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Cell Reports

Molecular mechanism by which prominent human gut Bacteroidetes utilize mixedlinkage beta-glucans, major health-promoting cereal polysaccharides --Manuscript Draft--

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Abstract:	Microbial utilization of complex polysaccharides is a major driving force in shaping the composition of the human gut microbiota. There is a growing appreciation that finely tuned polysaccharide utilization loci enable ubiquitous gut Bacteroidetes to thrive on the plethora of complex polysaccharides that constitute "dietary fiber". Mixed-linkage beta(1,3)/beta(1,4)-glucans (MLGs) are a key family of plant cell wall polysaccharides with recognized health benefits, but whose mechanism of utilization has remained unclear. Here, we provide molecular insight into the function of an archetypal MLG utilization locus (MLGUL) through a combination of biochemistry, enzymology, structural biology, and microbiology. Comparative genomics coupled with growth studies demonstrated further that syntenic MLGULs serve as genetic markers for MLG catabolism across commensal gut bacteria. In turn, we surveyed human gut metagenomes to reveal that MLGULs are ubiquitous in human populations globally, which underscores the importance of gut microbial metabolism of MLG as a common cereal polysaccharide.
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11 September 2017

Ruth Zearfoss, Ph.D. Associate Editor *Cell Reports*

Dear Dr. Zearfoss,

Thank you for your email on September 5 informing me that our manuscript ("Molecular mechanism by which prominent human-gut Bacteroidetes utilize mixed-linkage beta-glucans, major health-promoting cereal polysaccharides"; CELL-REPORTS-D-17-01660) has been accepted for publication in *Cell Reports*. We herewith submit our individual manuscript, figure, and supplementary files for production.

Please note that although we have endeavoured to comply with all of the publication guidelines for *Cell Reports*, our manuscript is now over the recommended character count following revision according to the reviewers' original comments (however, the main text minus references is under the limit at 49000 characters including spaces). Also to comply with the reviewers' requests, we have also moved one supplemental figure to the main text (Fig. 5 in the current version), and added a new supplemental figure (Fig. S5 in the current version), which brings the total number of main-text figures and table to eight, with an equivalent number of supplemental figures. We hope that you will kindly grant us an exception on the text and figure limits, since the extra content has been included in direct response to specific reviewer comments. Indeed, we feel that these additions bring significant value, and that trimming the existing content to meet the character and figure guidelines would erode the overall presentation of the work.

Yours sincerely,

Brand

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Reviewers' comments:

With reference to the email from Ruth Zearfoss, Associate Editor, on Sept. 5, there were no reviewer comments on our revised manuscript.

- 1 Molecular mechanism by which prominent human-gut Bacteroidetes utilize mixed-linkage beta-
- 2 glucans, major health-promoting cereal polysaccharides
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20 Summary

21 Microbial utilization of complex polysaccharides is a major driving force in shaping the composition of 22 the human gut microbiota. There is a growing appreciation that finely tuned polysaccharide utilization 23 loci enable ubiquitous gut Bacteroidetes to thrive on the plethora of complex polysaccharides that 24 constitute "dietary fiber". Mixed-linkage $\beta(1,3)/\beta(1,4)$ -glucans (MLGs) are a key family of plant cell wall 25 polysaccharides with recognized health benefits, but whose mechanism of utilization has remained 26 unclear. Here, we provide molecular insight into the function of an archetypal MLG utilization locus 27 (MLGUL) through a combination of biochemistry, enzymology, structural biology, and microbiology. 28 Comparative genomics coupled with growth studies demonstrated further that syntenic MLGULs serve as 29 genetic markers for MLG catabolism across commensal gut bacteria. In turn, we surveyed human gut 30 metagenomes to reveal that MLGULs are ubiquitous in human populations globally, which underscores 31 the importance of gut microbial metabolism of MLG as a common cereal polysaccharide.

32 Introduction

33 The composition and homeostasis of the human gut microbiota has a profound and intimate 34 connection to various aspects of our physiology, health and wellbeing (Littman and Pamer, 2011). Indeed, 35 a multitude of diseases such as type 2 diabetes, inflammatory bowel diseases (IBDs), and cancer have been linked to alterations in the population and proportion of microbes in this highly complex and 36 37 dynamic ecosystem that exists in our large intestine (Biedermann and Rogler, 2015; Fujimura et al., 2010; Kau et al., 2011; Schwabe and Jobin, 2013). The molecular mechanisms by which the microbiota exerts 38 influence on human health are largely unresolved and undoubtedly complex, yet may hold the key to 39 personalized medicine through therapeutics that target the gut microbial ecosystem (Blanton et al., 2016; 40 41 Haak et al., 2017; Kootte et al., 2012; Subramanian et al., 2015).

42 A major factor in shaping the composition and physiology of the gut microbiota is the influx of 43 complex glycans – popularly known as "dietary fibre" – that evade degradation by the limited set of human genome-encoded glycoside hydrolases (Hamaker and Tuncil, 2014; El Kaoutari et al., 2013; 44 45 Koropatkin et al., 2012). Indeed, regular ingestion of plant polysaccharides is integral to maintaining a 46 healthy balance of microbes in our lower gastrointestinal tract (De Filippo et al., 2010; Sonnenburg and 47 Sonnenburg, 2014). Members of the Bacteroidetes, a dominant phylum in the human gut, possess an 48 arsenal of Polysaccharide Utilization Loci (PUL) to target a wide range of complex glycans (El Kaoutari et al., 2013). Analogous to the archetypal *Bacteroides thetaiotaomicron* starch utilization system (Sus), a 49 hallmark of all Bacteroidetes PULs is the organization of genes clustered around tandem susC/susD 50 51 homologs (encoding a TonB dependent transporter, TBDT, and a cell-surface glycan-binding protein, 52 SGBP, respectively) (Martens et al., 2009). Additional co-localized and co-regulated SGBP(s), a cohort of 53 enzymes, and a transcriptional regulator typically make up a machinery that acts in concert to sense, break 54 down, and import complex glycans (Grondin et al., 2017; Hemsworth et al., 2016). Many such PULs, each targeting specific glycan structures, have been identified by genomics and transcriptomics (see, e.g., 55 the seminal study by (Martens et al., 2011), but detailed functional characterization lags severely behind 56 57 (reviewed in (Grondin et al., 2017; Martens et al., 2014)). Developing a precise understanding of the 58 molecular details of complex glycan utilization by individual members of the microbiota is essential to designing targeted therapies based on prebiotics, probiotics, and synbiotics (Ciorba, 2012; Slavin, 2013), 59 60 as well as novel therapeutic interventions.

Recently, comprehensive functional analysis has revealed the detailed molecular mechanisms by which individual PULs enable human gut Bacteroidetes to utilize predominant plant polysaccharides, including the matrix glycans, xyloglucan (Hemsworth et al., 2016; Larsbrink et al., 2014; Tauzin et al., 2016), xylan (Rogowski et al., 2015), β -mannan (Bågenholm et al., 2017), and rhamnogalacturonan II (Ndeh et al., 2017). Mixed-linkage $\beta(1,3)/\beta(1,4)$ -glucans (MLGs, Fig. 1A) from cereal grains constitute an additional key group of dietary glycans, whose utilization by gut microbes was previously unresolved 67 at the molecular level. MLGs are abundant in the aleurone layer of common cereals, including oats (3-8 68 % dry weight) and barley (2-20 % dry weight) (El Khoury et al., 2012). Beyond their obvious potential to 69 contribute to energy intake (Cummings and Macfarlane, 1997; McNeil, 1984), MLGs have been linked to 70 a range of health benefits, *e.g.* promoting healthy cholesterol and blood glucose levels, ameliorating 71 insulin resistance, and mitigating metabolic syndrome (El Khoury et al., 2012). In particular, the 72 cholesterol lowering effect of oat MLG has long been recognized by the United States Food and Drug 73 Administration (FDA) as well as the United Kingdom Joint Health Claims Initiative (JHCI), and been confirmed by subsequent studies (Othman et al., 2011). 74

The mechanisms behind these systemic benefits of MLG are, however, incompletely understood, in part due to a lack of understanding of MLG metabolism by individual members of the human gut microbiota. Thus, we report here the molecular characterization of a mixed-linkage glucan utilization locus (MLGUL) in the common symbiont *B. ovatus*. Identifying syntenic MLGUL in other Bacteroidetes revealed that as the archetype, this MLGUL serves as a molecular marker for MLG utilization across the Bacteroidetes phylum, thereby enabling future functional prediction across species.

81 Results

82 Identification of a multi-gene locus responsible for MLG utilization by *B. ovatus*

A putative MLGUL was previously identified in *B. ovatus* (Fig. 1B) based on the presence of a tandem *susC/susD* homolog signature (Martens et al., 2009) and high-level expression of select genes in the presence of bMLG (Martens et al., 2011). Individual genes in the locus, BACOVA_02741-02745, were all substantially upgregulated (125 to 298-fold) during growth on bMLG vs. glucose as sole carbon sources (Table S1). BACOVA_02742 and BACOVA_02743 encode the signature TBDT/SGBP pair with 28% and 19% protein sequence identity to SusC and SusD, respectively. The putative MLGUL was additionally predicted to encode a second, non-homologous SGBP (BACOVA_02744), a hybrid two90 component sensor/transcriptional regulator (HTCS, BACOVA_02740), and up to three glycoside
91 hydrolases (GHs).

92	BoGH16 _{MLG} (BACOVA_02741) is a member of Glycoside Hydrolase Family 16 (GH16), a
93	family of <i>endo</i> -β-glucanases in the Carbohydrate Active Enzymes (CAZy) classification (Cantarel et al.,
94	2009). GH16 notably includes canonical bacterial MLG endo-glucanases (endo-MLGase) (Planas, 2000),
95	along with a diversity of <i>endo</i> -glucanases and <i>endo</i> -galactanases (Eklof and Hehemann). BoGH 3_{MLG}
96	(BACOVA_02745) is classified into Glycoside Hydrolase Family 3 (GH3), whose members include exo-
97	β -glucosidases (Fincher et al.). Notably, we have determined that BACOVA_02738, which is predicted to
98	encode a second GH3 <i>exo</i> - β -glucosidase, is unlikely to be part of the MLGUL for three reasons: (1)
99	BACOVA_02738 was not significantly upregulated on MLG (1.6-fold vs. glucose control, Table S1), (2)
100	a corresponding gene is not found among syntenic loci (Fig. 1B), and (3) the encoded enzyme was
101	catalytically feeble compared to BoGH3 _{MLG} on β -glucosides relevant to MLG saccharification (vide
102	infra).

103To determine the correlation between the presence of the predicted MLGUL and growth of *B*.104*ovatus* on MLG, we constructed an isogenic mutant of *B. ovatus* Δtdk (Larsbrink et al., 2014) in which a105contiguous region of DNA encoding genes BACOVA_02738-02745 was deleted (*B. ovatus* $\Delta MLGUL$).106Vis-à-vis the parent strain, the *B. ovatus* $\Delta MLGUL$ was able to grow equally well on glucose as the sole107carbon source, however the ability to grow on bMLG was completely abolished (Fig. S1). Thus, the108putative MLGUL is necessary to confer *B. ovatus* the ability to utilize MLG.

109 Enzymology and structural biology of BoGH16_{MLG}, the vanguard MLGase

110 Cellular localization

The GH family membership of BoGH16_{MLG} suggested a potential role as the vanguard enzyme
 catalyzing polysaccharide backbone cleavage at the cell surface as the essential first step in MLG
 utilization. Indeed, the presence of a predicted Type II signal sequence (determined with LipoP 1.0
 Page 5 of 30

114 (Juncker and Willenbrock, 2003)) suggested that the protein is membrane-anchored via lipidation of the 115 N-terminal cysteine residue (Paetzel et al., 2002). To validate this prediction, B. ovatus Δtdk was grown 116 on minimal medium with either glucose or bMLG as a sole carbon source prior to immunolocalization of 117 BoGH16_{MLG}. As shown in Fig. 2A, BoGH16_{MLG} was clearly visualized on the outer surface of cells in 118 which the presence of the polysaccharide induced MLGUL expression, but was absent from cells grown on glucose (Fig. S2C, S2F). Further analysis of cellular fractions by Western blotting revealed the 119 120 presence of $BoGH16_{MLG}$ in the membrane fraction, corroborating its attachment to the outer membrane (Fig. 2C). Interestingly, BoGH16_{MLG} was also detected in the lysate supernatant (soluble periplasmic or 121 122 cytoplasmic proteins) and in the culture supernatant (secreted protein) (Fig. 2C). While the former may 123 represent anchored protein released into the soluble fraction during cell lysis, detection in the culture 124 supernatant could result from packaging and release in outer membrane vesicles, which has previously 125 been observed for other Bacteroidetes glycoside hydrolases (Elhenawy et al., 2014).

126 Substrate and product specificity

127 To verify the leading catalytic role of $BoGH16_{MLG}$ in MLG utilization and determine the 128 specificity of the enzyme for individual β -glucans, recombinant BoGH16_{MLG} produced in *E. coli* 129 (recBoGH16_{MLG}, Fig. S3A, S3B) was screened for hydrolytic activity against a library of polysaccharides. 130 No activity was observed on tamarind xyloglucan, beechwood xylan, wheat arabinoxylan, carob galactomannan, konjac glucomannan, synthetic carboxymethylcellulose, synthetic hydroxyethylcellulose, 131 132 Xanthomonas campestris xanthan gum, or Ulva sp. ulvan. In this initial screen, BoGH16_{MLG} was minimally active on all- $\beta(1,3)$ -glucans, including Laminaria digitata laminarin, yeast β -glucan, and 133 134 Alcaligenes faecalis curdlan, but exhibited high specific activity on bMLG. The optimum pH of 6.5 135 (consistent with function in the distal human colon) and maximum temperature range of 45 to 55 °C was 136 determined using bMLG as substrate (data not shown).

137	Subsequent Michaelis-Menten kinetic analysis at the pH optimum and 37 °C demonstrated that
138	BoGH16 _{MLG} is a highly predominant mixed-linkage $\beta(1,3)/\beta(1,4)$ -glucanase (MLGase), with a 33-fold
139	higher specificity constant, k_{cat}/K_m , for bMLG (Fig. 1A) over laminarin, an all- $\beta(1,3)$ -glucan with single
140	$\beta(1,6)$ -linked glucosyl branches (Fig. 3A, Table S2) (Martin et al., 2007). BoGH16 _{MLG} was even less
141	efficient on the other two all- $\beta(1,3)$ -glucans for which activity was initially observed: The k_{cat}/K_m , was
142	147-fold higher for bMLG than yeast β -glucan (similar in structure to laminarin but with longer $\beta(1,6)$ -
143	linked glucose branches (Lowman et al., 2011)) and nearly four orders of magnitude higher than that of
144	high curdlan, a 22 kDa, non-branched $\beta(1,3)$ -glucan (Harada et al., 1968) (Fig. 3A, Table S2).
145	Detailed product analysis was employed to determine the mode of hydrolysis, endo vs. exo, and
146	linkage specificity of recBoGH16 $_{MLG}$ to gain information on the nature of the MLG cleavage products at
147	the <i>B. ovatus</i> cell surface. HPLC analysis at selected time points in the hydrolysis showed the initial
148	production of very large oligosaccharide fragments, which were progressively converted into shorter
149	species and ultimately to two distinct oligosaccharides in the limit-digest (Fig. 3B). This product
150	evolution indicates that $BoGH16_{MLG}$ operates through an <i>endo</i> -dissociative mode of action in which the
151	MLG polysaccharide is stochastically cleaved along the backbone.
152	Comparison with oligosaccharide standards (Fig. 3B) and additional LC-MS analysis (data not
153	shown) revealed that the limit-digest products were the mixed-linkage trisaccharide, G4G3G [Glc β (1-4)
154	$Glc\beta(1-3)$ Glc], and the mixed-linkage tetrasaccharide, G4G4G3G [Glc $\beta(1-4)$ Glc $\beta(1-4)$ Glc $\beta(1-3)$ Glc].
155	Thus, BoGH16 _{MLG} specifically hydrolyzes $\beta(1,4)$ -linkages of glycosyl residues that are immediately
156	preceded by a $\beta(1,3)$ -linked glucosyl residue (toward the non-reducing chain end). This specificity is
157	typical of bacterial endo-MLGases within GH16 (Gaiser et al., 2006; Mcgregor et al., 2017; Planas,
158	2000).

159 To provide more refined insight into $BoGH16_{MLG}$ substrate specificity, Michaelis-Menten kinetics 160 were determined for a series of chromogenic glycosides (Fig. S4A, S4B; Table S3). recBoGH16_{MLG} had 161 no activity on the *ortho*-chloro-*para*-nitrophenyl (CNP) β-glycosides of glucose (G-CNP), cellobiose 162 (G4G-CNP), cellotriose (G4G4G-CNP), nor on *para*-nitrophenyl (*pNP*) β-glucoside (G-*pNP*). Weak 163 activity was observed on the pNP and CNP β -glycosides of laminaribiose (G3G), consistent with a requirement for a $\beta(1,3)$ linkage spanning the -2 to -1 active-site subsites (GH subsite nomenclature 164 165 according to (Davies et al., 1997)), as was indicated by the bMLG limit-digest analysis (vide supra). Likewise, G4G3G-CNP and G4G4G3G-CNP were specifically and efficiently hydrolyzed to release the 166 167 aglycone, with no cleavage of the internal glycosidic bonds. The specificity constants (k_{cat}/K_m values) for 168 CNP release from these mixed-linkage tri- and tetrasaccharides were 800- and 1500-fold greater than that 169 of G3G-CNP, respectively, which indicate that potential -3 and -4 subsites contribute 17 kJ/mol and 1.6 170 kJ/mol to transition state stabilization ($\Delta\Delta G^{\ddagger}$). Indeed, a very significant contribution from the -3 subsite 171 is a common feature of GH16 endo-MLGases (Gaiser et al., 2006; McGregor et al., 2016; Planas, 2000).

172 BoGH16_{MLG} tertiary structure

173 Three-dimensional structures of recBoGH16_{MLG} were solved by X-ray crystallography to reveal 174 the molecular basis for MLG recognition and hydrolysis. The apo structure of recBoGH16_{MLG} was 175 determined to a resolution of 1.8 Å by molecular replacement using the structure of Zobellia 176 galactanivorans laminarinase ZgLamC_{GH16-E1425} (PDB code 4CRQ) (Labourel et al., 2015) as a search 177 model (See Table S4 for processing and refinement statistics). The crystal contained two polypeptide chains in the asymmetric unit corresponding to residues I35-L271 of wild-type BoGH16_{MLG} for both 178 chains (residue numbering is from transcriptional start site according to the genomic sequence). No 179 180 electron density was observed for the N-terminal His6-tag and subsequent 15 amino acids in either chain 181 of the recombinant protein, which suggests that residues C19-D34 of the wild-type enzyme constitute a 182 flexible linker sequence to distance the catalytic module from the outer membrane surface (residues M1-183 S18 comprise the predicted signal peptide); the sidechain of C19 would constitute the site of N-terminal lipidation (Paetzel et al., 2002). The overall fold of BoGH16_{MLG} consists of a β -jelly roll architecture 184 185 typical of other GH16 members (Davies and Sinnot, 2008): Two antiparallel β -sheets stack on top of each Page 8 of 30

other with the concave face forming the polysaccharide substrate binding cleft. The end-on arrangement
of the two chains in the asymmetric unit hinted at the possibility of the formation of a dimer (Fig 4A).
Size-exclusion chromatography, however, indicated that BoGH16_{MLG} exists as a monomer in solution
(data not shown) which, together with steric considerations of polysaccharide binding through the activesite cleft, indicates that end-on contacts observed between Chains A and B are artifacts of crystal packing.

191The sidechains of the conserved GH16 catalytic residues (Planas, 2000), comprising Glu-143192(nucleophile), Asp-145 (electrostatic "helper") and Glu-148 (acid/base) are presented on the same face of193one β-strand (β 8), pointing into the active-site cleft. Notably, these residues are contained in a EXDXXE194consensus sequence that is typical of bacterial GH16 laminarinases (β (1,3)-specific endo-glucanases). The195insertion of an extra amino acid (underlined), typically methionine, results in a so-called "β-bulge"196secondary structural motif that is not found among canonical bacterial MLGases, which instead possess a197regular β-strand (Barbeyron et al., 1998; Michel et al., 2001).

198 Commensurate with this observation, the closest eight structural homologs identified using the 199 Dali server (Holm and Rosenstrom, 2010) feature a β -bulge active-site motif (Table S5). Specifically, the top match (Z-score = 29.3) was the structure of laminarinase "ZgLamC_{GH16-E1428}" from Zobellia 200 galactanivorans (PDB code 4CTE) (Labourel et al., 2015), which has 38% amino acid identity and 201 202 superimposed with BoGH16_{MLG} with a root mean square deviation (RMSD) value of 2.0 Å over 211 out of 231 C α pairs. In comparison, the closest GH16 homolog with a regular active-site β -strand was the 203 204 lichenase (MLGase) from Paenibacillus macerans (PDB code 1MAC) (Hahn et al., 1995), which has a comparable Z-score of 25.1 and an RMSD value of also 2.0 Å over 200 out of 212 Cα pairs, despite 205 having only 22% amino acid identity with BoGH16_{MLG}. 206

Soaking crystals of the wild-type enzyme with G4G4G3G yielded a product complex with 1.8 Å
 resolution (Table S4). The complete tetrasaccharide was modelled in electron density spanning subsites -1
 to -4 in the active-site cleft of Chain A, while the electron density for the fourth glucosyl residue in

Page **9** of **30**

subsite -4 was not resolved in chain B. This is most likely due to disorder of this residue since the
corresponding -4 Glc in Chain A is fully solvent exposed, makes no contact with the enzyme, and has
significantly higher B-factors (Fig. 4B). These structural observations are consistent with kinetic data for
chromogenic MLG oligosaccharides (Table 1), which likewise suggest the existence of three primary
negative subsites, -1 to -3, and a weakly interacting -4 subsite.

In both Chain A and B, the three glucosyl residues spanning subsites -1 to -3 are well defined and 215 216 virtually identical. The reducing-end glucosyl residue in the -1 subsite is in the β -conformation, with the 217 C1 hydroxyl hydrogen bonded to Tyr-181, which is observed in a dual conformation in both chains of the 218 G4G4G3G-complex (Fig. 4C). Interestingly, this dual conformation is not observed in the apo-form of the 219 enzyme; Tyr-181 is "swung in" to the active site in chain B, while it is "swung out" in chain A, the 220 sidechain from chain A stacking on top of the chain B sidechain (Fig. 4D). The conformation of this 221 sidechain will be key to determining the nature of the positive substrate binding subsites, indeed, comparison with other GH16 endo-glucanases clearly suggests the presence of two positive subsites 222 223 (Gaiser et al., 2006; Planas, 2000). Whether the dynamics observed for Tyr-181 are an artefact of 224 crystallisation, or perhaps play a role in substrate binding and product release is unclear in the absence of 225 an enzyme-substrate complex spanning the positive subsites.

With regard to specific interactions in the negative subsites, subsite -1 is further characterized by 226 hydrogen bonds between Glu-143 and the C2 hydroxyl, Trp-125 and the C6 hydroxyl, as well as Glu-148 227 228 and the ring oxygen and the C1 hydroxyl. This glucose is also positioned by a stacking interaction with 229 Trp-125 and Trp-129 (Fig. 4E), both of which are conserved across all GH16 laminarinases. At subsite -2, 230 highly conserved Arg-97 forms a hydrogen bond with the C6 hydroxyl, and Asn-60 hydrogen bonds to 231 the C2 hydroxyl as well as to the glucosidic bond oxygen between the -1 and -2 sugars. Another 232 conserved residue, Trp-138, serves as a platform that stacks with the subsite -2 glucose. In subsite -3, the 233 main interaction is stacking against Trp-58, which also forms a hydrogen bond to the glucosidic bond

oxygen between the -3 and -4 sugars (Fig. 4E). Together, these interactions in subsite -3 are responsible
for 17 kJ/mol of transition-state stabilization (*vide supra*).

236 Downstream saccharificaiton of mixed-linkage oligosaccharides produced by BoGH16_{MLG}

- To elucidate the mechanism for the downstream conversion of the oligosaccharide products of
- 238 BoGH16_{MLG} to glucose for primary metabolism, we examined the biochemistry of the two predicted exo-
- β -glucosidases, BoGH3_{MLG} and BACOVA 02738(GH3) associated with the MLGUL.

240 Cellular localization of BoGH3_{MLG} and the BACOVA_02738(GH3) gene product

241 BoGH3_{MLG} and BACOVA_02738(GH3) were unambiguously predicted by SignalP 4.0 (Petersen et al.,

242 2011) to contain a secretion signal peptide, while LipoP 1.0 (Juncker and Willenbrock, 2003) additionally

indicated a Type II lipoprotein signal sequence (Paetzel et al., 2002) in BoGH3_{MLG} only. The same *B*.

244 *ovatus* Δtdk cultures used for BoGH16_{MLG} localization, containing glucose or bMLG as the sole carbon

source, were probed using polyclonal antibodies independently raised against recombinant BoGH3_{MLG}

and the BACOVA 02738(GH3) gene product. Neither protein was detected on the cell surface by

fluorescence microscopy, especially in the presence of bMLG which induces BoGH16_{MLG} production

 $\label{eq:general} \mbox{248} \qquad (Fig. 2B, Fig. S2A). \mbox{ BoGH3}_{MLG} \mbox{ induction by bMLG was confirmed by a Western blot of cellular}$

249 fractions, which also confirmed that this enzyme is membrane anchored (Fig. 2C).

In contrast, the BACOVA_02738(GH3) gene product was detected to a higher degree in *B. ovatus* cells grown in minimal medium with glucose as a sole carbon source compared to cells induced with bMLG (Fig. S2B). The lack of upregulation by bMLG is consistent with transcriptional analysis which showed a limited change in expression in bMLG vs. glucose (1.6-fold), which was two orders of magnitude lower than definitive MLGUL genes (Table S1). The higher detection in uninduced cells is explained by the high basal expression of BACOVA_02738(GH3) (more than an order of magnitude greater than all MLGUL members; Table S1). The lack of detection in minimal medium containing

- bMLG is due to high amounts of induced MLGUL proteins diminishing the presence of the
- 258 BACOVA_02738(GH3) gene product when normalized to total protein (Fig. S2B).

259 Substrate product specificity of BoGH3_{MLG} and BACOVA_02738(GH3)

Initial activity screening on chromogenic *p*NP glycosides (see Experimental Procedures) revealed that both recBoGH3_{MLG} and recBACOVA_02738(GH3) are specific *exo*- β -glucosidases (activity on other *p*NP glycosides was undetectable at micromolar enzyme concentrations). However,

263 recBACOVA_02738(GH3) is strikingly feeble compared to recBoGH3_{MLG} on G- β -pNP (k_{cat}/K_m values of

264 0.084 mM⁻¹ s⁻¹ versus 20 mM⁻¹ s⁻¹; Fig. S4C, S4D, Table 1). Further, measuring Michealis-Menten

265 kinetic parameters on cello- and laminari-oligosaccharides was not feasible due to overall poor activity

and low production yields (data not shown). These kinetic results corroborate the above comparative

267 genetic and transcriptional analyses, collectively suggesting BACOVA_02738(GH3) is not integral to the

268 MLGUL. Hence, this enzyme was not characterized further.

269 To investigate oligosaccharide substrate preference of the $BoGH3_{MLG}$, we conducted initial-rate 270 kinetics analyses on a series of gluco-oligosaccharides of distinct linkage composition and degrees of 271 polymerization (d.p.). The non-reducing-end glucose was hydrolyzed from all- $\beta(1,4)$ -linked cello-272 oligosaccharides (d.p. 2-6), all- $\beta(1,3)$ -linked laminari-oligosaccharides (d.p. 2-5), and mixed-linkage 273 $\beta(1,3)/\beta(1,4)$ -gluco-oligosaccharides (d.p. 3-4, 5 examples) with comparable efficiencies, according to 274 classic Michaelis-Menten saturation kinetics (Fig. S4E, S4F; Table 1). In this series, only cellobiose (G4G) was poorly hydrolyzed by BoGH3_{MLG} vis-à-vis the $\beta(1,3)$ -linked congener laminaribiose (G3G) 275 276 and all other gluco-oligosaccharides (e.g., G4G has a k_{cat}/K_m value 20-fold lower than G3G, Table 1). The 277 $\beta(1,6)$ -linked diglucoside gentiobiose (G6G) was also a very poor substrate, with a $k_{\text{cat}}/K_{\text{m}}$ value 260-fold 278 lower than that of G3G. Gluco-oligosaccharides with a β (1,3)-linked glucosyl unit at the non-reducing 279 end all have slightly higher k_{cat} values compared to those with a $\beta(1,4)$ -linkage in this position, which 280 typically contributes to higher k_{cat}/K_m values for the former, when substrates of equal d.p. are compared.

However, the magnitude of these differences, which are often less than 2-fold, indicate that BoGH3_{MLG} is essentially agnostic to $\beta(1,3)$ versus $\beta(1,4)$ linkages. These results also suggest that in addition to a single negative subsite (-1) commensurate with its *exo*-activity, BoGH3_{MLG} has only two positive subsites that contribute to catalysis: in each gluco-oligosaccharide series, tetrasaccharides and larger are hydrolyzed with identical k_{cat}/K_m values to the corresponding trisaccharides.

Product analysis following extended incubation of BoGH3_{MLG} with G4G4G3G and G4G3G demonstrated that BoGH3_{MLG} completely degrades the BoGH16_{MLG} limit-digest products to glucose. HPLC also revealed that laminaribiose (G3G) is the only new intermediate formed during the course of hydrolysis (Fig. 3B). This demonstrates that BoGH3_{MLG} sequentially hydrolyzes one glucose unit at a time from the non-reducing end of MLG oligosaccharides, *viz*.: G4G4G3G \rightarrow G + G4G3G (also present in the starting mixture) \rightarrow G+ G3G \rightarrow G + G. Notably, the individual *k*_{cat} and *K*_m values for each step are nearly identical (Table 1).

293 BoGH3_{MLG} and BACOVA_02738(GH3) primary structures

294 Despite extensive efforts, we were unable to crystallize the key β -glucosidase BoGH3A_{MLG} for 295 experimental tertiary structure determination. However, $BoGH3_{MLG}$ has 63% sequence identity to a B. 296 ovatus β-glucosidase (BoGH3B, PDB code 5JP0 (Hemsworth et al., 2016)), from the xyloglucan utilization locus (Fig. S5A) and, as such, was amenable to tertiary structure homology modelling. Phyre2 297 298 (Kelley et al., 2015) utilized PDB code 5JP0 as the sole template and 728 out of 742 residues (98% of the 299 sequence, excluding the signal peptide) were modelled with 100 % confidence. The model suggests that 300 $BoGH3_{MLG}$ possesses a homologous three-domain architecture with the active site being formed at the 301 interface of the $(\alpha/\beta)_8$ TIM barrel and α/β sandwich domains (Fig. S5B). The predicted catalytic 302 nucleophile and acid/base residues are Asp-309 and Glu-453, respectively, based on primary and tertiary 303 alignment (Fig. S5A, S5C). Two tryptophan residues were modelled on opposite sides of the entrance to the active site pocket (Fig. S5D), forming a narrow "coin slot", which may effect a preference towards 304

 $\beta(1,3)$ - and $\beta(1,4)$ -linked glucans and poor activity against $\beta(1,6)$ -linked gentiobiose (Table 1). In

306 constrast, enzymes that lack a homologous Trp-453 have a more open entrance to the active site and show

broad activity against $\beta(1,2)$ - and $\beta(1,6)$ -linked glucans in addition to $\beta(1,3)$ - and $\beta(1,4)$ -linked glucans

308 (Karkehabadi et al., 2014; Pozzo et al., 2010).

In comparison, BACOVA_02738(GH3) possess catalytic residues homologous to BoGH3_{MLG} and similar GH3 members, despite having only 31% sequence identity to BoGH3_{MLG} (Fig. S5A). The most similar characterized GH3 member to BACOVA_02738(GH3) among ca. 300 members identified in the CAZy is a *Chrysosporium lucknowense* β -glucosidase with 39% sequence identity (Dotsenko et al., 2012).

314 Syntenic MLGUL are molecular markers of MLG utilization across the Bacteroidetes

Refined functional characterization of the catalytic specificity of GH components significantly increases confidence in the use of individual PULs as genetic markers of complex carbohydrate metabolism among Bacteroidetes (Cuskin et al., 2015; Larsbrink et al., 2014; Rogowski et al., 2015; Sonnenburg et al., 2010). The MLGUL characterized here represents the only PUL in *B. ovatus* that confers growth on MLG from cereals. To understand the wider distribution of MLG metabolic capacity among symbiotic Bacteroidetes in the human gut, we correlated the presence of a syntenic MLGUL with growth on bMLG for 354 individual Bacteroidetes strains representing 29 different species.

A total of 121 strains across just 7 of the species were able to grow on bMLG (Fig. 5). In particular, 33 of 33 *B. ovatus* strains (including the type strain ATCC 8483) grew well on bMLG, as well as 44 of 45 strains of the closely related *B. xylanisolvens*. The majority of *B. uniformis* strains tested (33 out of 35) were also competent bMLG utilizers. The limited penetrance of the MLGUL across the genus clearly demonstrates nutrient-niche specialization among individual *Bacteroides* species. Comparative analysis of available genomic sequences revealed that strains able to grow on

bMLG as the sole carbon source harbor a syntenic MLGUL (Fig. 1B). Previous transcriptional analysis

demonstrated that the syntenic MLGUL found in *B. cellulosilyticus* is also activated during growth on
bMLG (McNulty et al., 2013). Concordance between the presence of a syntenic MLGUL and the ability
to utilize MLG is further highlighted by the lack of a MLGUL in the *B. uniformis* ATCC 8492, one of
only two strains of *B. uniformis* that could not grow on bMLG. Based on this analysis, we can also predict
MLG utilization ability in two sequenced species of *Prevotella*, *Pr. copri* DSM 18205 and *Pr. multiformis*DSM16608, important members of the Bacteroidetes from the human gut and oral cavity, respectively
(Fig. 1B).

336 Discussion

337 A molecular model for MLG utilization by *B. ovatus*

Our current suite of data suggests a model by which the MLGUL gene products work in concert 338 339 to enable the utilization of MLG (Fig. 6), analogous to that of other PUL-encoded systems (Grondin et al., 340 2017). Thus, BoGH16_{MLG} is anchored to the outer membrane where it plays a leading role in fragmenting large MLG polysaccharide chains (typical d.p. 700 – 5000, depending upon the plant species of origin 341 (Lazaridou et al., 2004; Zheng et al., 2011)) into oligosaccharides that can be imported into the periplasm 342 343 via the TBDT. Notably, the specific limit-digest products of $BoGH16_{MLG}$ endo-hydrolysis identified here, 344 viz. the trisaccharide G4G3G and the tetrasaccharide G4G4G3G (Fig. 3B), have been shown previously to 345 bind the periplasmic sensor domain of the HTCS encoded by BACOVA 02740 ($K_{\rm D}$ 300 μ M and 400 μ M, 346 respectively), while the intact MLG polysaccharide does not (Martens et al., 2011). Monomeric glucose, all- $\beta(1,4)$ -linked cello-oligosaccharides, and all- $\beta(1,3)$ -linked laminari-oligosaccharides are also not 347 348 bound by the HTCS (Martens et al., 2011), indicating that the unique linkages present in MLG are central to inducing the MLGUL system. Thus, there is an essential, yet distant, interplay between the outer-349 membrane localized MLGase and the HTCS in specific nutrient sensing. 350 351 It is therefore likely that the BoGH16 $_{MLG}$ limit-digest products, or minimal repeats of these

352 structures [(G4G4G3G)_m(G4G3G)_n], comprise the main products transported through the TBDT *in vivo*.

353 Recent studies on inulin ($\beta(2,1)$ -fructan) utilization suggest that some TBDTs are able to import longer 354 polysaccharide chains (e.g. d.p. >20) (Rakoff-Nahoum et al., 2016). Regardless of length, the efficient 355 exo-hydrolytic activity of BoGH3_{MLG} in the periplasm is sufficient to completely saccharify all imported oligosaccharides to glucose (Fig. 3B), to feed primary metabolism in the cytosol. In this process, the 356 357 trisaccharide substrate of the HTCS, G4G3G, will always be generated regardless of the imported 358 saccharide chain length, ensuring continual production of the MLGUL up-regulation signal until substrate 359 is exhausted. Interestingly, $BoGH3_{MLG}$ will never encounter cellobiose (G4G), towards which it has 360 relatively weak activity (Fig. S4F; Table 1), in this process; the final step of saccharification of MLGOs is the hydrolysis of the competent substrate laminaribiose (G3G). 361

362 Structural enzymology reveals complex trajectories for the evolution of MLG activity in GH16

Previous phylogenetic analyses of GH16 have suggested that the evolution of the active-site βbulge motif EXDXXE, which is widespread among Clan GH-B (comprising GH16 and GH7), to a regular β-strand motif EXDXE is a defining feature that delineates *endo*- β (1,3)-glucanases (laminarinases, EC 3.2.1.39 and EC 3.2.1.6) from mixed-linkage *endo*- β (1,3)/ β (1,4)-glucanases (licheninases, EC 3.2.1.73), respectively (Barbeyron et al., 1998; Michel et al., 2001). In this context, the observation that BoGH16_{MLG} is highly specific for MLG, despite having a β-bulge motif, was surprising.

Using the CAZy Database as a starting point (http://www.cazy.org/GH16 characterized.html) 369 370 together with mining of the primary literature, we generated a contemporary maximum-likelihood 371 phylogeny of all biochemically characterized GH16 members (Fig. S6). This analysis indicates that 372 although canonical, normal β -strand MLGases do form a monophyletic group as previously observed, 373 MLGase activity is in fact widespread among the historical "laminarinase" group, in which BoGH16_{MLG} 374 is itself positioned. Despite currently limited and disparate kinetic data for individual enzymes, it also appears that it is not possible to define further substrate-specific clades within this group based on 375 376 molecular phylogeny alone, in light of weak bootstrap support. This precludes defining any single

evolutionary event giving rise to unique trajectories for the further diversification of extant laminarinases
and MLGases in this clade. Instead, it appears that diverse, subtle mutations have allowed the independent
evolution of predominant laminarinase or MLGase activity numerous times across a flat evolutionary
landscape. As such, we suggest that this GH16 subgroup should be more generally referred to as the
"laminarinase/MLGase group" going forward.

Detailed tertiary structural comparison of 10 β-bulge-containing members of this 382 383 laminarinase/MLGase group revealed, however, that predominant laminarinases harbor a significantly 384 more protruding loop (which is often, but not always, longer) connecting strands $\beta 2$ and $\beta 3$ than 385 predominant MLGases (Fig. S7A, S7B). Structural superposition with the BoGH16_{MLG}:G4G4G3G 386 complex indicates that this loop in predominant laminarinases would clash with MLG in the negative subsites, instead favoring binding of an all- $\beta(1,3)$ -glucan that curves away from this loop. Such curvature 387 is exemplified by the ZgLamC_{GH16-E142S}:thio- β -1,3-trisaccharide structure (Fig. S7A, PDB code 4CTE) 388 389 (Labourel et al., 2015). Indeed, Ilari et al. observed that shortening the homologous loop in LamA from 390 the archeon *Pyrococcus furiosus* (Fig. S7A, PDB code 2VY0) by 4 amino acids increased the activity 391 towards MLG by 10-fold (Ilari et al., 2009). Likewise, BglF from Nocardiopsis sp. F96 (Fig. S6B, PDB 392 code 2HYK) and LamR from *Rhodothermus marinus* (Fig. S7B, PDB code 3ILN), which have a 3.3- and 393 8.5-fold greater specificity constant and specific activity, respectively, toward MLG than laminarin, also 394 have a smaller loop, similar to BoGH16_{MLG}, in this position. The canonical, regular- β -strand MLGase 395 from Paenibacillus macerans (Fig. S7C, PDB code 1MAC) and Bacillus licheniformis (Fig. S7C, PDB 396 code 1GBG), similarly have a small loop at this position.

Taken together, these analyses reveal a complex evolutionary landscape that computational
phylogenetic analysis fails to resolve. Despite using a manually curated, structure-based sequence
alignment as input, the maximum-likelihood numerical approach did not delineate the members of the
laminarinase/MLGase group on the basis of the distinct active-site loop differences observed in tertiary

401 structures (Fig. S7). Instead, the phylogeny was likely obfuscated by diverse, random variations in amino
402 acid composition across the entire β-sandwich domain, which clearly limits large-scale, unsupervised
403 phylogenetic analysis of these GH16 members. Moreover, analysis of both MLG and laminarin
404 specificity (as a minimum) for individual members of this group, in light of their tertiary structures, is
405 essential to avoid potential mis-annotation of these enzymes.

406 Mining metagenomic data reveals the ubiquity of MLG utilization in the human gut and beyond

407 Using syntenic MLGULs as genetic markers, we surveyed the publicly available metagenome 408 data of 426 adults to understand the capacity of human populations to derive nutrition from cereal MLGs. 409 We were able to distinguish the species of origin based on nucleotide sequence except for MLGULs from 410 B. ovatus and B. xylanisolvens, which were strikingly similar at 97% nucleotide identity. The B. ovatus/B. 411 xylanisolvens and B. uniformis MLGULs are the most prevalent; both are observed in about 70% of the total human cohort (Fig. 7). The Pr. copri MLGUL is more often the sole MLGUL of an individual than 412 413 that of B. cellulosilyticus when only one is present (Fig. 7, cyan lines), despite the latter being more 414 frequent in total. Overall, 92.5% of the subjects harbor at least one of the five different MLGULs 415 identified in this study, irrespective of nationality or whether they have been diagnosed with IBD. MLGULs are ubiquitously detectable despite variability in sampling depth across different metagenomics 416 sequencing projects (Fig. 7). The prevalence of MLGULs across different nationalities is consistent with 417 418 MLG from cereal grains being a ubiquitous component of the human diet. Indeed, the importance of 419 MLG utilization is underscored by the upregulation of the MLGUL in the ceca of mice fed a complex 420 plant cell wall diet (Martens et al., 2011). Similar widespread global distribution in human populations 421 has been observed for xyloglucan utilization loci (Larsbrink et al., 2014). These observations are sharply 422 contrasted by the PUL that mediates utilization of the red algal polysaccharide porphyran, which is 423 essentially confined to subjects from Japan, where seaweed is a common part of the diet (Hehemann et 424 al., 2010; Larsbrink et al., 2014). Interestingly, we were unable to detect MLGULs in four unweaned

infants sampled in the Japanese metagenome project (data not included in our analysis of adult
metagenomes). This is consistent with a dearth of Bacteroidetes in infants who receive the bulk of their
nutrition from milk and are not yet consuming plant polysaccharides (Urokawa et al., 2007)
Moving beyond the human microbiota, we can likewise predict MLG utilization ability in *Dysgonomonas gadei* and *Pr. oryzae* (formerly *Xylanibacter oryzae*) based on the presence of a syntenic
MLGUL. These species are commonly found in the termite hindgut and decomposing rice straw,

431 respectively. This provides direct evidence that an analogous MLG utilization system is employed by

432 Bacteroidetes operating in environments beyond the human gut.

433 Conclusion

434 Complex carbohydrates that promote the growth of beneficial microbes in our distal large 435 intestine are a cornerstone of a healthy diet. MLGs in particular have long been known to impart healthful effects (Othman et al., 2011), yet its mechanism of utilization for fermentation by gut microbes was 436 437 unknown. Our work here sheds light on the fine-tuned mechanism that B. ovatus and other Bacteroidetes 438 has evolved to efficiently utilize MLGs in the highly competitive environment of the human gut 439 microbiota. The finding that a majority of humans possess microbes that can utilize this ubiquitous cereal polysaccharide highlights the relevance of potential therapeutic interventions based on MLG utilization to 440 the general population. The present study also sets the stage for future work to understand the 441 442 quantitative contributions of individual members of the microbiota and their cognate enzymes to MLG 443 utilization in the human gut (Patrascu et al., 2017; Zhong et al., 2015).

444 Experimental Procedures

445 Microbiology. *B. ovatus* gene deletions were constructed using allelic exchange as previously described
446 (Koropatkin et al., 2008). Anaerobic growth profiles were measured as previously described (Martens et

447 al., 2011). Details of localization analysis by immunofluorescence microscopy and immunoblotting are448 provided in the Supplemental Experimental Procedures.

Cloning, expression, and purification of recombinant enzymes. The genes encoding BoGH16_{MLG},
BoGH3_{MLG}, and BACOVA_02738(GH3) were cloned into expression vectors for recombinant production
in *E. coli*. Details of cloning strategies, production, and purification are provided in the Supplemental
Experimental Procedures.

453 Enzyme kinetics and product analysis. Thorough kinetic analysis was conducted on a panel of

454 polysaccharides, oligosaccharides, and chromogenic substrates. Products of enzymatic reactions were

analyzed by HPAEC-PAD and HILIC-MS. Details of enzymatic assays, analytical methods, as well as

456 sources of commercial substrates are provided in the Supplemental Experimental Procedures.

X-ray crystallography. Crystals of BoGH16_{MLG} were screened and optimized by sitting drop vapor

diffusion method. The structures of the apo- and G4G4G3G-BoGH16_{MLG} were solved by molecular

459 replacement. Details of crystallization, data collection, and structure solution are provided in the

460 Supplemental Experimental Procedures.

Bioinformatics. Phylogenetic anlaysis of select GH16 and GH3 sequences was conducted based on
structure-guided alignment. Metagenomic survey was carried out by nucleotide BLAST of MLGUL
sequences against various metagenome sequence data. Details are provided in the Supplemental
Experimental Procedures.

465 Statistical analysis. All kinetic assays were done in triplicate. Michaelis-Menten parameters are reported
466 as fitted values ± standard deviation throughout the article. All growth experiment results are averages of
467 two biological replicates.

468 Author contributions

469 KT cloned, expressed, and purified recombinant enzymes, conducted and analyzed kinetics for hydrolysis

470 of polysaccharides, oligosaccharides, and chromogenic substrates, determined hydrolysis products,

471 conducted phylogenetic and structural analyses, carried out metagenomics survey, and wrote the article.

472 GRH determined the crystal structures of apo- and G4G4G3G-complexed BoGH16_{MLG}. GD conducted

473 enzyme localization analyses. TR, NP, and KU conducted reverse genetics and growth analyses. NJ

474 synthesized G3G-CNP. GJD, ECM, and HB designed and directed research and co-wrote the article with

475 input from all authors.

476 Accession numbers

477 The atomic coordinates and structure factors of apo- and G4G4G3G-complexed BoGH16_{MLG} reported in 478 this paper can be accessed through the PDB with identifiers 5NBO and 5NBP, respectively.

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652

653 Figure Legends

654 Figure 1. Cereal mixed-linkage β(1,3)/β(1,4)-glucan (MLG) and MLG Utilization Locus (MLGUL)

55 structures. A: Chemical structure of MLG, consisting of a linear glucan chain of $\beta(1,4)$ -linked

cellotriosyl and cellotetraosyl units linked by $\beta(1,3)$ bonds. MLGs from various sources (barley, oat,

lichenin, etc) vary in the ratio of cellotriose to cellotetraose units (Lazaridou et al., 2004). Arrows indicate

the specific site of hydrolysis by the vanguard endo-glucanase of the MLGUL, BoGH16_{MLG}. B: Genetic

organization of the *B. ovatus* MLGUL and syntenic loci in select Bacteroidetes species. Homologous

genes are connected by colored bars and the locus tag of the TBDT of each syntenic MLGUL is given on

the right. See also Figure S1; Table S1.

Figure 2. Enzyme localization analysis. Phase contrast microscopy and corresponding fluorescence microscopy images of *B. ovatus* Δtdk cells grown in minimal medium with bMLG as the sole carbon source probed with custom polyclonal antibodies against recBoGH16_{MLG} (A) and recBoGH3_{MLG} (B). C: Western blots of protein collected from the culture supernatant, cell lysate supernatant, and cell lysate membrane fraction of *B. ovatus* Δtdk cells grown in minimal medium with glucose or bMLG as a sole carbon source. See also Figure S2.

668 Figure 3. BoGH16_{MLG} kinetics and MLGUL GHs product analysis. A: BoGH16_{MLG} initial-rate 669 kinetics curves fitted to the Michaelis-Menten equation for β -glucan polysaccharide substrates on which it 670 is active. Laminarin was reduced to laminaritol by sodium borohydride reduction to reduce background in the BCA assay. Curve fitting was done on OriginPro 2015 and error bars represent standard deviations 671 672 from the mean. B: Chromatograms of bMLG and its hydrolysis products by BoGH16_{MLG} and BoGH3_{MLG} 673 separated by HPAEC-PAD. Red: full length bMLG polysaccharide. Dark blue: reaction progress time 674 course and limit digest of bMLG hydrolysis by 10 nM BoGH16_{MLG}. Cyan: reaction progress time course 675 and limit digest of BoGH16_{MLG} products hydrolysis by 25 nM BoGH3_{MLG}. Standards are shown below in

black: solid lines are those corresponding to limit digest products and dotted line to intermediate products.
See also Figures S3, S4 and S5; Tables S2 and S3.

678 **Figure 4. BoGH16**_{MLG} structural biology. A: the overall structure of the BoGH16_{MLG}:G4G4G3G 679 asymmetric unit containing two polypeptide chains shown from orthogonal views with the bound 680 oligosaccharides in yellow and the transparent surface representation in white. Chain A cartoon is shown in cyan, and chain B cartoon is shown in slate blue throughout the figure. B: Mixed-linkage 681 682 tetrasaccharide ligand modelled into chain A of $BoGH16_{MLG}$ with the opaque surface representation in 683 gray and the oligosaccharide colored according to B-factors. The glucose in subsite -4 is outside of the 684 active site cleft and has significantly higher B factor than the glucose units in subsites -1 to -3. C: Tyr-181 685 rotamers observed in the complex structure with the 2Fo-Fc map of the tyrosines shown contoured at 0.5σ in grey. D: Tyr-181 residues observed in the apo structure with the 2Fo-Fc map of the tyrosines shown 686 687 contoured at 0.5σ in grey. E: Wall-eyed stero view of the active site of chain A of the 688 BoGH16_{MLG}:G4G4G3G complex. Hydrogen bonding interactions are shown as dashed black lines, sugars 689 are shown in yellow with its 2Fo-Fc map contoured at 1σ in orange, and the conserved GH16 active site 690 residues shown in purple. Hydrophobic stacking interactions in addition to hydrogen bonds position the 691 mixed-linkage oligosaccharide in the negative subsite of BoGH16_{MLG}. See also Figures S5 and S6; Tables 692 S4 and S5.

Figure 5. Penetrance map of MLG utilization ability across diverse human gut Bacteroidetes. The
phylogenetic tree was constructed from fully sequenced strains of the species shown. The number of
strains of each species tested for growth is depicted to scale as a black circle at each leaf. The number of

those strains that grew on bMLG as a sole carbon source is shown to scale in red within the black circle.

697 Figure 6. Model of mixed-linkage β-glucan saccharification by the concerted action of the MLGUL

698 **machinery.** Gene products are colored analogously to the gene locus in Fig. 1. The cell surface localized 699 endo-MLGase BoGH16_{MLG} cleave large mixed-linkage β -glucan polysaccharides into shorter fragments which are imported into the periplasm via the TonB dependent transporter, BoTBDT. This glycan capture and transport process at the cell surface is aided by the two surface glycan binding proteins BoSGBP-A and BoSGBP-B. The smaller mixed-linkage β -glucan fragments in the periplasm bind the sensor domain of the hybrid two-component sensor BoHTCS to induce upregulation of the system. Periplasmic exo- β glucosidases BoGH3_{MLG} and BACOVA_02738(GH3) act from the non-reducing ends to liberate individual glucose monomers which are imported into the cell and metabolized.

Figure 7. Bacteroidetes MLGULs from a survey of 426 adult human gut metagenomes. Vertical

707 lines represent the presence (cyan when unique, blue when one of multiple) or absence (black) of a

corresponding species-related MLGUL in a single individual. The total number of MLGULs observed in

an individual is shown in the bottom row, colored according to the legend in the top left corner. The

frequency of MLGUL occurrence across all 426 individuals is shown on the right. Variation in

sequencing depth in megabase pair is illustrated in the graph below: grey lines show the depth for

712 individual subjects and black lines show the average depth of each metagenomics project.

713

714 Table

715 Table 1.

Enzyme	Substrate	kcat (s ⁻¹)	K _m (mM)	$k_{\rm cat}/K_{\rm m}~({\rm s}^{-1}~{ m mM}^{-1})$	Assay
BoGH3 _{MLG}	β-Glc- <i>p</i> NP	59.5 ± 1.46	2.95 ± 0.14	20.2	pNP
	gentiobiose (G6G)	ND	ND	0.0571	HK/G6PDH
	cellobiose	5.52 ± 0.19	7.47 ± 0.48	0.739	HK/G6PDH
	cellotriose	22.1 ± 0.3	0.859 ± 0.033	25.7	HK/G6PDH
	cellotetraose	17.3 ± 0.5	0.687 ± 0.044	25.2	HK/G6PDH
	cellopentaose	19.4 ± 0.8	0.777 ± 0.060	25.0	HK/G6PDH
	cellohexaose	17.4 ± 0.4	0.747 ± 0.041	23.3	HK/G6PDH
	laminaribiose	28.0 ± 1.1	1.90 ± 0.12	14.7	HK/G6PDH
	laminaritriose	34.2 ± 1.0	0.911 ± 0.052	37.5	HK/G6PDH
	laminaritetraose	31.3 ± 2.3	0.898 ± 0.135	34.9	HK/G6PDH
	laminaripentaose	39.5 ± 3.4	1.27 ± 0.20	31.1	HK/G6PDH
	MLGO ₃ A (G3G4G)	61.6 ± 1.6	0.997 ± 0.040	61.8	HK/G6PDH
	MLGO ₃ B (G4G3G)	24.7 ± 1.3	0.521 ± 0.064	47.4	HK/G6PDH
	MLGO4 A (G3G4G4G)	55.7 ± 2.7	1.33 ± 0.12	41.9	HK/G6PDH
	MLGO4 B (G4G4G3G)	30.8 ± 2.0	0.736 ± 0.106	41.8	HK/G6PDH
	MLGO ₄ C (G4G3G4G)	15.7 ± 0.3	0.601 ± 0.031	26.1	HK/G6PDH
BACOVA_02738 (GH3)	β-Glc- <i>p</i> NP	0.212 ± 0.004	2.53 ± 0.13	0.0838	pNP

ND: not determined (in cases where Michealis-Menten curve fitting was not feasible, individual k_{cat} and K_m values

717 are not reported and k_{cat}/K_m value was determined from linear curve fit to initial-rate data in the [S] << $K_{m(apparent)}$

range). Data are represented as the fitted parameters \pm standard deviation. Highlighted in bold are the biologically

relevant substrates that $BoGH3_{MLG}$ encounters in the periplasmic space. See also Figure S4.

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Β



TBDT

SGBP-A

SGBP-B

GH3

HTCS

GH16

TBDT locus tag:

XylorDRAFT_1481





 \cap

Culture supernatant

Lysate supernatant

Total membranes

Culture supernatant

Lysate supernatant

Total membranes

Purified recombinant protein



Β

















Supplemental Information





Figure S1, related to figure 1. Growth profile of *B. ovatus* strains.

B. ovatus Δtdk (wt) and *B. ovatus* $\Delta MLGUL$ (whole-PUL knockout) were cultured in minimal medium containing either glucose or bMLG as the sole carbon source (average of n = 2 growths per strain).



Minimal medium - glucose

B. ovatus ZMLGUL Minimal medium - glucose

Figure S2, related to Figure 2. Enzyme localization analysis.

A: phase contrast and corresponding fluorescence microscope images of *B. ovatus* Δ tdk cells grown in minimal medium with bMLG as the sole carbon source probed with custom polyclonal antibodies against recBACOVA_02738 (GH3). B: Western blot of protein collected from the culture supernatant, cell lysate supernatant, and cell lysate membrane fraction of *B. ovatus* Δ tdk cells grown in minimal medium with glucose or bMLG as a sole carbon source. Phase contrast and corresponding fluorescence microscope images of wild type *B. ovatus* cells grown in minimal medium with glucose as the sole carbon source probed with custom polyclonal antibodies against recBoGH16_{MLG} (C), recBoGH3_{MLG} (D), and recBACOVA_02738(GH3) (E). Phase contrast and corresponding fluorescence microscope images of *B. ovatus* Δ MLGUL cells grown in minimal medium with glucose as the sole carbon source probed with custom polyclonal antibodies against recBoGH16_{MLG} (F), recBoGH3_{MLG} (G), and recBACOVA_02738(GH3) (H).



Figure S3, related to Figure 3. Purity and molecular mass of recombinant MLGUL proteins.

A: SDS-PAGE of recBoGH16_{MLG}, recBoGH3_{MLG}, and recBACOVA_02738(GH3). B: Reconstructed mass spectrum of intact recBoGH16_{MLG}. The second peak at +178.2 Da from the main peak is a species that has been spontaneously phosphogluconoylated at the N-terminal his-tag during production in *E. coli* (Geoghegan et al., 1999). C: Reconstructed mass spectrum of intact recBoGH3_{MLG}. D: Reconstructed mass spectrum of intact recBACO-VA_02738(GH3).



Figure S4, related to Figure 3. Initial-rate kinetic analysis of MLGUL GHs.

A: BoGH16_{MLG} against G3G- β -CNP and G3G- β -pNP fitted to a linear equation. B: BoGH16_{MLG} against G4G3G- β -CNP and G4G4G3G- β -CNP fitted to the Michealis-Menten equation. C: BoGH3_{MLG} against glucose- β -pNP fitted to the Michealis-Menten equation. D: BACOVA_02738(GH3) against glucose- β -pNP fitted to the Michealis-Menten equation. E: BoGH3_{MLG} against oligosaccharides with a $\beta(1,3)$ bond at the non-reducing end. D: BoGH3_{MLG} with oligosaccharides with a $\beta(1,4)$ bond at the non-reducing end. Curve fitting was done on OriginPro 2015 and error bars represent standard deviations from the mean.









Figure S5, related to Figure 3. Primary and tertiary structure analysis of BoGH3_{MLG}.

A: Structure-based sequence aligmment of BoGH3_{MLG} and BACOVA_02738(GH3) with structurally characterized GH3 β -glucosidases. Portions of the alignment have been removed for brevity and breaks are indicated by double hash lines. Red highlights indicate invariant positions, blue outlines indicate similar positions and green arrows indicate catalytic residues. Alignment illustration created with ESPript (Gouet et al., 2003). B: Homology model of BoGH3_{MLG} generated by Phyre2. The (α/β)₈ TIM barrel (blue) is connected by a linker (teal) to a central α/β sandwich (orange), in turn connected by a linker (pink) to a C-terminal fibronectin type-III (FN-III) domain (red). The black box indicates the location of the active site. C: Catalytic site of BoGH3_{MLG} with a bound glucose from the XyGUL BoGH3B overlay in the active site pocket. The catalytic nucleophile (Asp-309) and catalytic acid/base (Glu-527) are shown as sticks. D: Surface representation of the entrance to the BoGH3_{MLG} active site with the two tryptophan residues that line the positive subsite shown in white.



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Figure S6, related to Figure 4. Phylogenetic tree of characterized Glycoside Hydrolase Family 16 sequences.

Clades distinguishing major substrate specificities (see https://www.cazypedia.org/index.php/GH16) are represented by at least 5 members. Leaf names contain GenBank accession number, enzyme name, organism of origin, and a PDB code where available. Bootstrap values are shown for each node. The dotted horizontal line separates GH Family 16 enzymes with active-site residues on a regular β -strand from those with a bulged β -strand. The clade highlighted with a blue background is traditionally referred to as a "laminarinase" (EC 3.2.1.39) group, yet our current literature analysis also indicates the presence of predominant MLGases (EC 3.2.1.6). Where known, biochemically determined activities are shown next to select sequences, with the predominant activity displayed in bold type; (++) indicates better activity; (+) indicates poorer activity, (0) indicates no activity, and (?) indicates that specific activity data is unavailable. Reported activities are based on the following references, A: (Labourel et al., 2014), B: (Kitamura et al., 2002), C: (Kobayashi et al., 2016), D: (Hong et al., 2008), E: (Li et al., 2015), F: (Masuda et al., 2006), G: (Krah et al., 1998), H: (Ilari et al., 2009), I: (Fuchs et al., 2003), J: (Zverlov et al., 1997), K: (Gorlach et al., 1998), L: (Nakajima et al., 2012), M: (Kawai et al., 2006), N: (Labourel et al., 2015), O: (Hong and Meng, 2003), P: (Pauchet et al., 2009), Q: (Genta et al., 2009), R: (Kumagai and Ojima, 2009), S: (Kovalchuk et al., 2006)



Figure S7, related to Figure 4. BoGH16_{MLG} loop comparison with laminarinases and MLGases.

BoGH16_{MLG} (cyan) is structurally aligned with all 10 available β -bulge-containing laminarinases/MLGases and two representative regular β -stranded canonical lichenases (white); PDB ID of the compared structure shown below each alignment. The BoGH16_{MLG} loop is colored blue and the loop of the compared structure is colored orange. The BoGH16_{MLG} catalytic residues and mixed-linkage oligosaccharide G4G4G3G in complex with BoGH16_{MLG} are also shown in cyan. The thio- β -1,3-trisaccharide in complex with ZgLamC_{GH16-E142S} (PDB code 4CTE) is shown in green A: β -bulge-containing laminarinases, B: β -bulge-containing MLGases, C: regular β -stranded MLGases (canonical lichenases).

Supplemental Tables

Locus tag	Putative protein ID	bMLG	MM-Glc	bMLG/MM-Glc
BACOVA_02736		879.4 ± 29.4	511.6 ± 94.3	1.7
BACOVA_02737		1072.3 ± 23.0	590.4 ± 147.6	1.8
BACOVA_02738	GH3	355.7 ± 46.9	229.0 ± 55.5	1.6
BACOVA_02739	Sigma 70, region 4	22.7 ± 0.2	37.2 ± 8.9	0.6
BACOVA_02740	HTCS	$\textbf{376.8} \pm \textbf{4.4}$	197.6 ± 0.1	1.9
BACOVA_02741	GH16 _{MLG}	$\textbf{3657.6} \pm \textbf{77.7}$	16.0 ± 16.5	228.8
BACOVA_02742	TBDT	3401.1 ± 375.5	13.1 ± 5.7	259.7
BACOVA_02743	SGBP-A	1837.5 ± 111.1	14.7 ± 6.3	124.9
BACOVA_02744	SGBP-B	$\textbf{2748.9} \pm \textbf{103.9}$	9.2 ± 1.0	298.3
BACOVA_02745	GH3 _{MLG}	2988.3 ± 53.7	16.5 ± 0.4	180.7
BACOVA_02746 ^b	Transposase	2763.9 ± 111.5	11.9 ± 9.7	232.8
BACOVA_02747 ^b	Helicase	2821.0 ± 91.9	16.8 ± 5.8	167.6
BACOVA_02748		54.5 ± 10.5	25.8 ± 9.1	2.1
BACOVA_02749		62.3 ± 6.0	30.1 ± 6.8	2.1

Table S1, related to Figure 1. Transcriptional expression of MLGUL and neighboring genes.^a

^a Microarray results of upregulation under bMLG induction normalized to a glucose background are shown with MLGUL genes in bold type. Values reported as averages and standard deviations of two biological replicates. Data are from (Martens et al., 2011).

^b Loci BACOVA_02746 and BACOVA_02747 are predicted to encoded proteins of only 63 and 44 amino acids, respectively, and were therefore likely to have been originally mis-annotated based on limited regional sequence similarity. Despite apparently high transcript levels, which may result from read-through downstream of highly active operons whether there is protein coding function or not, these loci are not considered to be part of the MLGUL.

Substrate	$k_{\rm cat}~({\rm s}^{-1})$	$K_{\rm m} ({\rm mg} {\rm mL}^{-1})$	$k \operatorname{cat}/K_{\mathrm{m}}$ (s ⁻¹ mg ⁻¹ mL)	Assay
barley MLG	85.8 ± 4.6	0.364 ± 0.051	238	BCA
laminarin	18.1 ± 5.25	2.54 ± 0.97	7.12	BCA
yeast β-glucan	0.875 ± 0.101	0.541 ± 0.169	1.61	BCA
curdlan	ND	ND	0.042	BCA

Table S2, related to Figure 3. Kinetic parameters of BoGH16_{MLG} on polysaccharide substrates.

Data is only presented for substrates on which BoGH16_{MLG} showed activity (no detectable activity on tamarind xyloglucan, beechwood xylan, wheat arabinoxylan, carob galactomannan, konjac glucomannan, carboxymethyl cellulose, hydroxyethylcellulose, xanthan gum, and ulvan as determined by BCA and HPLC analyses). ND: not determined (in cases where Michealis-Menten curve fitting was not feasible, individual k_{cat} and K_m values are not reported and k_{cat}/K_m value was determined from linear curve fit to initial rate data in the [S] $\leq K_{m(apparent)}$ range). Data are represented as curve fit parameters \pm standard deviation.

Substrate	$k_{\rm cat}~({\rm s}^{-1})$	$K_{\rm m}$ (mM)	$k \operatorname{cat}/K_{\mathrm{m}} (\mathrm{s}^{-1} \mathrm{m} \mathrm{M}^{-1})$	Assay
G-pNP	NA	NA	NA	pNP
G-CNP	NA	NA	NA	CNP
G4G-CNP	NA	NA	NA	CNP
G4G4G-CNP	NA	NA	NA	CNP
G3G-pNP	ND	ND	0.0298	pNP
G3G-CNP	ND	ND	0.768	CNP
G4G3G-CNP	82.3 ± 3.8	0.134 ± 0.018	614	CNP
G4G4G3G-CNP	103.7 ± 3.4	0.0895 ± 0.0097	1160	CNP

Table S3, related to Figure 3. Kinetic parameters of BoGH16_{MLG} on chromogenic substrates.

NA: no detectable activity. ND: not determined (in cases where Michealis-Menten curve fitting was not feasible, individual k_{cat} and K_m values are not reported and k_{cat}/K_m value was determined from linear curve fit to initial rate data in the [S] $\leq K_{m(apparent)}$ range). Data are represented as curve fit parameters \pm standard deviation.

	Apo-BoGH16 _{MLG} (5NBO)	G4G4G3G-BoGH16 _{MLG}
Data collection		
Space group	C2	C2
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	167.5, 60.8, 49.4	167.3, 60.2 49.7
α, β, γ (°)	90.0, 94.5, 90.0	90.0, 93.5, 90.0
Wavelength (Å)	0.976	0.979
Resolution (Å)	49.23-1.80 (1.84-1.80)	49.57-1.80 (1.84-1.80)
R _{merge}	0.067 (0.446)	0.094 (0.735)
Ι/σΙ	10.0 (2.0)	10.8 (1.7)
Completeness (%)	98.9 (99.9)	99.0 (99.0)
Redundancy	3.0 (3.0)	3.9 (3.9)
Half-set correlation CC(1/2)	0.996 (0.669)	0.994 (0.516)
R _{p.i.m.}	0.053 (0.365)	0.066 (0.499)
Refinement		
Resolution (Å)	49.23-1.80	49.57-1.80
No. reflections (Work/Free)	43,072/2,392	45,346/2,384
$R_{\rm work}$ / $R_{\rm free}$	0.161/0.204	0.175/0.212
No. atoms		
Protein	3,766	3,767
Ligand/solvent/ion	8	93
Water	353	262
Average B-factors (Å ²)		
Protein	18.6	21.6
Ligand/ion	25.4	29.9
Water	29.2	28.6
R.m.s deviations		
Bond lengths (Å)	0.018	0.013
Bond angles (°)	1.83	1.62

Table S4, related to Figure 4. Data collection and refinement statistics for $BoGH16_{MLG}$ structures.

Values in parentheses represent data in the highest resolution shell.

PDB ID	Organism	Protein name	Z score	RMSD (Å)	% ID	Active site	Predominant activity
4CTE	Zobellia galac- tanivorans	ZgLamC	29.3	2.0	38	β-bulge	laminarinase ^a
2НҮК	Nocardiopsis sp. F96	BglF	28.8	1.9	32	β-bulge	MLGase ^b
3ATG	Cellulosimicrobium cellulans	BglII	28.3	1.9	34	β-bulge	laminarinase ^c
3AZY	Thermotoga mariti- ma	Lam16	27.8	1.9	32	β-bulge	laminarinase ^d
2VY0	Pyrococcus furiosus	LamA	27.3	1.8	31	β-bulge	laminarinase ^e
4DFS	Thermotoga petrophila	TpLam	27.3	2.1	31	β-bulge	laminarinase ^f
4BOW	Zobellia galac- tanivorans	ZgLamA	26.9	2.0	30	β-bulge	laminarinase ^g
3ILN	Rhodothermus mari- nus	LamR	26.4	2.2	29	β-bulge	MLGase ^h
1MAC	Paenibacillus mac- erans	Bgi	25.1	2.0	22	regular β- strand	MLGase ⁱ
3DGT	Streptomyces sioyaensis	Curd1	25.0	2.4	28	β-bulge	laminarinase ^j
1GBG	Bacillus licheni- formis	Bg1	25.0	2.1	23	regular β- strand	MLGase ^k
3058	Bacillus subtilis	BglS	25.0	2.0	24	regular β- strand	MLGase ¹

Table S5, related to Figure 4. Dali search results.

- ^a (Labourel et al., 2014) ^b (Masuda et al., 2006) ^c (Ferrer, 2006) ^d (Jeng et al., 2011) ^e (Ilari et al., 2009) ^f (Cota et al., 2011) ^g (Labourel et al., 2015) ^h (Krah et al., 1998) ⁱ (Hahn et al., 1995a) ^j (Hong et al., 2008) ^k (Hahn et al., 1995b) ¹ (Furtado et al., 2011)

Supplemental Experimental Procedures

Microbiology

Bacteroidetes reverse genetics and growth analysis

Flat bottom 96-well plates (Costar) were loaded with 100 μ L of sterilized carbohydrate stock at 2× concentration. A 24-hour culture was centrifuged to pellet bacteria, resuspended in 2× MM-no carbohydrate (MM-NC) and used to inoculate MM-NC at a ratio of 1:50. Each carbohydrate array was loaded with 100 μ L of the inoculated 2× medium to produce 200 μ L cultures at a final bacteria ratio of 1:100. Assay plates were sealed with an optically clear gas-permeable polyurethane membrane (Diversified Biotech, Boston, MA) in an anaerobic chamber (Coy manufacturing, Grass Lake, MI). Plates were loaded into a Biostack automated plate handling device coupled to a Powerwave HT absorbance reader (both devices from Biotek Instruments, Winooski, VT). Absorbance at 600 nm (A_{600}) was measured for each well at 10–15 minute intervals.

Enzyme localization

Immunofluorescence microscopy.

Fluorescence microscopy was performed on fixed *Bacteroides ovatus Atdk* and *AMLGUL* cells. The cells were grown to mid-exponential phase (A₆₀₀ = 0.5-0.6) in Minimal Media (MM) with bMLG or glucose (0.5% w/v) as the sole carbon source. The cultures were then pelleted, and washed with phosphate-buffered saline (PBS). The cells were then fixed by incubation in formalin (4.5% formaldehyde in PBS) for 1.5 h at room temperature, washed with PBS, and blocked for 16 hours at 4 °C in blocking solution (2% goat serum (Sigma-Aldrich), 0.02% NaN₃, PBS). The cells were then incubated with individual polyclonal antibodies raised against recBoGH16_{MLG}, recBoGH3_{MLG}, and recBACOVA_02738(GH3) (Cedarlane Laboratories, Burlington, ON) for 2 hours at room temperature (1:500 dilution of the antibody in blocking solution). For secondary labelling, cells were pelleted, washed three times in 1 mL of PBS and resuspended in 0.4 mL goat anti-rabbit IgG Alexa-Fluor 488 (Thermo Fisher Scientific), diluted 1:500 in blocking solution, and incubated 1 hour at room temperature in the dark. The cells were then washed three more times and resuspended in 0.05 mL of PBS containing ProLong Gold Antifade (Thermo Fisher Scientific). Cells were mounted on agarose pads on glass slides and capped with coverslips. Fluorescence was imaged on an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan) at 100 X magnification.

Immunoblotting analysis.

Bacteroides ovatus Δtdk cells were grown as described above in MM on bMLG (0.5% w/v) or glucose (0.5% w/v) as a sole carbon source. The cells were then centrifuged at 10000g for 45 minutes, resuspended in Trisbuffered saline (TBS), and lysed. After cell disruption, the membranes and cell debris were harvested by centrifuga-

tion for 1 hour at 42000 rpm (TLA 100.3 Beckman) at 4 °C. To prepare the total membrane fraction, the pellet was resuspended in 60 mM of n-octyl β -D-glucopyranoside, agitated for 1 hour at room temperature and centrifuged at 35000 rpm for 45 minutes at 4 °C. The supernatant was then harvested for further analysis.

The appropriate dilution of the culture supernatant, the lysate supernatant, and the total membrane fraction were added to 4X Laemmli buffer, boiled for 10 minutes, and run on an SDS polyacrylamide gel (Mini-PROTEAN® TGXTM gels, Bio-Rad). Transfer to a western blot Polyvinylidene difluoride (PVDF) membrane (Immobilon®-P) was performed for 45 minutes at 20 volts using a semi-dry transfer cells (Trans-Blot SD, Bio-Rad). The membranes were then blocked for 1 hour at room temperature with blocking buffer (5% milk in TBST buffer (Tris-Buffered Sa-line (TBS) with 0.1% Tween20)). The membranes were then washed three times with TBST buffer and the proteins of interest were revealed by incubation with the primary antibodies generated for BoGH16_{MLG}, BoGH3_{MLG}, and BA-COVA_02738(GH3), diluted in blocking buffer (1:15000, 1:20000, and 1:15000 dilution respectively). After three more washes, the membranes were incubated for 1 hour at room temperature with the secondary antibody goat antirabbit IgG H&L (Alkaline Phosphatase; Abcam), diluted 1:25000 in blocking buffer solution. The membranes were then washed another three times and the immunodetection of the alkaline phosphatase enzyme on the membrane was revealed with Novex® AP Chromogenic Substrate (ThermoFisher Scientific).

Cloning, expression, and purification of recombinant enzymes

Gene sequences were obtained from the *B. ovatus* ATCC 8483 draft genome available on the Integrated Microbial Genomes database from the Joint Genome Institute. PCR primers were synthesized by Integrated DNA technologies.

Cloning

Open reading frames encoding BACOVA_02738, BACOVA_02742, and BACOVA_02745 were amplified by PCR using Q5 high fidelity polymerase (NEB) with appropriate primers (see below) and genomic *B. ovatus* DNA as template. All primers were designed to amplify constructs truncated to exclude predicted signal peptides (prediction by SignalP 4.1) and N-terminal lipidation cysteine residues (prediction by LipoP 1.0). NdeI and XhoI restriction sites were included in the forward and reverse primers of BACOVA_02742 for subsequent digestion (NdeI and XhoI from NEB) and ligation (T4 ligase from Thermo Scientific) into the pET28 vector. pMCSG complementary sequences were included in the forward and reverse primers of BACOVA_02738 and BACOVA_02745 for subsequent ligation independent cloning into pMCSG53 plasmids as per Eschenfeldt *et al.* (Eschenfeldt, William H., Stols, Lucy, Millard, Cynthia Sanville, Joachimiak, Andrzej, Donnelly, 2009). All three constructs were designed to harbor an N-terminal His₆-tag fusion in the translated recombinant peptide. Successful cloning was confirmed by colony PCR (GoTaq polymerase from Promega) and sequencing (Genewiz).

List of primers used for cloning.

Primer Name	Primer sequence $(5' \rightarrow 3')$	Vector
BoGH16 _{MLG} _F	GACGAC <u>CATATG</u> TCGGATTCTGTTGGAACG	pET28
BoGH16 _{MLG} _R	GACGAC <u>CTCGAG</u> CTATAATATTTTCACCCA	pET28
BoGH3 _{MLG} _F	TACTTCCAATCCAATGCCATGGTTCCCACTGCCATTCCTGAA	pMCSG53
BoGH3 _{MLG} _R	TTATCCACTTCCAATGTTATTACTTGCATATAATATTCAGTGTTTGA	pMCSG53
BACOVA_02738_F	TACTTCCAATCCAATGCCATGAACAACAAACCTACTGATAACA	pMCSG53
BACOVA_02738_R	TTATCCACTTCCAATGTTATTGGACCTCAAAACTCCCCT	pMCSG53

Restriction sites are underlined and pMCSG LIC vector complementary sequences are double underlined.

Expression

Plasmids harboring the gene of interest were transformed into chemically competent *E. coli* BL21 (DE3) and cultured in lysogeny broth (LB) containing 50 μ g/mL kanamycin for BACOVA_02742 or 100 μ g/mL ampicillin for BACOVA_02738 and BACOVA_02745. Cells were grown on a large scale at 37 ° C until mid-logarithmic growth phase was reached (A₆₀₀ = 0.4-0.6) at which point protein expression was induced by addition of isopropyl β -D-thioglactopyranoside to a final concentration of 0.5 mM and temperature was lowered to 16 ° C. Induction of recombinant protein production continued overnight after which the cells were collected by centrifugation at 4000 g for 20 minutes.

Purification

The harvested cell pellet was resuspended in binding buffer (20 mM sodium phosphate pH 7.4, 500 mM sodium chloride, 20 mM imidazole) and lysed using a Sonic Dismembrator F550 Ultrasonic Homogenizer (Fisher Scientific). Cell debris was pelleted by centrifugation at 15000 rpm for 45 minutes and the supernatant was loaded onto a 2×1 mL HisTrap IMAC FF nickel-nitrilotriacetic acid column (GE Healthcare), using a BioLogic FPLC system (BioRad). After washing with 10 column volumes of binding buffer, His₆-tagged protein was eluted using a linear gradient of 0 - 100% elution buffer (20 mM sodium phosphate pH 7.4, 500 mM sodium chloride, 500mM imidazole) over 10 column volumes. Fractions were monitored by A₂₈₀ and eluted protein fractions were pooled and buffer

exchanged into 50 mM sodium phosphate pH 7.0 using Vivaspin centrifugal filters (GE Healthcare). After concentrating, aliquots were flash frozen in liquid nitrogen and stored at -80 ° C. Protein purity was determined by SDS-PAGE analysis and mass was confirmed by intact protein mass spectrometry on a Waters Xevo Q-TOF with nanoACQUITY UPLC system, as described previously (Sundqvist et al., 2007). Protein concentrations were determined by spectrophotometry on an Epoch Microplate Spectrophotometer (BioTek) using the following molar extinction coefficients, which were calculated using ProtParam tool from the ExPASy Bioinformatics Resource Portal (Gasteiger et al., 2005): 108555 M⁻¹cm⁻¹ for BACOVA_02738, 54890 M⁻¹cm⁻¹ for BACOVA_02742, and 108180 M⁻¹ cm⁻¹ for BACOVA_02745.

Typical yields after purification were 80 mg of $recBoGH16_{MLG}$ from 1 L of lysogeny broth (LB) culture, 70 mg of $recBoGH3_{MLG}$ from 1 L of LB culture, and 8 mg of $recBACOVA_02738(GH3)$ from 1 L terrific broth culture (Fig. S3).

Enzyme kinetics and product analysis

Substrates and polysaccharides

Polysaccharides

Beta-glucan (barley) high viscosity (bMLG), yeast beta-glucan, curdlan, tamarind xyloglucan, konjac glucomannan, carob galactomannan, wheat arabinoxylan, beechwood xylan were purchased from Megazyme International (Bray, Ireland). Laminarin (from *Laminaria digitata*) was purchased from Sigma Aldrich (St. Louis, MO, USA). Carboxymethyl cellulose was purchased from Acros Organics (Morris Plains, NJ, USA). Hydroxyethyl cellulose was purchased from Amresco (Solon, OH, USA). Xanthan gum was purchased from Spectrum (New Brunswick, NJ, USA). Ulvan (from Ulva sp.) was purchased from Elicityl (Crolles, France). Laminarin was reduced to laminaritol as described previously (Abdel-Akher and Smith, 1951).

Oligosaccharides

Cellobiose (G4G) was purchased from Acros Organics. Cellotriose (G4G4G), cellotetraose (G4G4G4G4G), cellopentaose (G4G4G4G4G), cellohexaose (G4G4G4G4G4G), laminaribiose (G3G), laminaritriose (G3G3G), laminaritetraose (G3G3G3G), laminaripentaose (G3G3G3G3G), mixed-linkage glucotriose A (G3G4G), mixed-linkage glucotetraose B (G4G3G), mixed-linkage glucotetraose A (G3G4G4G), mixed-linkage glucotetraose B (G4G4G3G), mixed-linkage glucotetraose C (G4G3G4G) were purchased from Megazyme. Gentiobiose (G6G) was purchased from Carbosynth (Compton, UK).

Chromogenic substrates

para-nitrophenyl (*p*NP) glycosides of β-glucoside (G-β-*p*NP), α-glucoside, β-galactoside, β-mannoside, and β-xyloside were purchased from Sigma Aldrich. *ortho*-chloro-*para*-nitrophenyl (CNP) glycosides of G4G3G (G4G3G-CNP) and G4G4G3G (G4G4G3G-CNP), and *p*NP β-laminaribioside (G3G-*p*NP) were purchased from Megazyme. CNP glycosides of cellobioside (G4G-CNP) and that of cellotriose (G4G4G-CNP) were purchased from Carbosynth. G3G-CNP was synthesized by glycosylation of the known α-laminaribiosyl bromide (Viladot et al., 1997) and the corresponding phenol under phase-transfer conditions (Ibatullin et al., 2008; Viladot et al., 1997), the details of which will be published elsewhere.

Enzyme kinetics

BCA endpoint assay

Polysaccharide hydrolysis was quantified using the bicinchoninic acid (BCA) reducing sugar assay. All reactions were carried out in 100 μ L volumes in the optimum pH buffer (50 mM sodium citrate pH 6.5 for BoGH16_{MLG}) at 37 ° C unless otherwise specified. Reactions were initiated by adding 10 μ L of enzyme solution to 90 μ L of the remaining assay mixture, which had been pre-incubated at 37 ° C. Reactions were terminated by addition of equal volume (100 μ L) of BCA reagent and developing the color by boiling at 80 ° C for 20 minutes. Absorbance at 563 nm (A₅₆₃) was measured in 96-well plates on an Epoch Microplate Spectrophotometer (BioTek). Blank absorbance readings were determined for each polysaccharide at each concentration by using inactivated enzyme (denatured by boiling at 100 ° C for 10 minutes). Reducing ends released were quantified with a glucose standard curve (25 – 150 μ M). All kinetic assays were conducted in technical triplicates.

Activity on a library of polysaccharides was initially screened by incubating 10 μ M BoGH16_{MLG} with 1.0 mg/mL substrate for 24 hours. The polysaccharide was determined to be a substrate for BoGH16_{MLG} if the A₅₆₃ increased significantly compared to the blank.

The pH optimum of $BoGH16_{MLG}$ was determined by incubating 7.5 nM enzyme with 1.0 mg/mL bMLG for 10 minutes in different buffers at 50 mM: sodium citrate (pH 3.0 – 6.5), sodium phosphate (pH 6.5 – 8.5), glycylgly-cine (pH 7.5 – 9.0), glycine (pH 9.0 – 10.5). Released reducing ends were measured as described above.

The temperature optimum of $BoGH16_{MLG}$ was determined by incubating 7.5 nM enzyme with 1.0 mg/mL bMLG for 10 minutes at various temperatures ranging from 30 to 70 ° C. Released reducing ends were measured as described above.

For initial-rate saturation kinetics, the following concentrations of enzyme were used: 4.9 nM for bMLG, 48.6 nM for laminarin, 485.6 nM for yeast beta-glucan, and 4.9 μ M for curdlan. These are the concentration that were optimized for the reaction to be in the initial, linear stage of the reaction (less than 10% conversion) after 12 minutes of hydrolysis. To determine Michaelis-Menten parameters, eight different concentrations of each substrate were hydrolyzed by appropriate concentration of enzyme for 10 minutes after which the reaction was quenched and reducing ends released were quantified as described above.

Chromogenic substrate assay

Reaction with *p*NP and CNP glycoside substrates was used to quantify the hydrolysis of chromophore from the aglycone. Enzyme concentrations used to maintain initial-rate conditions were 6.9 nM for BoGH3_{MLG}, 941 nM for BACOVA_02738(GH3), 9.4 nM for BoGH16_{MLG} against the G4G3G-CNP and G4G4G3G-CNP, 446 nM for BoGH16_{MLG} against G3G-CNP, and 942 nM for BoGH16_{MLG} against G3G-*p*NP.

Endpoint assays were used for pH and temperature optima of BoGH3_{MLG} and BACOVA_02738(GH3). Enzyme, 1 mM G-*p*NP, and 50mM of the same range of different pH buffers described above were mixed to a final volume of 100 μ L. The reactions were also carried out in optimal pH buffer (50 mM sodium phosphate pH 7.5 for BoGH3_{MLG} and 50 mM sodium citrate pH 6.5 for BACOVA_02738(GH3)) at various temperatures ranging from 30 to 70 ° C. Reactions were terminated after 10 minutes by addition of 100 μ L of 1 M Na₂CO₃ to raise the pH and absorbance at A₄₀₅ was measured in 96-well plates on an Epoch Microplate Spectrophotometer (BioTek). An extinction coefficient of 18,100 M⁻¹ cm⁻¹ was used for these assays.

Continuous assays were used for initial-rate saturation kinetics. Reactions, carried out in 250 μ L volumes in the optimum pH buffer at 37 ° C, were initiated by adding 25 μ L of enzyme solution to 225 μ L of the remaining assay mixture, pre-incubated at 37 ° C. Release of *p*NP or CNP was monitored by following absorbance at 405 nm in quartz cuvettes using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies). Eight different substrate concentrations were assayed and rate was calculated using an extinction coefficient of 15298 M⁻¹ cm⁻¹ for CNP in sodium citrate pH 6.5, 3311 M⁻¹ cm⁻¹ for *p*NP in sodium citrate pH 6.5, and 12511 M⁻¹ cm⁻¹ for *p*NP in sodium phosphate pH 7.5

HK/G6PDH coupled assay

Release of glucose monosaccharides was quantified using the D-Glucose HK Assay Kit from Megazyme, modified for use as a continuous assay. All reactions were carried out in 250 μ L volumes at 37 ° C in the triethylamine pH 7.6 buffer provided in the kit. BoGH3_{MLG} concentrations used to maintain initial-rate conditions were 9.2 nM for laminari-oligosaccharides and mixed-linkage oligosaccharides, 50.1 nM for cello-oligosaccharides, and 1.28 μ M for gentiobiose. Reactions were initiated by adding 25 μ L of enzyme solution to 225 μ L of the remaining assay mixture containing hexokinase, glucose-6-phosphate dehydrogenase, ATP, and NADP⁺, pre-incubated at 37 ° C. The release of glucose monosaccharides corresponds stoichiometrically with the reduction of a molecule of NADP⁺ to NADPH, which was monitored by following absorbance at 340 nm on a Cary 60 UV-Vis spectrophotometer. An extinction coefficient of 6,220 M⁻¹ cm⁻¹ was used to convert to rate of hydrolysis.

Enzyme limit digest assay

To determine limit-digestion products of $BoGH16_{MLG}$, 10 µM enzyme was incubated with 1.0 mg/mL polysaccharide in 1 mL of 50 mM sodium citrate pH 6.5 for 24 hours at 37 ° C. To determine limit-digestion products of $BoGH3_{MLG}$ and $BACOVA_02738(GH3)$, 10 µM enzyme was incubated with the limit digest product of $BoGH16_{MLG}$ hydrolysis of 1.0 mg/mL polysaccharide in 1 mL of the appropriate buffer for 24 hours at 37 ° C. 10 µL of the reaction was diluted into 1 mL of ultrapure water and analyzed on HPAEC-PAD and HILIC-MS as described below.

The same experiment was conducted to observe reaction progress, except 10 nM of BoGH16_{MLG} and 12 nM of BoGH3_{MLG} were used and reactions were stopped at various time points by taking 100 μ L of the reaction mixture and adding to 100 μ L of NH₄OH. 20 μ L of the reaction was diluted into 1 mL of ultrapure water and analyzed on HPAEC-PAD as described below.

Carbohydrate analytical methods

HPAEC-PAD product analysis

HPAEC-PAD was performed on a Dionex ICS-5000 HPLC system operated by Chromelion software version 7. Samples were separated on a 3×250 mm Dionex Carbopac PA200 column (Thermo Scientific). Solvent A was ultrapure water, solvent B was 1 M sodium hydroxide, and solvent C was 1 M sodium acetate. Conditions used were 0 - 5 min, 10 % B (initial conditions); 5 - 12 min, 10 % B, linear gradient from 0 - 30 % C; 12.0 - 12.1 min, linear gradient from 10 - 50 % B, linear gradient from 30 - 50 % C; 12.1 - 13.0 min, exponential gradient of B and C back to initial conditions; 13 - 17 min, initial conditions.

HILIC-MS product analysis

Samples were separated by hydrophilic interaction liquid chromatography on a TSKgel Amide-80 column (Tosoh Bioscience). Solvent A was ultrapure water and solvent B was 1 M acetonitrile. The mobile phase used was a linear gradient of 35 % A and 65 % B to 50 % A and 50 % B over 30 minutes. The eluent was split between an evaporative light scattering detector (ELSD) (Agilent Technologies) and the Bruker Esquire 3000 Plus ion trap mass spectrometer (Bruker Daltonics). The eluent was ionized in positive mode by electrospray ionization before detection by ion trap. The ELSD and total ion count chromatograms were identical to the HPAEC-PAD trace. Esquire HyStar software was used to process the mass spectrometry data (Bruker Daltonics).

X-ray crystallography

Pure BoGH16_{MLG} at 23 mg/ml in 50 mM sodium phosphate pH 7, was used to set up initial sitting drop crystal screens using a Mosquito robot (TTP Labtech). An initial hit condition was identified in the PACT screen (Qiagen) condition B9: 100 mM MES pH 6, 200 mM LiCl, 20 % w/v PEG-6000. Crystals were readily reproduced by hand in larger sitting drops by screening around this condition, varying only the PEG-6000 concentration from 15 to 25%. The crystals obtained from these optimizations were used in all subsequent work.

Crystals of the apo protein were cryo-cooled for data collection by first soaking in a solution of mother liquor supplemented with 18% ethylene glycol for 30 seconds before plunging in liquid nitrogen. Diffraction data were collected from these crystals at Diamond Light Source, beamline I03 at a wavelength of 0.976 Å. Data were indexed and integrated using XDS (Kabsch, 2010) with all subsequent data processing performed using the CCP4 software suite (Winn et al., 2011). A search model for molecular replacement was prepared using a single subunit from the *Zobellia galactanivorans* laminarinase ZgLamC_{GH16-E142S} (PDB code 4CRQ) (Labourel et al., 2015) and using CHAINSAW (Stein, 2008) to trim any sidechains in the model to the nearest common atom based on a sequence alignment. The structure was then determined using this model by molecular replacement in PHASER (McCoy et al., 2007). Following density modification in PARROT (Cowtan, 2010), BUCCANEER (Cowtan, 2007) was used to construct an initial model before further model building and refinement were performed in COOT (Emsley and Cowtan, 2004) and REFMAC5 (Murshudov et al., 2011) respectively.

To obtain the G4G4G3G complex structure, crystals were soaked for 30 minutes in cryo-protectant solution (100 mM MES pH 6, 200 mM LiCl, 25 % w/v PEG-6000, 18 % w/v ethylene glycol) in which the ligand had been dissolved at 50 mM. Crystals were then plunged in liquid nitrogen ready for data collection. X-ray data were collect-

ed from these crystals at Diamond Light Source, beamline I02 at a wavelength of 0.979 Å. Data were processed as above using XDS (Kabsch, 2010) for indexing and integration followed by subsequent processing in the CCP4 software suite (Winn et al., 2011). Since the crystals were isomorphous to the apo-structure, the apo model with waters and flexible loops removed was refined against these new data. The model was rebuilt and refined using COOT (Emsley and Cowtan, 2004) and REFMAC5 (Murshudov et al., 2011).

For both structures, the quality of the model was monitored throughout using MOLPROBITY (Davis et al., 2007) - the final models having no outliers, 98.5 % and 98.7 % of residues in the favored region of the Ramachandran plot for the apo- and G4G4G3G-complex respectively. Additionally, the sugar conformations in the G4G4G3G-BoGH16_{MLG} complex were all confirmed as ${}^{4}C_{1}$ chairs using PRIVATEER (Agirre et al., 2015) and the generated restraints applied during structure refinement. Data processing and refinement statistics for both structures can be found in Table S4. The apo- and G4G4G3G-complex structures have been deposited in the Protein Data Bank with accession codes 5NBO and 5NBP respectively.

Bioinformatics

Phylogenetic analysis

Glycoside Hydrolase Family 16 sequences with EC number 3.2.1.6 and 3.2.1.39 were extracted from the CAZy Database (URL http://www.cazy.org) using the Extract Sequences tool (URL http://research.ahv.dk/cazy/ extract). The sequences were initially aligned by MUSCLE (Edgar, 2004) in AliView (Larsson, 2014) and manually trimmed to remove amino acids outside of the GH16 catalytic domain. The resulting sequences were structurally aligned using T-Coffee Expresso (Armougom et al., 2006), then further manually refined in AliView, guided by available three-dimensional structures. A maximum-likelihood phylogenetic tree was constructed using MEGA6 v6.06 (Tamura et al., 2013) and reliability of the nodes was tested by bootstrap analysis using 100 resampling. Five cellulases from Glycoside Hydrolase Family 7 were used as an outgroup to root the tree.

All Glycoside Hydrolase Family 3 sequences listed as "characterized" as well as those that are structurally characterized with EC number 3.2.1.21 (β -glucosidases) were similarly extracted from the CAZy database. The roughly 300 characterized GH3 sequences were aligned by MUSCLE and a maximum-likelihood phylogenetic tree constructed using MEGA v6.06. The structurally characterized β -glucosidase sequences were combined with BoGH3_{MLG} and BACOVA_02738 and aligned by T-Coffee Expresso.

Survey of metagenomic data sets

Human metagenomic sequence data sets (Huttenhower et al., 2012; Junjie Qin, Yingrui Li, Zhiming Cai, Shenghui Li, Jianfeng Zhu, Fan Zhang, Suisha Liang, Wenwei Zhang, Yuanlin Guan, Dongqian Shen, Yangqing Peng, Dongya Zhang, Zhuye Jie, Wenxian Wu, Youwen Qin, Wenbin Xue, Junhua Li, Lingchuan Han, Donghui Lu, Peixian W, 2012; Qin et al., 2010; Urokawa et al., 2007) were searched by BLAST for the presence of MLGUL nucleotide sequences from *B. ovatus* (13.4kb), *B. uniformis* (14.4kb), *B. cellulosilyticus* (14.1kb), *B. finegoldii* (16.2kb), and *Pr. copri* (13.9kb). Each BLAST probe was first searched against the NCBI Refseq genomes database to determine the background thresholds for BLAST hits and subsequently trimmed to remove any sequences that may return off-target hits. *B. ovatus* and *B. xylanisolvens* MLGULs could not be distinguished due to their very high nucleotide identity (97%). Otherwise, this analysis failed to reveal any off-target hits with length >75 bp, nucleotide identity >90%, and E value <1⁻²⁰. Thus, we considered a metagenome to be positive for a particular MLGUL probe if it returned two or more hits >100 bp in length with >90% identity and E value <1⁻²⁰, or one hit >1000 bp in length with the same identity and E value cut-offs.

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