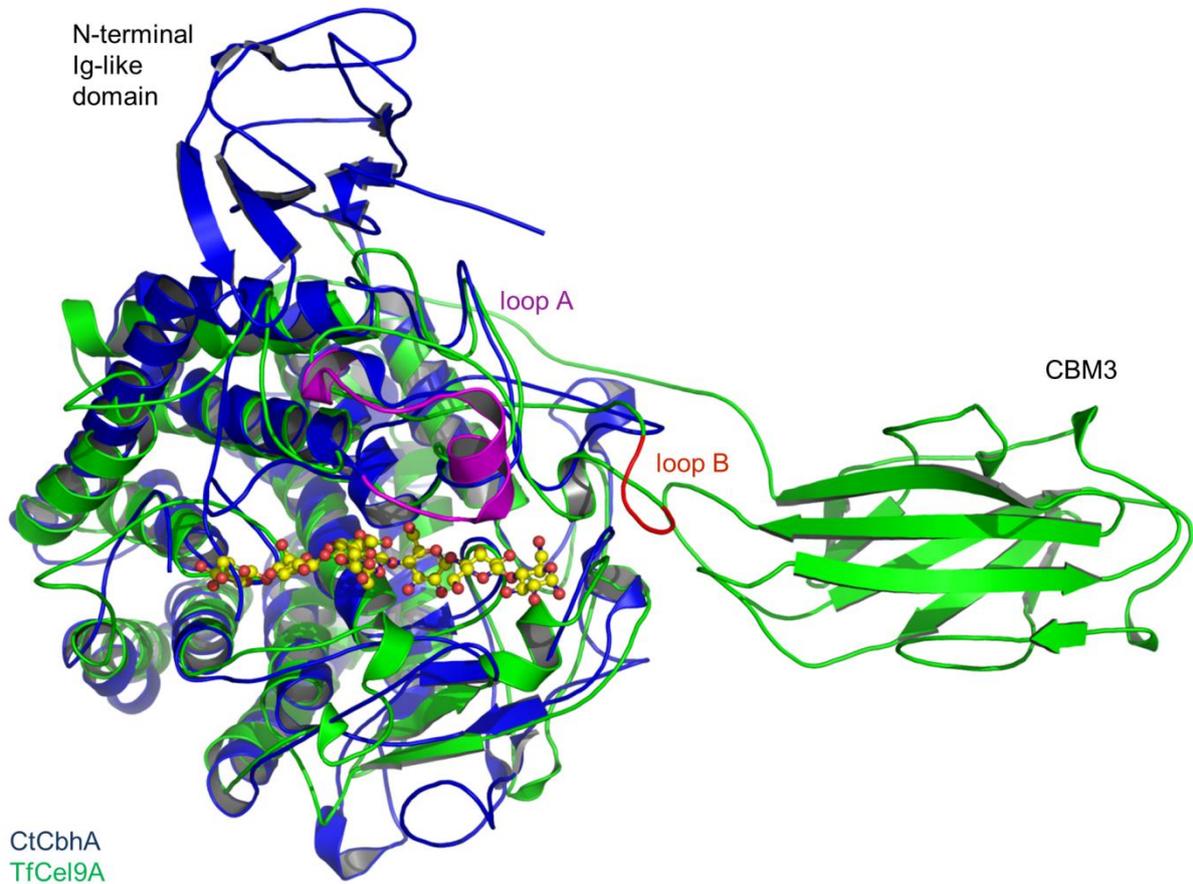


Fig. S4: Purification of recombinant metagenome-derived bacterial GH9s

(A) SDS-PAGE of recombinant GH9 enzymes purified by affinity chromatography on a nickel chelating resin (GH9e) followed by gel filtration (GH9b and GH9d). GH9b (65.8 kDa), GH9d (93.5 kDa) and GH9e (83.5 kDa) were purified from the 12 000 x g supernatant of the lysates of *E. coli* cultures and 0.5 μ g were separated by SDS-PAGE. (B) Gel filtration chromatography of recombinant GH9 enzymes. Purified GH9b, GH9d and GH9e (respectively 1.6 mg, 3.2 mg and 0.3 mg) were loaded on a Superdex 200 10/300 GL (GE Healthcare) using 25 mM triethanolamine pH 7.0, 150 mM NaCl, 5% (v/v) glycerol, as buffer. For calibration of the column, the following molecular weight markers (BioRad, pointed by black diamonds) were used: thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B12 (1.35 kDa). The elution was monitored by measuring the absorbance at 280 nm. GH9b (solid line), GH9d (dotted line) and GH9e (dashed line) main peak were eluted at respective apparent molecular weights of 28 kDa, 287 kDa and 40 kDa. MW, molecular weight

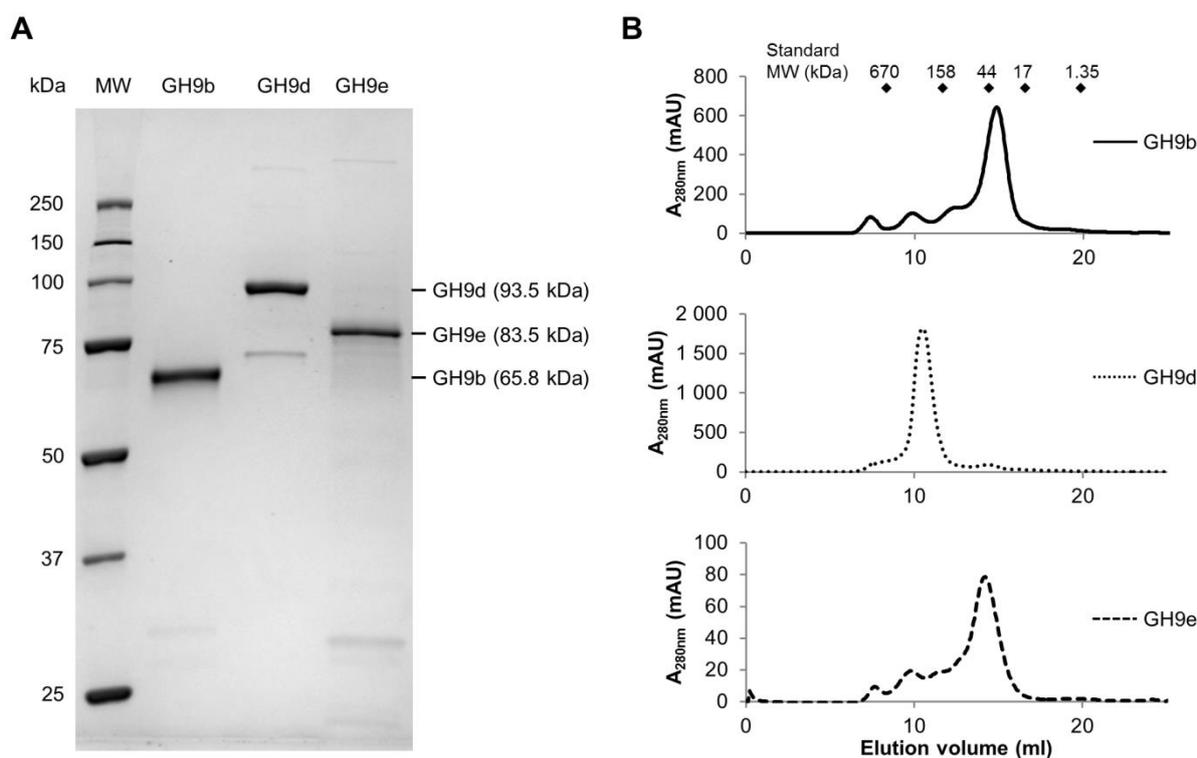


Figure S5: Purification and domain architecture of the GH48 enzyme retrieved from the metagenome

Right, schematic representation of the mature protein are presented according to its relative sequence length. Mature protein molecular weight was calculated using ExPASy ProtParam tool (Gasteiger et al., 2005). Taxonomic order was predicted by BLAST using the closest homolog corresponding organisms (Madeira et al. 2019). N, N-terminal, C. C-terminal. Left, SDS-PAGE of purified recombinant GH48 produced in *E. coli*.

