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***In vitro and in vivo* characterization of three *Cellvibrio japonicus* Glycoside Hydrolase  
Family 5 members reveals potent xyloglucan backbone-cleaving functions**

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***Dedication:*** This article is dedicated to *Cellvibrio japonicus* vanguard Prof. Harry J. Gilbert on  
the occasion of his retirement.

## Abstract

**Background:** Xyloglucan (XyG) is a ubiquitous and fundamental polysaccharide of plant cell walls. Due to its structural complexity, XyG requires a combination of backbone-cleaving and sidechain-debranching enzymes for complete deconstruction into its component monosaccharides. The soil saprophyte *Cellvibrio japonicus* has emerged as a genetically tractable model system to study biomass saccharification, in part due to an innate capacity to utilize a wide range of plant polysaccharides for growth. Whereas the downstream debranching enzymes of the xyloglucan utilization system of *C. japonicus* have been functionally characterized, the requisite backbone-cleaving *endo*-xyloglucanases were unresolved.

**Results:** Combined bioinformatic and transcriptomic analyses implicated three Glycoside Hydrolase Family 5 Subfamily 4 (GH5\_4) members, with distinct modular organization, as potential keystone *endo*-xyloglucanases in *C. japonicus*. Detailed biochemical and enzymatic characterization of the GH5\_4 modules of all three recombinant proteins confirmed particularly high specificities for the XyG polysaccharide versus a panel of other cell wall glycans, including mixed-linkage beta-glucan and cellulose. Moreover, product analysis demonstrated that all three enzymes generated XyG oligosaccharides required for subsequent saccharification by known exo-glycosidases. Crystallographic analysis of GH5D, which was the only GH5\_4 member specifically and highly upregulated during growth on XyG, in free, product-complex, and active-site affinity-labelled forms revealed the molecular basis for the exquisite XyG specificity among these GH5\_4 enzymes. Strikingly, exhaustive reverse-genetic analysis of all three GH5\_4 members and a previously biochemically characterized GH74 member failed to reveal a growth defect, thereby indicating functional compensation *in vivo*, both among members of this cohort and by other, yet unidentified, xyloglucanases in *C. japonicus*. Our systems-based analysis

indicates distinct substrate-sensing (GH74, GH5E, GH5F) and attack-mounting (GH5D) functions for the endo-xyloglucanases characterized here.

**Conclusions:** Through a multi-faceted, molecular systems-based approach, this study provides a new insight into the saccharification pathway of xyloglucan utilization system of *C. japonicus*.

The detailed structural-functional characterization of three distinct GH5\_4 endo-xyloglucanases will inform future bioinformatics predictions across species, and provides new CAZymes with defined specificity that may be harnessed in industrial and other biotechnological applications.

## Keywords

Xyloglucan, saccharification, glycoside hydrolase, *Cellvibrio japonicus*, saprophyte

## Background

Renewable plant biomass is envisioned as a promising alternative to fossil petroleum for the production of liquid fuels and high-value chemicals [1, 2]. Plant cell walls are, however, chemically and structurally complex in nature and require harsh thermo-chemical treatment to yield fermentable sugars. Such processes often generate undesirable by-products that inhibit subsequent microbial conversion [3]. In light of their ability to catalyze the degradation of recalcitrant plant cell walls under ambient conditions, enzymes from saprophytic micro-organisms constitute an attractive palette of biocatalysts for improved biomass saccharification [4]. The discovery and characterization of new enzymes from saprophytes is thus central to advancing biotechnology and, not least, underpins fundamental understanding of the biological roles of these micro-organisms in the global carbon cycle.

The Gram-negative bacterium, *Cellvibrio japonicus* Ueda107 (formerly, *Pseudomonas fluorescens* subsp. *cellulosa*) has emerged as a model saprophytic micro-organism with a demonstrated ability to utilize nearly all plant cell wall polysaccharides, including cellulose, xylans, mannans, arabinans, and pectins [5, 6]. Indeed, sequencing of the *C. japonicus* genome in 2008 revealed vast array of carbohydrate-active enzymes (CAZymes [7]) predicted to be involved in plant cell wall saccharification [8]. The recent development of genome editing techniques for *C. japonicus* has further advanced the biology and bioengineering of this bacterium in biomass conversion [9-13].

The xyloglucans (XyG) comprise an important family of cell wall matrix polysaccharides, which are ubiquitous and abundant across the plant kingdom [14, 15]. In dicots, XyGs may constitute up to 25% of the primary cell wall dry-weight, with lower amounts found in conifers

(10%) and grasses (<5%) [16, 17]. Structurally, XyGs have brush-like architectures built upon a linear, cellulosic  $\beta(1\rightarrow4)$ -D-glucan backbone that is extensively branched with  $\alpha(1\rightarrow6)$ -xylopyranosyl residues at regular intervals. Further elaboration of these branch points with diverse monosaccharides and acetyl groups is dependent on the species and tissue of origin [18, 19]; presently *ca.* 20 distinct sidechain saccharide compositions are known [20, 21]. The structure of the canonical dicot (fucogalacto)xyloglucan is shown in Fig. 1A. Due to this structural complexity, complete XyG saccharification requires the concerted action of numerous backbone-cleaving *endo*-xyloglucanases and side-chain-cleaving *exo*-glycosidases [22, 23].

As part of our ongoing effort to elucidate the xyloglucan (XyG) utilization system of *C. japonicus*, we functionally characterized a multi-gene XyG utilization locus (XyGUL) in the *C. japonicus* genome via a combination of genetics, enzymology, and structural biology. This XyGUL encodes the three *exo*-glycosidases required for (fucogalacto)xyloglucan sidechain cleavage (a GH95  $\alpha$ -L-fucosidase, a GH35  $\beta$ -galactosidase, and a GH31  $\alpha$ -xylosidase) together with a predicted TonB-dependent transporter (TBDT) (Fig. 1B & C) [13, 24, 25]. A highly specific  $\beta$ -glucosidase, Bgl3D, which is encoded elsewhere in the genome, works in concert with the *exo*-glycosidases of the XyGUL to effect the complete saccharification of XyG oligosaccharides (XyGOs) in the periplasm (Fig. 1B & 1C) [26]. Noting that this locus likewise lacked an associated *endo*-xyloglucanase, we also provided biochemical and structural evidence that the lone, secreted *C. japonicus* GH74 member (Fig. 1B & C) could efficiently generate the Glc<sub>4</sub>-based XyGOs required by the downstream *exo*-glycosidases [27].

As we now show, genetic deletion of this GH74 *endo*-xyloglucanase did not, however, impede the growth of *C. japonicus* on the polysaccharide, which suggested the involvement of additional, unidentified *endo*-xyloglucanases. Hence, we also explored the *in vitro* and *in vivo*

function of three candidate *endo*-xyloglucanases from GH5 subfamily 4 (GH5\_4) [28], guided by bioinformatic and transcriptomic analyses. Utilizing a combination of reverse genetics, enzymology, and structural biology, the present study provides a new insight into the upstream deconstruction of XyG.

## Results and Discussion

### Transcriptomic analysis reveals a potential keystone *endo*-xyloglucanase from Glycoside Hydrolase (GH) Family 5, subfamily 4.

We previously showed via quantitative PCR (qPCR) that the *C. japonicus* gene cluster containing *xyl31A* (CJA\_2706), *bgl35A* (CJA\_2707), CJA\_2709, and *afc95A* (CJA\_2710) (Fig. 1B), was up-regulated during growth on xyloglucan-containing medium [13]. Biochemical characterization confirmed that *xyl31A*, *bgl35A*, and *afc95A* encode a XyGO-specific GH31  $\alpha$ -xylosidase, GH35  $\beta$ -galactosidase, and GH95  $\alpha$ -L-fucosidase, respectively, while CJA\_2709 was predicted to encode a TonB-dependent transporter (TBDT) [13, 24, 25]. To aid identification of potential *C. japonicus* *endo*-xyloglucanases acting upstream of these enzymes, a comprehensive expression analysis via RNAseq was performed in the present study. Samples were collected from both exponentially growing and stationary phase cells grown on glucose or xyloglucan as the sole carbon source to allow for analyses of gene expression based on early-stage substrate detection (Additional file 1: Figure S1), late-stage substrate detection (Additional file 1: Figure S2A), or growth rate (Additional file 1: Figure S2B).

During exponential growth, there were 27 CAZyme-encoding genes significantly up-regulated on XyG, including the four genes of the *C. japonicus* XyG cluster, which corroborated

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4 previous qPCR results (Additional file 1: Table S1). Notably, CJA\_3010, which encodes a GH5  
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6 subfamily 4 (GH5\_4) member previously annotated as *cel5D*, was the highest upregulated gene  
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8 followed by the XyG cluster genes CJA\_2709 (encoding a predicted TBDT) and CJA\_2706  
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11 125 (*xyl31A*, encoding a GH31  $\alpha$ -xylosidase) [8]. Among the large and functionally diverse GH5  
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13 family [28], subfamily 4 is the only subfamily known to contain predominant *endo*-  
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15 xyloglucanases [23], which suggested a keystone role for this enzyme in xyloglucan utilization  
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17 by *C. japonicus*. Notably, CJA\_2477 (previously annotated as *gly74* [8]; Fig. 1B) was not  
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19 significantly up-regulated during growth on XyG, despite the encoded GH74 *endo*-  
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21 xyloglucanase being previously shown to have high, specific activity for this polysaccharide  
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23 130 [27]. Instead, CJA\_2477 appeared to be constitutively expressed at a low level (RPKM levels in  
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25 the 100-200 range), as were 14 other predicted CAZyme-encoding genes (Additional file 1:  
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27 Table S2).  
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35 The remaining CAZyme genes up-regulated during exponential growth are predicted to  
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37 135 have roles in the degradation of a diverse set of polysaccharides, which suggests that there is  
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39 complex cross-regulation of expression. As xyloglucan is unlikely to be encountered alone  
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41 during the saprophytic growth habit of *C. japonicus*, these results are suggestive of xyloglucan  
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43 degradation being one component of a sophisticated plant cell wall degradation response.  
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45 Congruently, when comparing the stationary phase *C. japonicus* cells growing on XyG and  
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47 glucose, only two genes of the XyG cluster, *bgl35A* and *afc95A* were still up-regulated on XyG,  
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49 140 together with 33 additional predicted and confirmed hemicellulase- and pectinase-encoding  
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51 genes (Additional file 1: Figure S2A, Additional file 1: Table S3). Additionally, when comparing  
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53 the exponential phase to the stationary phase for xyloglucan-grown cells, we observed that there  
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55 was a growth-phase-dependent response manifested as a significant shift in the suite of expressed  
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CAZyme genes (Additional file 1: Figure S2B, Additional file 1: Table S4). Specifically, these differentially expressed CAZyme genes were not predicted to be XyG-specific, based on their CAZy family membership, which suggested they are part of regulatory circuit that responds generally to polysaccharides. Similar growth-phase-dependent responses have been previously observed during cellulose utilization by *C. japonicus* [12] and *Clostridium thermocellum* (now *Ruminiclostridium thermocellum*) [29, 30].

### Bioinformatic analysis and recombinant production of GH5\_4 members from *C. japonicus*

Spurred-on by the implication of the GH5 subfamily 4 (GH5\_4) member encoded by CJA\_3010 in xyloglucan utilization by *C. japonicus*, we searched the genome for potential homologs. *C. japonicus* encodes 15 GH5 members, of which only three belong to subfamily 4 ([8] see <http://www.cazy.org/b776.html>): The aforementioned CJA\_3010 (GenBank ACE84905.1, previously annotated as *cel5D* [8]), CJA\_3337 (GenBank ACE83841.1, previously annotated as *cel5E* [8]), and CJA\_2959 (GenBank ACE86198.1, previously annotated as *cel5F* [8]). Protein sequence analysis revealed that each of these gene products had a unique, multi-modular architecture that suggested the possibility of distinct cellular localization and biological function (Additional file 1: Figure S3). Considering the lack of demonstrable activity on cellulose and high activity on xyloglucan (*vide infra*), the corresponding encoded enzymes are referred to as *CjGH5D*, *CjGH5E*, and *CjGH5F* hereafter.

The highly up-regulated CJA\_3010 encodes a signal peptidase II lipoprotein signal peptide (predicted by LipoP 1.0 [31]), followed by a serine-rich linker and a GH5\_4 catalytic module, and was thus predicted to be anchored extracellularly in the outer membrane by N-terminal

cysteine lipidation (Additional file 1: Figure S3). CJA\_3337 encodes an N-terminal signal peptide (predicted by SignalP 4.0 [32]) and two carbohydrate-binding modules (CBMs [33]), CBM2 and CBM10, in train with a GH5\_4 catalytic module (Additional file 1: Figure S3). CJA\_2959 encodes a signal peptide (predicted by SignalP 4.0 [32]) a Fibronectin type 3 (FN3) domain, an undefined region, and a C-terminal GH5\_4 catalytic module (Additional file 1: Figure S3). The presence of signal peptides, and CBMs in the case of *CjGH5E*, is indicative of extracellular secretion of both *CjGH5E* and *CjGH5F*.

#### Amino acid alignment of the catalytic modules of *CjGH5D*, *CjGH5E*, and *CjGH5F*

demonstrate conservation of the two catalytic glutamate residues, but low to moderate overall sequence conservation (26 to 45% identity). Notably, alignment with endo-xyloglucanases from *Bacteroides ovatus* [22], *Paenibacillus pabuli* [34], and a rumen metagenome [35] suggests that the *C. japonicus* proteins are members of Subfamily 4 (Additional file 1: Figure S4, Additional file 1: Table S5). GH5\_4 is one of the largest GH5 subfamilies and contains, in addition to specific endo-xyloglucanases, promiscuous endo- $\beta$ (1,4)-glucanases, strict cellulases, and mixed-linkage endo- $\beta$ (1,3)/ $\beta$ (1,4)glucanases (reviewed in [23, 28]). As such, we undertook the recombinant production and enzymological characterization of the three *C. japonicus* GH5\_4 members to precisely define their catalytic activities in the context of potential biological function.

Our initial attempts to produce the full-length, multi-modular proteins recombinantly in *E. coli* by replacement of the native signal peptides with an N-terminal hexahistidine (His<sub>6</sub>) purification tag were consistently unsuccessful: Intact protein mass spectrometry revealed proteolytic instability of His<sub>6</sub>-SRL-GH5D, while His<sub>6</sub>-CBM2-CBM10-GH5E and His<sub>6</sub>-FN3-GH5F had very poor production yields (data not shown). In contrast, His<sub>6</sub>-GH5D (Additional

file 1: Figure S3A) was produced as a stable, intact, active protein (calculated mass, 44222.2 Da; observed by ESI-MS, 44222.6 Da) in excellent yield (150 mg L<sup>-1</sup>). Likewise, our attempts to produce the individual catalytic modules of *CjGH5E* and *CjGH5F* as N-terminally His<sub>6</sub>-tagged constructs (Additional file 1: Figure S3B & C) were successful (*His<sub>6</sub>-CjGH5E* calculated mass, 41367.1 Da; observed by ESI-MS, 41370.1 Da, *His<sub>6</sub>-CjGH5F* calculated mass, 40253.8 Da; observed by ESI-MS 40253.9 Da) with approximate production yields of 14 and 9 mg L<sup>-1</sup>, respectively.

### ***CjGH5\_4* enzymes are highly efficient, specific endo-xyloglucanases.**

Informed by the subfamily membership of the three GH5\_4 members, we anticipated that these enzymes might exhibit significant *endo*-hydrolytic activity towards XyG. Hence, this polysaccharide was used to determine pH and temperature optima. *CjGH5D*, *CjGH5E*, and *CjGH5F* each exhibited approximately bell-shaped pH profiles, with the highest activity achieved in 50 mM phosphate buffer (pH 7.5 in case of *CjGH5D* and *CjGH5E*, and pH 7.0 in case of *CjGH5F*; Additional file 1: Figure S5). When the 3 enzymes were incubated with XyG at different temperatures over the course of 10 minutes, optimum temperature was identified as 50 °C (*CjGH5D* and *CjGH5F*) and 55 °C (*CjGH5E*) (Additional file 1: Figure S5). To determine substrate specificity of the three GH5\_4 members, a panel of nine soluble polysaccharide substrates were screened under these optimal conditions. Indeed *CjGH5D*, *CjGH5E*, and *CjGH5F* all displayed high specific activity toward XyG (Table 1). No detectable activity toward barley mixed-linkage 1,3/1,4-β-glucan, guar galactomannan, konjac glucomannan, beechwood xylan, wheat flour arabinoxylan, or xanthan for any of the three enzymes was observed. *CjGH5D* appeared to strictly require the branched XyG structure, while *CjGH5E* demonstrated trace activities against the artificial 1,4-β-glucans hydroxyethylcellulose (HEC)

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4 and carboxymethylcellulose (CMC) at the highest tested substrate concentration (2 mg mL<sup>-1</sup>);  
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6 specific activities were 200-1500-fold less than XyG, respectively (Table 1). Similarly, CjGH5F  
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9 215 was able to hydrolyze HEC with an 800-fold lower specific activity than XyG, while no activity  
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11 towards CMC was detected. Michaelis-Menten analysis for XyG further underscored the high  
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13 XyG specificity of the three enzymes: remarkably low  $K_m$  values were observed and high  $k_{cat}$   
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15 values recapitulated those previously observed for predominant *endo*-xyloglucanases, including  
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17 CjGH74 [22, 27, 36, 37] (Table 1, Additional file 1: Figure S6).  
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22 220 Time-course analyses of native XyG polysaccharide hydrolysis products by HPAEC-PAD  
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24 revealed that all three GH5\_4 enzymes generated products of intermediate retention time in the  
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26 early stages of the reactions, with no significant generation of the Glc<sub>4</sub>-based XXXG, XLXG,  
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28 XXLG, and XLLG limit-digestion products (Additional file 1: Figure S7, Additional file 1:  
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30 Figure S8; *cf.* Fig. 1). These results indicate that the three enzymes hydrolyze XyG through a  
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32 dissociative, rather than processive [38] mechanism, and are thus canonical *endo*-xyloglucanases  
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34 225 (EC 3.2.1.151; *cf.* EC 3.2.1.150, EC 3.2.1.155). The limit-digestion products further revealed  
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36 that all *C. japonicus* GH5\_4 enzymes specifically catalyze hydrolysis at the anomeric position of  
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38 the unbranched glucose residues of the (galacto)XyG polysaccharide chain (Fig. 1). This  
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40 cleavage pattern is typical for many GH5 [22, 34, 37], GH9 [39, 40], GH12 [34, 41-44], GH16  
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42 [45] and GH74 [46-50] *endo*-xyloglucanases, although certain GH5 [35, 36], GH7 [51], GH44  
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44 230 [40, 52] and GH74 [53-55] members preferentially hydrolyze the XyG backbone between  
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46 branched glucosyl residues. The canonical XXXG-type XyGOs produced by CjGH5D,  
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48 CjGH5E, and CjGH5F are direct substrates for the *exo*-glycosidases of the XyG gene cluster  
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With knowledge of the cleavage specificity of the GH5\_4 members, we determined kinetic parameters for the hydrolysis of a panel of chromogenic oligosaccharides to reveal the contribution of side chain substitution on substrate recognition and catalysis (Table 2). All three enzymes were only weakly active on 2-chloro-4-nitrophenyl cellotrioside (GGG- $\beta$ -CNP) and 2-chloro-4-nitrophenyl cellotetraoside (GGGG- $\beta$ -CNP), with meagre increases in  $k_{\text{cat}}/K_{\text{m}}$  values arising from the addition of potential -4 subsite binding for the cellotetraoside (Table 2, Additional file 1: Figure S9) GH subsite nomenclature according to [56]. Strikingly, the addition of three  $\alpha(1\rightarrow6)$ -xylopyranosyl residues to the glucan backbone resulted in significant increases in catalytic efficiency for all GH5\_4 members, which was manifested as 65-, 700-, and 150-fold higher  $k_{\text{cat}}/K_{\text{m}}$  values for XXXG- $\beta$ -CNP vis-à-vis GGGG- $\beta$ -CNP with CjGH5D, CjGH5E, and CjGH5F, respectively (Table 2, Additional file 1: Figure S9). These values correspond to 11, 18, and 13 kJ/mol, respectively, of additional transition state stabilization in the formation of the covalent glycosyl-enzyme in these anomeric-configuration-retaining GH5 enzymes (calculated using the formula:  $\Delta\Delta G^\ddagger = -RT \ln[(k_{\text{cat}}/K_{\text{m}} \text{ XXXG}) / (k_{\text{cat}}/K_{\text{m}} \text{ GGGG})]$ ) [57]. With XLLG- $\beta$ -CNP, the specificity constants ( $k_{\text{cat}}/K_{\text{m}}$ ) were only increased 1.5- to 5-fold for the three *endo*-xyloglucanases, thereby indicating that extending  $\beta(1\rightarrow2)$ -galactopyranosyl residues (Fig. 1) have little additional effect on catalysis (Table 2).

### Covalent labeling of CjGH5D with an active-site-directed inhibitor

Active-site affinity-based inhibitors are important tools for the detailed kinetic analysis of GH enzymes [58]. In particular, *N*-bromoacetyl-glycosylamine derivatives of xyloglucan oligosaccharides have been previously demonstrated to be specific active-site affinity labels for *endo*-xyloglucanases [59] [36]. A time- and concentration-dependent inactivation of the enzyme CjGH5D was observed upon incubation with XXXG-NHCOCH<sub>2</sub>Br, which followed pseudo-

first-order kinetics (Fig. 2). The dissociation constant  $K_i$  and the irreversible inactivation constant  $k_i$  for towards *CjGH5D* were  $1.78 \pm 0.17$  mM and  $0.17 \pm 0.01$  min<sup>-1</sup>, respectively, resulting in a  $k_i/K_i$  value ( $9.3 \times 10^{-2}$  mM<sup>-1</sup>.min<sup>-1</sup>) that was comparable to that previously observed for a *Prevotella bryantii* GH5\_4 member (*PbGH5A*) [36]. Notably, intact protein mass spectrometry of *CjGH5D* following incubation with the inhibitor indicated covalent labelling with 1:1 stoichiometry and no over-labelling of the enzyme (Additional file 1: Figure S10).

### *CjGH5D* crystallography

A tertiary structure of the catalytic domain of *CjGH5D* was determined at 1.6 Å resolution in uncomplexed “apo” form by X-ray crystallography and molecular replacement with *BoGH5A* (pdb: 3ZMR, [22]). The overall structure of *CjGH5D* (residues Gly96 to Gln468) is an ( $\beta/\alpha$ )<sub>8</sub> barrel as is typical for GH5 family members (Fig. 3A). Despite sequence identities in the 25-40% range, the structure is similar to the catalytic domains of many GH5 enzymes, most of which are annotated as xyloglucanases, glucanases and lichenases, with typical alignment values of approximately 310 residues aligning with an rmsd of 1.3 Å [60]. For example, the structure used for molecular replacement, *BoGH5A*, overlaps with an r.m.s.d. of 1.1 Å over 332 equivalent Ca atoms with 40% identity. Minor differences are observed between the two structures in loops at the end of core helices. *BoGH5A* has an extra loop Val170-Gly180 (residues equivalent to Ile137-Gly138 in *CjGH5D*) which enables the formation of a hydrogen bond to the -4'-xylosyl residue of ligand XXXG (between N Val182 and the sugar ring O atom, *vide infra*).

A 1.9 Å-resolution product complex of *CjGH5D* was obtained by soaking crystals with a mixture of Glc<sub>12</sub>-based XyGOs of variable sidechain galactosylation. Here, we anticipated that the substrate mixture would be hydrolyzed and that the enzyme would selectively bind the oligosaccharide for which it had the best affinity. Commensurate with limit-digest analysis, we

observed a Glc<sub>4</sub>-based oligosaccharide backbone spanning the -4 to -1 subsites for both molecules in the asymmetric unit: GXLG in molecule A (with glucose in the -4 subsite and the -3'-xylosyl group modelled at occupancies of 0.5 and 0.7 respectively) and GXXG in molecule B (here, there was insufficient electron density in the F<sub>o</sub>-F<sub>c</sub> difference map to allow unambiguous modelling of a galactose on the -2'-xylosyl unit). In the -1 subsite, the glucosyl residue interacts with the catalytic acid base Glu255 (via O1), and nucleophile Glu390 via O2. In addition, O3 is hydrogen bonded to His208. In molecule B, the equivalent glucose also hydrogen bonds via O2 to Asn254 and His208 (Fig. 3B & C).

A second oligosaccharide complex was obtained at 2.1 Å resolution by soaking *CjGH5D* crystals with the *N*-bromoacetyl affinity label XXXG-NHCOCH<sub>2</sub>Br, in which the reagent had indeed reacted through attack of the catalytic general acid/base sidechain to displace the bromide nucleofuge. In molecule A of the asymmetric unit there is electron density for GXXG-NHCOCH<sub>2</sub>-*CjGH5D*, whilst in molecule B, XXXG-NHCOCH<sub>2</sub>-*CjGH5D* is modeled, but with the -3'- and -4'-xylosyl sugars modelled at half occupancy. The carboxyl oxygen of the *N*-acetyl moiety forms a hydrogen bond with His323. There are hydrogen bonds between this subsite -1 sugar and the catalytic nucleophile Glu390, and also to His208 and Asn254 (Fig. 3B & D). These are similar to interactions observed in the structure of an analogous XXXG-NHCOCH<sub>2</sub>-*PbGH5A* complex structure (pdb: 5D9P, [36]). Glucose in the -2 subsite is hydrogen bonded via O3 to ND2 Asn132 and via O2 to NE1 Trp432; this latter interaction is notably long (ca. 3.2 Å), which may reflect the positioning of the tryptophan as the -1 subsite stacking residue. The equivalent Asn/Trp interactions are also seen in related enzymes: Asn28 and Trp324 in the XXXG-NHCOCH<sub>2</sub>-*PbGH5A* complex (pdb: 5D9P) and Asn165 and Trp472 in the *BoGH5A*-XXXG complex (PDB 3ZMR). In addition to Trp432, Trp143 provides aromatic stacking

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4 interactions with the glucose in the -3 subsite (homologous to Trp324 and Trp48 in *PbGH5A*,  
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6 305 and Trp472 and Trp185 in *BoGH5A*, respectively), while Trp209 lies against the -2'-xylosyl  
7  
8 residue (as does the equivalent Trp252 in *BoGH5A*). This pattern of conserved/ highly invariant  
9  
10 residues interacting with the xyloglucan chain presumably accounts for the fact that despite  
11  
12 sharing amino acid identity as low as 30%, these enzymes are all tailored for xyloglucan as a  
13  
14 substrate (Fig. 4). None of these three GH5D structures exhibits direct interactions with glucosyl  
15  
16 units in the -3 and -4 subsites with the protein. The -3'-xylosyl unit is tethered by two hydrogen  
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18 310 bonds between O3 and O4 and Asp438, which hold the sugar perpendicular to the orientation of  
19  
20 the equivalent xylose in the XXXG-NHCOCH<sub>2</sub>-*PbGH5A* and *BoGH5A*:XXXG complexes (in  
21  
22 the latter, the xylose lies parallel to the side chain of Tyr476).  
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30 The covalent adduct formation through the reactivity of the *N*-bromoacetyl reagent is  
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32 315 fascinating given that in the structures observed here, the attack is made by the acid-base  
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34 Glu255, as opposed to the enzymatic nucleophile of the enzymatic reaction, Glu390. This latter  
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36 residue is poised for nucleophilic attack at the anomeric carbon, C1, of the -1 subsite glucoside.  
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38 However, Glu390 is too distant (6-7 Å) from the reactive carbon of the *N*-bromoacetyl moiety,  
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40 and has impossible geometry and steric hindrance, to permit nucleophilic interception. The  
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42 320 reactive group, however is located in the +1 subsite – some 3.8 Å from C1 – thus can be  
43  
44 fortuitously attacked by the acid/base which is in almost ideal position for S<sub>N</sub>2 attack on the  
45  
46 reactive carbon to displace the bromide. Such a reaction is facilitated, either prior, or subsequent  
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48 to attack by rotation around the CB-CG bond, which leaves the side-chain in a different rotamer  
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50 after the reaction relative to its “normal” position in unreacted complexes (Figure 3D).  
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## Mutational analyses of *C. japonicus* GH5\_4 genes indicate a complex mode of action for the initial stages of xyloglucan degradation.

Facilitated by knowledge of their broadly similar catalytic properties, we embarked on a comprehensive reverse-genetic analysis in an attempt to delineate the biological functions of the individual GH5\_4 and GH74 endo-xyloglucanases, using recently developed in-frame gene deletion techniques [9].

In-frame deletion mutants were first generated in the XyG gene cluster encoding the three *exo*-glycosidases and the TBDT (Fig. 1B) to provide benchmark controls for subsequent analysis of *endo*-xyloglucanase deletion mutants. Recapitulating our previous work using insertional mutants [13], an in-frame  $\Delta xyl31A$  ( $\alpha$ -xylosidase) mutant was unable to grow on XyG due to an inability to remove non-reducing-terminal xylosyl residues as the first essential step in XyGO saccharification (Fig. 5A *cf.* Fig. 1). A  $\Delta CJA\_2709$  (TBDT) single mutant strain had a significant growth defect, presumably resulting from a decreased ability to uptake extracellularly produced XyGOs into the periplasm. The deletion of *bgl35A* also attenuated growth, due to an inability of the strain to access the full complement of sidechain monosaccharides. As expected, growth of the  $\Delta afc95A$  ( $\alpha$ -L-fucosidase) mutant on tamarind (galacto)xyloglucan was identical to the wild-type strain, because this readily available substrate lacks the terminal fucosyl residues typically found in dicot primary cell wall XyG (Fig. 1A). Moreover, all XyG gene cluster mutant strains grew similarly to wild type in glucose containing medium (Additional file 1: Figure S11).

With these control experiments complete, we next analyzed the effect of deleting the individual GH5\_4- and GH74-encoding genes. Despite original indications by RNAseq analysis of a potential lead role for *CjGH5D* in XyG utilization, in-frame deletion of *CJA\_3010*

surprisingly did not elicit a statistically significant growth defect (Fig. 5B). Likewise, strains containing single in-frame deletions of CJA\_3337, CJA\_2959, and CJA\_2477 grew identically to the wild-type strain. Moreover, comprehensive combinatorial mutagenesis did not yield a strain with a substantial growth defect for any combination of double, triple, or quadruple mutants (Fig. 5C & D). Furthermore, the growth traits (maximum OD and growth rate) of both the wild-type and the quadruple deletion mutant were similar when reduced (0.25%) or limiting (0.125%) concentrations of XyG were used (Additional file 1: Figure S12 and Table S6). Collectively, these results suggest that despite their individual high activities and specificities toward XyG, as defined by the biochemical and structural analyses described above, the GH5\_4 and GH74 members are not the only enzymes encoded by *C. japonicus* with sufficient xyloglucanase activity to support growth.

*CjGH5D* is predicted to be attached to the exterior face of the outer membrane by N-terminal lipidation, while *CjGH5E*, *CjGH5F*, and *CjGH74* are likely secreted enzymes (*vide supra*). Thus, we hypothesized that other secreted enzymes, with either predominant or side hydrolytic activities toward XyG, may be enabling growth of the quadruple mutant. Deletion of the Type-Two Secretion System (T2SS) in the  $\Delta gsp$  mutant has been previously shown to abolish the ability of *C. japonicus* to secrete cellulases [11], and constitutes a powerful tool to restrict extracellular secretion of CAZymes in general. Interestingly, introduction of the CJA\_3010 deletion into the  $\Delta gsp$  background resulted in only limited growth attenuation on xyloglucan (Additional file 1: Figure S13). With the T2SS extracellular secretion pathway disabled, the ability of the  $\Delta gsp \Delta CJA\_3010$  strain to grow on XyG strongly suggests the presence of other membrane-bound XGases that effect XyG depolymerization in a physiologically relevant manner. These data sharply contrast observations for the human gut

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4 370 symbiont *Bacteroides ovatus*, for which deletion of a single GH5\_4 member from the XyGUL  
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7 resulted in complete loss of growth on XyG [22].  
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10 Predominant xyloglucanase activity has been demonstrated previously in members of  
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12 CAZyme families GH5, GH7, GH9, GH12, GH16, GH44, and GH74, and potentially may  
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14 constitute a side activity in other *endo*- $\beta$ (1,4)glucanases (cellulases) [23, 61]. Examination of *C.*  
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17 375 *japonicus* genome indicates the presence of multiple GH5 ( $n = 15$ ), GH9 ( $n = 3$ ), and GH16 ( $n =$   
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19 9) encoding genes, in addition to the single GH74 member ([8]; for a summary table, see  
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21 <http://www.cazy.org/b776.html>). Further, Deboy, *et al.* [8] predicted that there are  
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23 approximately 45 membrane-bound CAZymes. Although it constitutes a significant undertaking  
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25 that is beyond the scope of the present study, our future investigations will focus on scrutinizing  
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29 380 these additional CAZymes in the context of XyG utilization by *C. japonicus*.  
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## 35 Conclusions

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38 We previously proposed a model of XyG utilization by *C. japonicus*, in which an  
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40 extracellular *endo*-xyloglucanase mediates degradation of the polysaccharide to XyGOs for  
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42 uptake via the TBDT, followed by complete hydrolysis to monosaccharides in the periplasm by  
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45 385 the *exo*-glycosidases encoded by the XyG gene cluster [13]. Our present study, combining  
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47 biochemical and reverse-genetic analyses, reveals that the number of actors in the initial cleavage  
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49 event is significantly greater than originally anticipated by bioinformatics. We propose that the  
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51 existence of so many extracellular *endo*-xyloglucanases of apparently overlapping biochemical  
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53 function can be explained by a physiological interplay of secreted reconnaissance enzymes and  
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57 390 cell-surface-bound, proximal XyG degraders (Fig. 1B).  
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Thus, the secreted GH74 and two secreted GH5\_4 enzymes may act as highly mobile, primary “unravellers” of the plant cell, liberating XyG fragments from the lignocellulose matrix (Fig. 1C). Indeed, the concept of “sensing” polysaccharidases playing a lead role in generating inducers has been previously proposed [62]. As plant cell wall polysaccharide degradation advances, more intimate contact between the bacterial cell surface and the substrate may ensue, engaging the outer-membrane-bound *CjGH5D* and a more efficient interplay between XyG backbone hydrolysis and direct TBDT-mediated uptake of the oligosaccharide products. The coordinated capture, hydrolysis, and uptake of partially hydrolyzed polysaccharides as a successful competitive strategy has considerable precedent in the Polysaccharide Utilization Loci of the Bacteroidetes [63]. Moreover, the need to initiate cell wall “unravelling” has been suggested to explain why saprophytes such as *C. thermocellum*, which are unable to utilize xyloglucan or xylan for growth, contain *endo*-xyloglucanases and *endo*-xylanases within their cellulosomes [39, 64].

## Methods

### Transcriptomic analysis

RNAseq sampling and analysis was performed as previously described [9, 12]. Briefly, *C. japonicus* cultures were grown in 500 mL flasks at 30 °C shaking at 200 RPM. OD<sub>600</sub> was measured every hour to monitor growth and samples were taken during exponential and stationary phase. Within two minutes of sampling metabolism was stopped using a phenol/ethanol solution (5%/ 95%). The samples were immediately pelleted by centrifugation at 8000 *g* at 4 °C for five minutes. The supernatant was discarded and cell pellets were then flash frozen using a dry ice/ethanol bath and stored at -80 °C. RNA extraction, library preparation, and sequencing was performed by GeneWIZ (South Plainfield, NJ). Illumina HiSeq2500 was performed as 50 bp single-reads with at least 10 million reads generated per sample. The raw data have been submitted to the NCBI Gene Expression Omnibus (Accession GSE109594).

### Bioinformatic analysis

The full-length proteins encoded by ORFs CJA\_3010 (*CjGH5D*), CJA\_3337 (*CjCBM2-CBM10-GH5E*) and CJA\_2959 (*CjFN3-GH5F*) in *C. japonicus* genome were screened for the presence of a signal peptide using SignalP 4.0 [32] and LipoP 1.0 [65]. The modular architecture of the three enzymes was obtained from BLASTP analysis and additional alignment with representative GH and CBM modules from the CAZy Database [7] using ClustalW [66].

### Cloning of cDNA encoding protein modules

cDNA encoding the full-length enzymes *CjSRL-GH5D*, *CjCBM2-CBM10-GH5E* and *CjFN3-GH5F*, in addition to the catalytic domains *CjGH5D*, *CjGH5E* and *CjGH5F* were PCR amplified from *C. japonicus* genomic DNA; all constructs were designed such that the native

predicted signal peptide was removed (PCR primers are listed in Additional file 1: Table S7).

The amplified *CjSRL*-GH5D, *CjCBM2*-CBM10-GH5E, *CjFN3*-GH5F, *CjGH5D* and *CjGH5F* products were double-digested with *NheI* and *XhoI*, gel purified and ligated to the respective sites of *pET28a* to fuse an N-terminal 6x His-Tag. The amplified *CjGH5E* product was ligated in an *SspI* linearized pMCSG53 vector using Ligation Independent Cloning (LIC) strategy [67]. Successful cloning was confirmed by PCR and plasmid DNA sequencing. Q5 high fidelity DNA polymerase was used for all the PCR amplifications.

## Gene expression and protein purification

Constructs were individually transformed into the chemically competent *E. coli* Rosetta DE3 cells. Colonies were grown on LB solid media containing kanamycin (50  $\mu\text{g mL}^{-1}$ ) and chloramphenicol (30  $\mu\text{g mL}^{-1}$ ) [*CjSRL*-GH5D, *CjCBM2*-CBM10-GH5E, *CjFN3*-GH5F, *CjGH5D* and *CjGH5F*], or containing ampicillin (50  $\mu\text{g mL}^{-1}$ ) and chloramphenicol (30  $\mu\text{g mL}^{-1}$ ) [*CjGH5E*]. One colony of the transformed *E. coli* cells was inoculated in 5 mL of LB medium containing the same antibiotics and grown overnight at 37 °C (200 rpm). The whole overnight culture was used to inoculate 500 mL of TB liquid medium containing the proper antibiotics. Cultures were grown at 37 °C (200 rpm) until  $D_{600} = 0.6$ . Overexpression was induced by adding IPTG to a final concentration of 0.1 mM. After induction, cultures were grown overnight at 16 °C (200 rpm). Cultures were then centrifuged and pellets were resuspended in 5 mL of *E. coli* lysis buffer containing 20 mM HEPES, pH 7.0, 500 mM NaCl, 40 mM imidazole, 5% glycerol, 1 mM DTT and 1 mM PMSF. Cells were then disrupted by sonication and the clear supernatant was separated by centrifugation at 4 °C (4400 rpm for 45 minutes). Recombinant proteins were purified from the clear soluble lysates using a  $\text{Ni}^{+2}$ - affinity column utilizing a gradient elution up to 100% elution buffer containing 20 mM HEPES, pH 7.0, 100 mM NaCl, 500 mM

imidazole, and 5% glycerol in an FPLC system. Purity of the recombinant proteins was determined by visualizing the protein contents of the fractions on SDS-PAGE. Pure fractions were pooled, concentrated, and buffer exchanged against 50 mM phosphate buffer (pH 7.0) containing 10% glycerol. Protein concentrations were then determined using Epoch Micro-Volume Spectrophotometer System (BioTek®, USA) at 280 nm, and identities of the expressed proteins were confirmed by intact mass spectrometry [68]. Purified proteins were then aliquoted and stored at -80 °C until needed.

## Carbohydrate sources

Tamarind seed XyG, konjac glucomannan (KGM), barley  $\beta$ -glucan (BBG), wheat flour arabinoxylan, and beechwood xylan were purchased from Megazyme® (Bray, Ireland). Hydroxyethylcellulose (HEC) was purchased from Amresco® (Solon, USA). Carboxymethyl cellulose was purchased from Acros Organics (New Jersey, USA). Guar gum was purchased from Sigma Aldrich® (St. Louise, USA). Xanthan gum was purchased from Spectrum® (New Brunswick, USA). 2-Chloro-4-nitrophenyl (CNP)- $\beta$ -D-cellobioside (GGG- $\beta$ -CNP) and CNP- $\beta$ -D-cellobioside (GGGG- $\beta$ -CNP) were purchased from Megazyme®. XXXG- $\beta$ -CNP and XLLG- $\beta$ -CNP were prepared as previously described [69]. Glc<sub>4</sub>-based XyGOs (XXXG, XLXG, XXLXG, and XLLG; nomenclature according to [20]) and Glc<sub>8</sub>-based XyGOs were prepared from XyG powder (Innovassynth Technologies, Maharashtra, India) as previously described [61]. XXXG-NHCOCH<sub>2</sub>Br was synthesized as previously described [59].

## Carbohydrate analytics

High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) was performed on a Dionex ICS-5000 DC HPLC system operated by the Chromeleon software version 7 (Dionex) using a Dionex Carbopac PA200 column. Solvent A

was double-distilled water, solvent B was 1 M sodium hydroxide (NaOH), and solvent C was 1 M sodium acetate (NaOAc). The gradient used was: 0–4 min, 10% solvent B and 2.5% solvent C; 4–24 min, 10% B and a linear gradient from 2.5–25% C; 24–24.1 min, 50% B and 50% C; 24.1 – 25 min, an exponential gradient of NaOH and NaOAc back to initial conditions; and 25–31 min, initial conditions.

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) was performed on a Bruker Daltonics Autoflex System (Billerica, USA). The matrix, 2,5-dihydroxy benzoic acid, was dissolved in 50% methanol in water to a final concentration of 10 mg mL<sup>-1</sup>. Oligosaccharide samples were mixed 1:1 (v/v) with the matrix solution. One µl of this solution was placed on a Bruker MTP 384 ground steel MALDI plate and left to air dry for two hours prior to analysis.

### Enzyme kinetic analysis

All enzyme activities toward polysaccharides were determined using a bicinchoninic acid (BCA) reducing-sugar assay [70]. The effect of temperature on xyloglucanase activity was determined by incubating the recombinant catalytic domain: *CjGH5D* (0.098 µg), *CjGH5E* (0.086 µg), *CjGH5F* (0.017 µg) with tamarind seed xyloglucan at a final concentration of 1 mg mL<sup>-1</sup>. Citrate buffer (pH 6, *CjGH5D* and *CjGH5F*) or phosphate buffer (pH 7.5, *CjGH5E*) was used to a final concentration of 50 mM in a total reaction volume of 200 µL. Reaction mixtures were incubated for 10 minutes at temperatures ranging from 25 °C to 80 °C prior to the BCA assay. To determine the pH-rate profile, the same XyG concentration was incubated with the same enzyme amounts, except for *CjGH5D* (0.049 µg), for 10 minutes at 50 °C (*CjGH5D*, *CjGH5F*), or 55 °C (*CjGH5E*), with 50 mM final concentration of the following buffers: citrate (pH 3-6.5), phosphate (pH 6.5-8), and glycine (pH 8.5-9).

For qualitative activity assessment against the other polysaccharide substrates, 1  $\mu\text{g}$  of each recombinant enzyme was added to XyG, HEC, CMC, BBG, KGM, wheat flour arabinoxylan, beechwood xylan, xanthan gum, and guar gum to a final concentration of 2  $\text{mg mL}^{-1}$  in 200  $\mu\text{L}$  reaction volumes containing 50 mM phosphate buffer (pH 7.5: *CjGH5D* and *CjGH5E*, or pH 7: *CjGH5F*). Mixtures were then incubated at 50  $^{\circ}\text{C}$  (*CjGH5D* and *CjGH5F*) or 55  $^{\circ}\text{C}$  (*CjGH5E*) for 10 minutes before the generated reducing ends were detected using BCA assay.

To determine specific activity values of *CjGH5* enzymes toward XyG, final concentration of 0.75, 2.59, and 0.71 nM of the recombinant purified catalytic modules *CjGH5D*, *CjGH5E*, and *CjGH5F*, respectively, was incubated with tamarind seed XyG (1  $\text{mg mL}^{-1}$ ) in 200  $\mu\text{L}$  reaction mixtures containing 50 mM phosphate buffer (pH 7.5: *CjGH5D* and *CjGH5E*, or pH 7: *CjGH5F*). Likewise, specific activity values of *CjGH5* enzymes toward HEC were obtained by incubating *CjGH5E* and *CjGH5F* at a final concentration of 1.04 and 0.65  $\mu\text{M}$ , respectively, with 2  $\text{mg mL}^{-1}$  HEC in 200  $\mu\text{L}$  reaction mixtures containing 50 mM phosphate buffer (pH 7.5: *CjGH5E* or pH 7: *CjGH5F*). For specific activity toward CMC, final concentration of 1.04  $\mu\text{M}$  of the purified catalytic module *CjGH5E* was incubated with CMC (2  $\text{mg mL}^{-1}$ ) in 200  $\mu\text{L}$  reaction volume containing 50 mM phosphate buffer (pH 7.5). All reaction mixtures were incubated at 50  $^{\circ}\text{C}$  (*CjGH5D* and *CjGH5F*) or 55  $^{\circ}\text{C}$  (*CjGH5E*) for 10 minutes prior to the BCA assay and all assays were performed in triplicates.

To determine Michaelis-Menten parameters for XyG, eight different concentrations of XyG solutions were used over the range 0.025 to 1  $\text{mg mL}^{-1}$ . The recombinant enzyme *CjGH5D* (0.007  $\mu\text{g}$ ), *CjGH5E* (0.022  $\mu\text{g}$ ), and *CjGH5F* (0.006  $\mu\text{g}$ ) was individually incubated with each XyG concentration at 50  $^{\circ}\text{C}$  (*CjGH5D* and *CjGH5F*) or 55  $^{\circ}\text{C}$  (*CjGH5E*) for 10 min in a 200  $\mu\text{L}$

final reaction mixture containing 50 mM phosphate buffer (pH 7.5: *CjGH5D* and *CjGH5E*, or pH 7: *CjGH5F*).  $K_m$  and  $k_{cat}$  values were determined by non-linear fitting of the Michaelis-Menten equation to the data in Sigmaplot® (Systat software Inc.)

To identify Michaelis-Menten constants for the chromogenic substrates, different dilution series were established to give final concentration ranges of 0.0625- 8 mM (CNP- $\beta$ -GGG), 0.0625- 8 mM (CNP- $\beta$ -GGGG), 0.002-4 mM (XXXG- $\beta$ -CNP), and 0.002- 2 mM (XLLG- $\beta$ -CNP). Substrate mixtures (225  $\mu$ L) containing 50 mM phosphate buffer with the optimum pH of the enzyme were pre-incubated for 10 minutes at the optimum temperature of the enzyme (*vide supra*). 25  $\mu$ L of 10X *CjGH5D* (to give 30-3800 nM final concentration according to the tested substrate), *CjGH5E* (3- 260 nM), and *CjGH5F* (4- 650 nM) was added to the substrate mixtures before the release of the aglycone was continuously monitored by measuring the change in absorbance at 405 nM for 2 minutes in a Cary50 UV–visible spectrophotometer (Varian). CNP molar extinction coefficients were determined to be 17288 M<sup>-1</sup>cm<sup>-1</sup> in 50 mM phosphate buffer pH 7 and 17741 M<sup>-1</sup>cm<sup>-1</sup> in 50 mM phosphate buffer pH 7.5.

### Enzyme product analysis

To determine the limit-digest products of the *CjGH5s*, 5  $\mu$ g of each recombinant enzyme was incubated with tamarind seed XyG at final concentration of 0.25 mg mL<sup>-1</sup> for 7 hours (40 °C) in a 200  $\mu$ L reaction mixture that contained 50 mM phosphate buffer of the optimum pH of the tested enzyme (pH 7.5: *CjGH5D* and *CjGH5E*, or pH 7: *CjGH5F*). The reaction mixture was then diluted 5 times prior to product analysis by HPAEC-PAD. To determine the mode of action of the enzyme, 0.01  $\mu$ g of *CjGH5s* was incubated at 40 °C with 1 mg mL<sup>-1</sup> final concentration of tamarind seed XyG in 200  $\mu$ L reaction volumes containing the same buffers used in limit-digest

analysis. The reaction was stopped at different time points by adding 100  $\mu\text{L}$  of  $\text{NH}_4\text{OH}$ .

Reaction mixtures were then diluted 2 times with water prior to product analysis by HPAEC-PAD.

### Inhibition kinetics and active-site labeling

Inhibition kinetic parameters were determined as previously described [59]. Briefly, a final concentration of 0.23  $\mu\text{M}$  of CjGH5D was incubated with a series of different concentrations (0.5-16 mM) of XXXG-NHCOCH<sub>2</sub>Br at 40 °C in 20 mM phosphate buffer (pH 7.5) for up to 90 minutes. BSA to a final concentration of 0.1 mg. mL<sup>-1</sup> was added to the inhibition mix to prevent the non-specific loss of activity. Small samples (10  $\mu\text{L}$ ) of the incubate were periodically diluted 1:100 in 20 mM phosphate buffer (pH 7.5), and 100  $\mu\text{L}$  of the diluted incubate was added to 100  $\mu\text{L}$  of the pre-incubated substrate XXXG-CNP at 40 °C (0.1 mM final substrate concentration in the assay). Residual activities of the enzyme was determined by measuring the rate of the release of the chromophore 2-chloro-4-nitrophenolate [69] at 405 nm in Agilent Cary 60 UV-Vis Spectrophotometer. Initial-rate kinetics were measured in the strictly linear range of the enzyme. Equations 1 and 2 were used to determine  $K_i$  and  $k_i$  values by non-linear regression curve-fitting using OriginPro 2015 software as previously described [59].

$$V = V_0 \exp(-k_{\text{app}}t) + y_{\text{offset}} \quad (1)$$

$$k_{\text{app}} = \frac{k_i [I]}{K_i + [I]} \quad (2)$$

Intact protein masses were determined on a Waters Xevo Q-TOF with a nanoACQUITY UPLC system, according to the method previously published [68], with 2.5 mM inhibitor and 4.52  $\mu$ M of enzyme.

### Construction of *C. japonicus* mutants and growth conditions

In-frame deletion mutants were made as previously described [9]. Briefly, 500bp regions up- and down-stream of the genes of interest were amplified by PCR (CJA\_3010, CJA\_3337, and CJA\_2959) or synthesized by GeneWIZ (South Plainfield, NJ) (CJA\_2477) and assembled into pK18*mobsacB* by the method of Gibson, *et al.* [71]. Deletions were confirmed by PCR. For a complete list of primers used see Additional file 1: Table S8. Cultures were grown at 30 °C with 200 RPM shaking in MOPS minimal media containing 0.25% (w:v) glucose or 0.5% (w:v) tamarind seed xyloglucan (Megazyme) as the sole carbon source in 18 mm test tubes or in 96 well flat bottom polystyrene plates (Corning). Growth was measured using a Spec20D+ spectrophotometer (Thermo Scientific) or a Tecan Plate reader (Tecan, Switzerland). All experiments were performed in biological triplicate. Statistical analysis was performed using Graphpad Prism 6 software package (La Jolla, CA) where appropriate.

### Crystallization, X-ray crystallography and structure solution

*CjGH5D* was crystallized in sitting drops by the Vapour Diffusion method, using protein at 21 mg mL<sup>-1</sup> in 50 mM sodium citrate pH 6.5, 10 % glycerol over a well solution comprised of 1.9 M ammonium sulfate, 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.5. The drop consisted of 0.5  $\mu$ l enzyme, 0.1  $\mu$ l seed stock and 0.4  $\mu$ l well solution, and the seed stock was prepared by vortexing crystals, grown in 1.4 M ammonium sulfate, 0.1 M MES pH 5.5, 1 % (w/v) polyethylene glycol 1,000, in an Eppendorf tube with a polystyrene bead. Crystals were

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4 harvested into liquid nitrogen using nylon CryoLoops™ (Hampton Research). A non-ligand  
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6 complexed “apo” dataset was collected from a crystal after immersing for a few minutes in a  
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8 cryoprotectant solution, comprised of the mother liquor supplemented with 20 % (v/v) glycerol.  
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10 Data were collected at Diamond beamline I04, and processed using *DIALS* [72], and scaled using  
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14 585 *AIMLESS* [73] to 1.6 Å. The space group is P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> with unit cell dimensions 55.0, 96.4, 159.0  
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16 Å, and there are 2 molecules in the asymmetric unit.  
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20 The structure was solved by molecular replacement using *Phaser* [74] using residues 138 to  
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22 500 of PDB entry 3zmr as the search model [22], which align to residues 106 to 464 of CjGH5D,  
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24 with which they share 38% identity (using the program *lalign* from the FASTA package [75]).  
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27 590 The structure was built automatically using *Buccaneer* [76] and refined using cycles of manual  
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29 model rebuilding using *Coot* [77] followed by refinement with *REFMAC* [78], including cycles  
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31 using anisotropic B-factor refinement. In addition to the two protein chains, there are 20  
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33 molecules of glycerol, 5 sulfate ions and 3 molecules of PEG (introduced from the seed stock  
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35 solution). Data collection and refinement statistics for all structures are given in Additional file  
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39 595 1: Table S9.  
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43 A crystal of CjGH5D, grown as above over a well solution comprised of 2.3 M ammonium  
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45 sulfate, 0.1 M MES pH 5.5, was soaked for 27.5 hours in 1.85 M ammonium sulfate, 0.1 M MES  
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47 pH 5.5 with 4.5 mM XXXG-NHCOCH<sub>2</sub>Br, and fished directly into liquid nitrogen. Data were  
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49 collected at Diamond beamline I03, and processed using *DIALS* [72]. After scaling with  
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52 600 *AIMLESS* [73] the data were cut off at a resolution of 2.1 Å, as although the X-ray images  
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54 showed significant spot smearing, the R<sub>merge</sub> and CC<sub>1/2</sub> values were good (6.2% overall, 54.7% in  
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56 the outer shell and 0.998 overall, 0.933 in the outer shell respectively).  
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Crystals grown under similar conditions, over a well containing 1.6 M ammonium sulfate, 0.1 M MES pH 5.5, were soaked in the presence of a mixture of Glc<sub>12</sub>-based XyGOs (produced as described in [46] at a concentration of 5 mM for 5 hours, before fishing into liquid nitrogen via a cryoprotectant solution, as for the apo crystal. A dataset was collected at Diamond beamline I04 and processed using *DIALS* and scaled using *AIMLESS* to 1.9 Å.

Both ligand structures were solved initially using the apo structure as a model for *REFMAC*, and the ligand was placed after the protein chain had been rebuilt (using cycles of Coot interspersed with refinement in *REFMAC*) and some water molecules added. All models were validated using *MolProbity* [79] and the sugar conformations of the ligand in the complex structures were checked using Privateer [80]. Problems with diffraction anisotropy in both ligand datasets limited the possibility of refining the structures to R/Rfree lower than 0.22/0.28 and 0.23/0.30 for the complexes with XXXG-NHCOCH<sub>2</sub> and GXLG (produced after the Glc<sub>12</sub> soak) respectively.

## Additional file

Additional file 1. Supporting Tables 1-9 and Supporting Figures 1-13.

## Abbreviations

XyG, tamarind seed xyloglucan; XyGO, xylogluco-oligosaccharides; CAZy, carbohydrate active enzymes; GH5, glycoside hydrolase family 5; CBM, carbohydrate binding module; SRL, serine rich linker; HPAEC-PAD, high performance anion exchange chromatography- pulsed amperometric detector; MALDI-TOF, matrix assisted laser desorption ionization- time of flight; KGM, konjac glucomannan; BBG, barley β-glucan; HEC, hydroxyethylcellulose; CNP 2-chloro-

4-nitrophenyl; BCA, bicinchoninic acid; TBDT, TonB-dependent transporter; XyGUL,  
xyloglucan utilization locus; MES, 0.1 M 2-(N-morpholino)ethanesulfonic acid.

## Authors' contributions

MA performed the bioinformatic analysis, recombinant protein production, and biochemical characterization. CN performed the RNAseq analysis and all mutational analysis. NJ synthesized the active-site affinity label and, together with MA, performed inhibition and protein MS analysis. JGG designed the RNAseq and mutational analysis experiments, and assisted in analyzing the RNAseq and mutational data. WAO solved X-ray structures which were analysed under supervision of GJD. HB devised the overall study and supervised research. All authors contributed to writing the article.

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## Competing interests

The authors declare that they have no competing interests.

## Availability of data and materials

The structural datasets generated during the current study are available in the RCSB protein repository under the following PDB IDs: *CjGH5D*: 5OYC, XXXG-NHCOCH<sub>2</sub>-*CjGH5D*: 5OYD, *CjGH5D*-GXLG: 5OYE (<https://www.rcsb.org/>). The RNAseq datasets generated during the current study are available in the NCBI Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>) repository under accession GSE109594. Other datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Consent for publication

Not applicable.

## Ethical approval and consent to participate

Not applicable.

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## Supporting information

### Additional file 1: Supporting tables

**Table S1.** List of genes up-regulated during exponential growth on xyloglucan compared to glucose.

**Table S2.** List of genes with low-level constitutive expression on xyloglucan.

**Table S3.** List of genes up-regulated during stationary phase on xyloglucan compared to glucose.

**Table S4.** List of genes up-regulated during stationary phase compared to exponential phase on xyloglucan.

**Table S5.** Identity matrix between *CjGH5\_4* enzymes and other specific previously characterized endo-xyloglucanases.

**Table S6.** Maximum growth and growth rate of *C. japonicus* strains using reduced or limiting concentrations of XyG.

**Table S7.** Primer sequences used for recombinant protein production.

**Table S8.** Primers used for generation of in-frame deletion mutants.

**Table S9.** X-ray data collection and refinement statistics for *CjGH5D*.

### Additional file 1: Supporting figures

**Figure S1. Volcano plots summarizing the RNAseq data for a comparative analysis of *C. japonicus* exponential phase cells grown on either glucose or xyloglucan.** The volcano plot represents a comparison between exponentially growing cells (glucose vs xyloglucan). Each gray circle denotes a single gene, and the blue-filled circles indicate up-regulated CAZyme genes. The complete list of up-regulated CAZyme genes can be found in Additional file 1: Table S1. Fold change in gene expression ( $\log_2$  scale) is plotted on the x-axis and p-value ( $-\log_{10}$  scale) is plotted on the y-axis. For orientation on the x-axis (fold change), positive values indicate genes that are up-regulated when grown using xyloglucan as the sole carbon source. The red dashed lines indicate significance cut-off values (2-fold for gene expression and p-value of 0.01).

**Figure S2. Volcano plots summarizing transcriptomic analysis of *C. japonicus* cells grown on either glucose or xyloglucan.** The volcano plots represent comparisons between **A)** stationary phase cells (glucose vs xyloglucan), or **B)** xyloglucan grown cells in either exponential

or stationary phase. Each gray circle denotes a single gene, and the blue-filled circles indicate up-regulated CAZyme genes. The complete list of up-regulated CAZyme genes for panel A can be found in Additional file 1: Table S3, and for panel B in Additional file 1: Table S4. The fold change ( $\log_2$  scale) is plotted on the x-axis and the p-value ( $-\log_{10}$  scale) is plotted on the y-axis. For orientation on the x-axis, positive values indicate genes that are up-regulated when grown using xyloglucan as the sole carbon source for panel A, and genes up-regulated during stationary phase for panel B. The red dashed lines indicate the significance cut-off values (2-fold for gene expression and p-value of 0.01).

**Figure S3. Modular architecture of the native *CjGH5\_4* enzymes with the different expression constructs used in the current study. A)** The locus CJA\_3010 (GenBank

ACE84905.1) encodes a signal peptide, a serine rich linker, and a GH5 catalytic domain

(*CjGH5D*). **B)** CJA\_3337 (GenBank ACE83841.1) encodes a signal peptide, two carbohydrate

binding modules (CBM2 and CBM10), and a GH5 catalytic domain (*CjGH5E*). **C)** CJA\_2959

(GenBank ACE86198.1) encodes a signal peptide, an FN3 domain and a GH5 catalytic domain

(*CjGH5F*). All expression constructs were designed to produce 6x His-Tag at the N-terminus of

the recombinant protein.

**Figure S4. Amino acid sequence alignment showing regions of structural similarity between the catalytic domains of *Cellvibrio japonicus* GH5\_4 enzymes (*CjGH5D*, *CjGH5E*, and *CjGH5F*) and other members exhibiting endo-xyloglucanase activity belonging to the same family.**

*Cellvibrio japonicus* GH5s (*CjGH5D*, Accession: ACE84905.1; *CjGH5E*, Accession:

ACE83841.1; and *CjGH5F*, Accession: ACE86198.1); *Bacteroides ovatus* GH5 (*BoGH5A*,

Accession: WP\_004298445.1); *Prevotella bryantii* B14 GH5 (*PbGH5A*, Accession:

EFI71705.1); *Paenibacillus Pabuli* GH5 (*PpGH5*, PDB: 2JEP\_A); ruminal metagenomic GH5s (XEG5A, Accession: ACZ54907.1; and XEG5B, Accession: ADB44000.1). Secondary structural elements are shown for *CjGH5D*, with  $\eta$  referring to  $3_{10}$ -helices, and  $\alpha$  to  $\alpha$ -helices (displayed as small and medium squiggles respectively), and  $\beta$ -strands shown as arrows, with TT and TTT representing strict  $\beta$ -turns and strict  $\alpha$ -turns respectively. Alignment was created using Clustal Omega and Esript 3.0. Catalytic residues are marked with red asterisks, *CjGH5D* active site residues interacting with the ligands (GXLG and XXXG-NHCOCH<sub>2</sub>Br) via hydrogen bond formation and stacking interactions are marked with blue and green asterisks, respectively.

**Figure S5. pH and temperature profiles of *CjGH5\_4* enzymes with tamarind seed XyG as a substrate. A) *CjGH5D*. B) *CjGH5E*. C) *CjGH5F*.** Left panels are pH rate profiles while right panels are temperature profiles. Black squares, citrate buffer; red circles, phosphate buffer; and blue triangle, glycine buffer. Error bars represent standard error of the mean for 3 replicates. Lines were drawn to guide the eye with no physical significance.

**Figure S6. Michaelis-Menten kinetics of *CjGH5\_4* enzymes on tamarind seed XyG. A) *CjGH5D*. B) *CjGH5E*. C) *CjGH5F*.** Error bars represent standard error based on 3 replicates.

**Figure S7. HPAEC-PAD analysis of the hydrolysis time course and limit-digest of *CjGH5\_4* enzyme-xyloglucan degradation products. A) *CjGH5D*. B) *CjGH5E*. C) *CjGH5F*.**

**Figure S8. MALDI-TOF analysis of the limit digest products of *CjGH5\_4* enzymes upon incubation with tamarind seed XyG. A) *CjGH5D*. B) *CjGH5E*. C) *CjGH5F*.** The observed molecular masses of the major 3 peaks were 1085.21, 1247.29 and 1409.37; which correspond to [M+Na]<sup>+</sup> of XXXG (calculated: 1085.9), XLXG/XXLG (calculated: 1248.05), and XLLG (calculated: 1410.19), respectively.

**Figure S9. Michaelis-Menten kinetics of *CjGH5\_4* enzymes on a panel of chromogenic (xylo)gluco-oligosaccharide glycosides. A-D) *CjGH5D*. E-H) *CjGH5E*. I-L) *CjGH5F*. Error bars represent standard errors of the mean for 2 replicates. Only one replicate was done on XLLG- $\beta$ -CNP due to limited availability of the substrate.**

**Figure S10. Intact protein mass spectrometry of *CjGH5D* with the XXXG-NHCOCH<sub>2</sub>Br inhibitor. A) *CjGH5D* negative control with no inhibitor. B) *CjGH5D* incubated with 2.5 mM of the inhibitor for 3 hours at 37 °C. The peak at 44226.5 Da corresponds to *CjGH5D* (calculated 44222.2 Da) and 45330.5 Da to *CjGH5D*-inhibitor covalent adduct (calculated: 45325.2 Da). Peak at 44403.1 Da (and correspondingly at 45509.5 Da) is attributed to the post translationally modified protein due to the N-gluconylation of the His-tag of the recombinant protein.**

**Figure S11. Growth analysis control experiments for in-frame deletions mutants of the GH5\_4, and GH74 genes on xyloglucan. Cultures were grown for 24 hours at 30 °C with high aeration (200 RPM) in MOPS defined media supplemented with 0.5% (w:v) glucose as the sole carbon source. Graphs represent the average of three biological replicates and error bars represent the standard deviation, A) XyGUL mutants, B) single, double, triple, and quadruple deletion mutants of the GH5\_4 and GH74 genes. The figure can be read as follows: *cel5D* (CJA\_3010) encodes *CjGH5D*, *cel5E* (CJA\_3337) encodes *CjGH5E*, *cel5F* (CJA\_2959) encodes *CjGH5F*, and *gly74A* (CJA\_2477) encodes *CjGH74A*.**

**Figure S12. Growth analysis of *C. japonicus* strains using reduced or limiting concentrations of XyG. Wild type, a  $\Delta xyl31A$  strain, and the quadruple mutant strain were grown using A) 0.2% glucose, B) 0.5% xyloglucan, C) 0.25% xyloglucan, or D) 0.125%**

xyloglucan as the sole carbon source. Standard deviation from biological triplicate experiments is shown. Maximum OD and growth rate calculations can be found in Table S6.

**Figure S13. Growth analysis of  $\Delta$ CJA\_3010 and  $\Delta$ gsp mutant strains when using glucose or xyloglucan.** Cultures were grown in 18 mm test tubes at 30 °C with shaking at 200 RPM using MOPS minimal media supplemented with **A**) 0.25% (w:v) glucose or **B**) 0.5% (w:v) xyloglucan as the sole carbon source. Open circles represent wild type,  $\Delta$ gsp is represented by closed squares,  $\Delta$ CJA\_3010 is represented by open triangles, and  $\Delta$ CJA\_3010  $\Delta$ gsp is represented by inverted closed triangles. Graphs depict the average of biological triplicate experiments, and the error bars represent the standard deviation. The *cel5D* gene (CJA\_3010) encodes CjGH5D.

## Tables

**Table 1.** Activity of *CjGH5\_4* enzymes against different polysaccharide substrates<sup>a</sup>.

Enzyme	Substrate	$K_m$	$k_{cat}$	Specific activity
Catalytic domains		mg. mL <sup>-1</sup>	sec <sup>-1</sup>	μmol/ min. mg
<b><i>CjGH5D</i></b>	XyG	<0.025	30.3 ± 0.4	43.3 ± 1.9
<b><i>CjGH5E</i></b>	XyG	0.020 ± 0.002	10.3 ± 0.1	15.1 ± 0.1
	hydroxyethylcellulose (HEC)	ND <sup>b</sup>	ND <sup>b</sup>	0.070 ± 0.004
	carboxymethylcellulose (CMC)	ND <sup>b</sup>	ND <sup>b</sup>	0.010 ± 0.002
<b><i>CjGH5F</i></b>	XyG	0.040 ± 0.003	52.4 ± 0.8	74.8 ± 4.1
	hydroxyethylcellulose (HEC)	ND <sup>b</sup>	ND <sup>b</sup>	0.090 ± 0.003

<sup>a</sup>Assays conducted at pH 7.5 (*CjGH5D* and *CjGH5E*) or pH 7 (*CjGH5F*). Recombinant enzymes were incubated at 50 °C (*CjGH5D* and *CjGH5F*) or 55 °C (*CjGH5E*) with the different tested substrates.

<sup>b</sup>Not determined due to poor specific activity.

**Table 2.** Kinetic parameters of *CjGH5\_4* enzymes for (xylo)gluco-oligosaccharide glycosides

Enzyme	Substrate	$K_m$	$k_{cat}$	$k_{cat}/K_m$
catalytic domains		mM	min <sup>-1</sup>	min <sup>-1</sup> · mM <sup>-1</sup>
<b><i>CjGH5D</i></b>	GGG-CNP	ND <sup>a</sup>	ND <sup>a</sup>	2.21 ± 0.05
	GGGG-CNP	ND <sup>a</sup>	ND <sup>a</sup>	5.36 ± 0.07
	XXXG-CNP	0.81 ± 0.10	281 ± 12	347 ± 45
	XLLG-CNP	0.18 ± 0.02	162 ± 4	900 ± 103
<b><i>CjGH5E</i></b>	GGG-CNP	11.8 ± 0.6	191 ± 7	16.2 ± 1.0
	GGGG-CNP	5.02 ± 0.35	180 ± 7	35.9 ± 2.8
	XXXG-CNP	0.010 ± 0.001	254 ± 4	(25.4 ± 2.6) × 10 <sup>3</sup>
	XLLG-CNP	0.010 ± 0.001	332 ± 9	(33.2 ± 3.4) × 10 <sup>3</sup>
<b><i>CjGH5F</i></b>	GGG-CNP	ND <sup>a</sup>	ND <sup>a</sup>	6.45 ± 0.30
	GGGG-CNP	ND <sup>a</sup>	ND <sup>a</sup>	16.5 ± 1.0
	XXXG-CNP	0.07 ± 0.01	169 ± 4	(2.41 ± 0.35) × 10 <sup>3</sup>
	XLLG-CNP	0.030 ± 0.002	393 ± 8	(13.1 ± 0.9) × 10 <sup>3</sup>

<sup>a</sup>not determined due to limited availability of substrate.

## Figure legends

**Fig. 1. Xyloglucan (XyG) and the xyloglucan utilization system in *C. japonicus*.** **A)** Structure of dicot XXXG- type fucogalacto-XyG. XyG substructure nomenclature is according to [20]. **B)** *C. japonicus* genes involved in XyG utilization. Genes encoding backbone-cleaving endo-xyloglucanases (GH5 and GH74) are indicated in navy blue, genes encoding side-chain-cleaving exo-glycosidases (GH35  $\beta$ -galactosidases; GH31  $\alpha$ -xylosidases and GH95  $\alpha$ -L-fucosidase) are in cyan, and the TonB dependent transporter (TBDT) is shown in green. **C)** Spatial model of XyG utilization in *C. japonicus*.

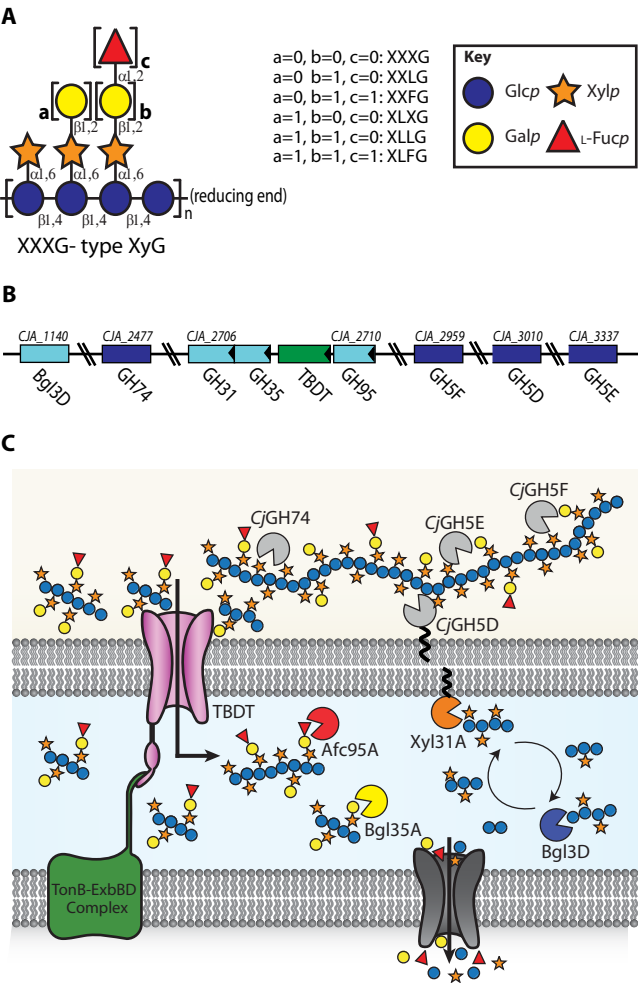
**Fig. 2. Inhibition kinetics of *Cj*GH5D with XXXG-NHCOCH<sub>2</sub>Br.** **A)** Initial-rate enzyme activity over time (single determinations). **B)** Pseudo-first-order rate constants ( $k_{app}$ ) obtained from the fitted curves shown in panel A. Bars represent errors in  $k_{app}$  values from curve-fitting. The 95% confidence interval is indicated (pink band) for the fitted curve (solid line).

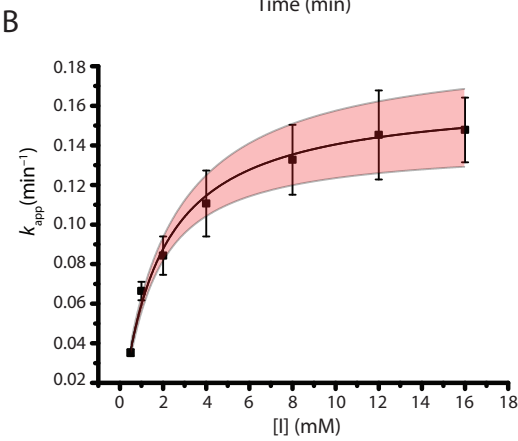
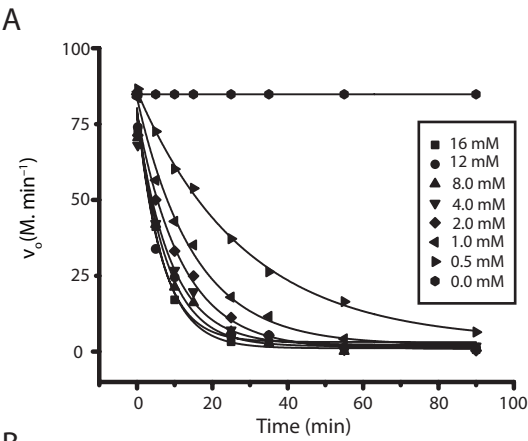
**Fig. 3. Three-dimensional structure of *Cj*GH5D in complex with XXXG-NHCOCH<sub>2</sub>Br and XyGOs.** **A)** Cartoon representation of the secondary structure of *Cj*GH5D colour ramped from the N-terminus (blue) to the C-terminus (Red). The two ligands XXXG-NHCOCH<sub>2</sub>Br and GXLG are overlaid in the active site cleft and shown in green and magenta sticks, respectively. **B)** A close-up view of the active site cleft with the overlaid ligands XXXG-NHCOCH<sub>2</sub>Br in green and GXLG in magenta showing different amino acids interacting with the carbohydrate ligands. **C)**  $2F_o - F_c$  ( $\sigma_A$ /maximum likelihood weighted) electron density contoured in blue around GXLG in the *Cj*GH5D-GXLG complex (left panel) and the chemical structure of the corresponding ligand (Right panel). Insufficient electron density was observed for the -4' xylosyl residue to allow modelling, therefore it is shown in grey. **D)**  $2F_o - F_c$  electron density at  $1\sigma$

(approx.  $0.2 \text{ e}^-/\text{\AA}^3$ ) contoured in blue around XXXG-NHCOCH<sub>2</sub> moiety in the CjGH5D-XXXG-NHCOCH<sub>2</sub>Br complex (Left panel) and chemical structure of the corresponding ligand (Right panel). The bromide leaving group is shown in grey.

**Fig. 4. Divergent (wall-eyed) stereo surface representation of CjGH5D-GXLG showing regions of sequence conservation.** Surfaces of conserved and non-conserved residues, shown in purple at reduced opacity and sea-green, respectively, were calculated from an amino acid sequence alignment of GH5 domains of CjGH5D, CjGH5E, CjGH5F and five additional GH5 members showing E.C. 3.2.1.151 activity (Additional file 1: Figure S4). Figure was generated using CCP4MG [81].

**Fig. 5. Growth analysis of in-frame deletions of GH5\_4, and GH74 mutant strains on xyloglucan.** **A)** Control experiment with XyGUL in-frame deletion mutant strains. **B)** Single, **C)** double, **D)** triple and quadruple deletion mutants were made with the GH5\_4 and GH74 genes; CJA\_3010 encodes CjGH5D, CJA\_3337 encodes CjGH5E, CJA\_2959 encodes CjGH5F, and CJA\_2477 encodes CjGH74. Graphs represent the average of three biological replicates and error bars represent the standard deviation. All strains grew like wild-type when grown with MOPS-glucose defined medium (Additional file 1: Figure S12).





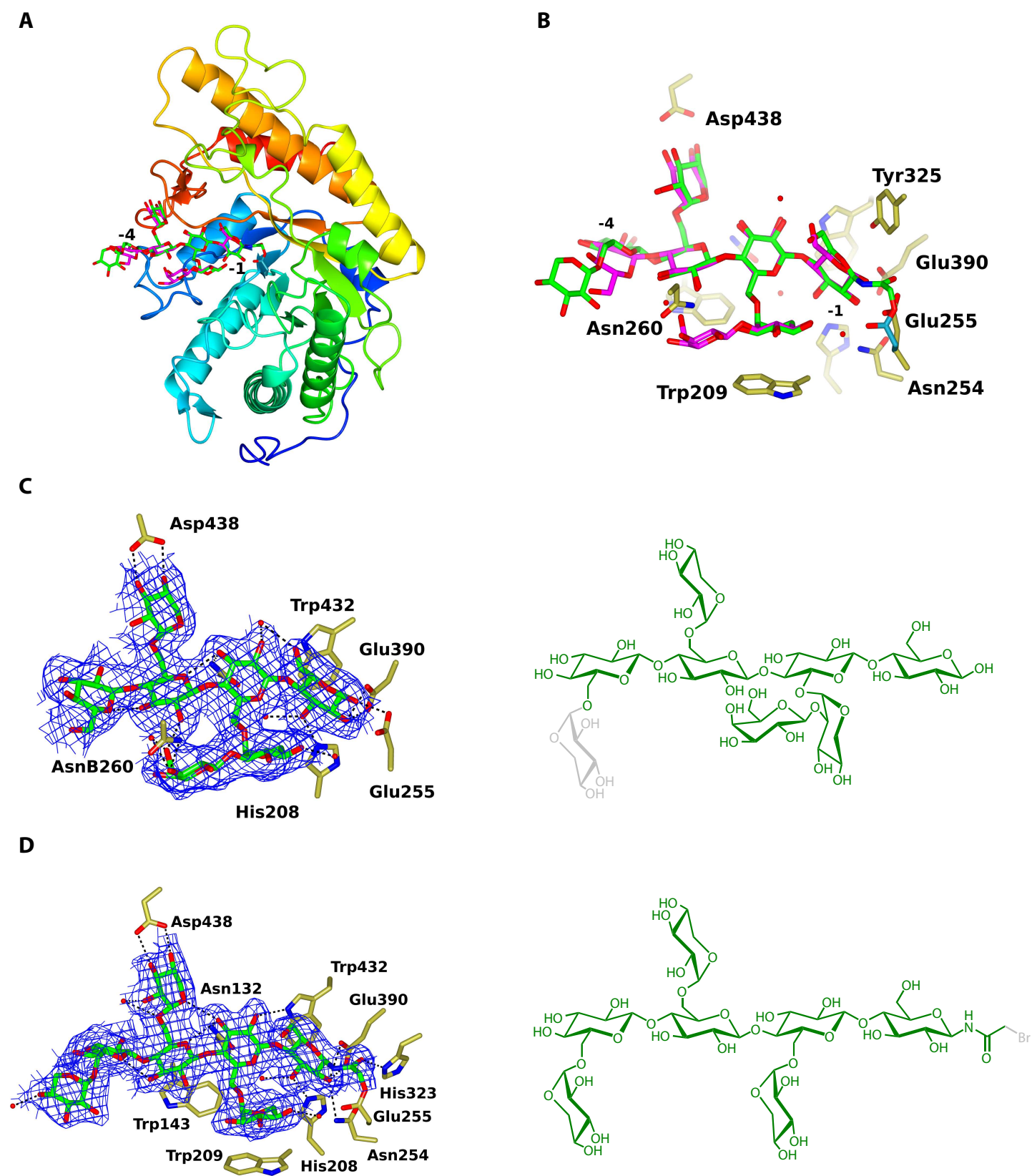


Figure 4

[Click here to download Figure Fig\\_4 stereo surface with labels only conserved purple.pdf](#)

