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Multiple enzymatic approaches to hydrolysis of fungal β-glucans by the soil bacterium *Chitinophaga pinensis*

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The genome of the soil Bacteroidota *Chitinophaga pinensis* encodes a large number of glycoside hydrolases (GHs) with noteworthy features and potentially novel functions. Several are predicted to be active on polysaccharide components of fungal and oomycete cell walls, such as chitin, β-1,3-glucan and β-1,6-glucan. While several fungal β-1,6-glucanase enzymes are known, relatively few bacterial examples have been characterised to date. We have previously demonstrated that *C. pinensis* shows strong growth using β-1,6-glucan as the sole carbon source, with the efficient release of oligosaccharides from the polymer. We here characterise the capacity of the *C. pinensis* secretome to hydrolyse the β-1,6-glucan pustulan and describe three distinct enzymes encoded by its genome, all of which show different levels of β-1,6-glucanase activity and which are classified into different GH families. Our data show that *C. pinensis* has multiple tools to deconstruct pustulan, allowing the species' broad utility of this substrate, with potential implications for bacterial biocontrol of pathogens via cell wall disruption. Oligosaccharides derived from fungal β-1,6-glucans are valuable in biomedical research and drug synthesis, and these enzymes could be useful tools for releasing such molecules from microbial biomass, an underexploited source of complex carbohydrates.

Introduction

The enzymatic deconstruction of complex carbohydrates is an integral part of the carbon cycle [1,2], contributing to plant health [3,4], soil fertility [5,6] and other biogeochemical processes [7,8]. The enzymes used by soil bacteria to break down biomass are mostly glycoside hydrolases (GHs) [9]. These enzymes have exquisite specificity for glycosidic linkages and structures, making them useful tools in research and industrial biotechnology [10].

Bacteria of the Bacteroidota phylum are dominant in essentially all studied microbial ecosystems where biomass recycling occurs [10–15], they tend to have

Abbreviations
CAZymes, carbohydrate active enzymes; CBM, carbohydrate-binding module; G2, gentiobiose (β-1,6-linked glucose disaccharide); G3, gentiotriose; G4, gentiotetraose; G5, gentiopentaose; GH, glycoside hydrolase; Glc, glucose; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; L2, laminaribiose (β-1,3-linked glucose disaccharide); L3, laminaritriose; L4, laminaritetraose; L5, laminaripentaose; YBG, yeast β-glucan.

large numbers of genes encoding carbohydrate active enzymes (CAZymes) [15]. An exemplar species, *Chitinophaga pinensis*, makes extensive use of secreted CAZymes for polysaccharide breakdown, and we have proposed the large genome of the species as a valuable source of new activities [16–19]. Specifically, a number of *C. pinensis* CAZymes are predicted to target glycans found in fungal biomass, including chitin, β-1,3-glucans and β-1,6-glucans. Such enzymes were identified in proteomic analysis of the *C. pinensis* secretome [17,18], and we have shown that the species is capable of extensive deconstruction of β-1,3-glucans and β-1,6-glucans. Indeed, β-1,6-glucan is a preferred carbon source [19], and the *Chitinophaga* genus is enriched where mycelium is degraded [20].

The β-1,6-glucan known as pustulan is water-extractable from lichenised *Umbilicariaceae* fungi [21]. The structure is also found in certain fungal and oomycete cell walls, often cross-linked to mannoproteins, chitin and/or β-1,3-glucans [22]. Enzymatic attack on the cell wall of potentially phytopathogenic fungi and oomycetes is a cornerstone of microbial biocontrol [23–25]. In addition, oligosaccharides produced by β-1,6-glucan hydrolysis represent an interesting set of bioactive molecules. They are thought to have immunostimulatory properties [26] and have been used to synthesise complex glycans with prebiotic and immune-modulatory functions [27,28].

β-1,6-Glucanases have largely been found in subfamily GH30_3 in the Carbohydrate Active enZymes (CAZy) database (http://www.cazy.org/, [29]). Most are fungal proteins thought to be transglucosidases used in cell wall remodelling [30]. The first bacterial β-1,6-glucanase discovered was Gly30B from the marine bacterium *Saccharophagus degradans* [31]. Others have since been found in a human gut symbiont [30] and in fungus-preying myxobacteria, where the activity restricts fungal infection of plants [25]. This echoes the ability of some GH30_3 enzymes from *Trichoderma* that contribute to mycoparasitism [32,33]. Fungal β-1,6-glucanases can also be found in family GH5, as is one bacterial enzyme, Gly5M (subfamily GH5_47) from *S. degradans* [34].

Here, we describe three enzymes produced by *C. pinensis*, each belonging to a different GH family and all displaying some degree of β-1,6-glucanase functionality. Cpin_4356 (GH30_3) displays the β-1,6-glucanase activity predicted for its subfamily and is appended to a pustulan-binding carbohydrate-binding module (CBM) from the recently established family 92 [35]. Cpin_2580 belongs to subfamily GH5_46 and has specificity for pustulan, albeit with low product formation. Finally, in addition to the expected ability to deconstruct β-1,3-glucan, the GH64 enzyme Cpin_3536 shows a surprising capacity for hydrolysing pustulan when appended to a CBM6 domain, a gain-of-function that likely derives from the binding specificity of the modular protein. The GH30_3 enzyme is by far the most efficient of those we have characterised here, while the GH5_46 and GH64 enzymes may be showing a 'side activity' in these assays. Nonetheless, the redundancy apparent in possessing three pustulan-degrading enzymes with different efficacies likely promotes the stable use of β-1,6-glucan as a carbon source. These data expand our view of which GH families can produce β-1,6-glucanases and offer new enzymes showcasing *C. pinensis* as an industrial toolbox and potential agent of biocontrol [25].

Results and discussion

Chitinophaga pinensis secretes a number of enzymes with predicted activities against fungal cell wall β-glucans

Analysis of the *C. pinensis* predicted proteome indicates the potential for extensive degradation of β-glucans deriving from microbial cell walls. We are particularly motivated to discover enzymes targeting β-1,6-glucans as these most commonly occur in fungi and oomycetes, representing a potential target for infection diagnostics in humans [36] and disease control in plants [25,32,33]. Our previous data showed that *C. pinensis* grows strongly on β-1,6-glucan, with extracellular deconstruction of the polysaccharide [19]. To check for activity, in this study the bacterium was grown in minimal medium containing either glucose, pustulan, curdlan (a linear β-1,3-glucan) or yeast β-glucan (YBG; a β-1,3-glucan with β-1,6 branches) as sole carbon source. Growth on the β-1,6-Glc-containing pustulan and YBG was stronger than growth on curdlan or glucose (Fig. 1A). After removing cells by centrifugation, protein content in the medium was measured (Fig. 1B), and the secretomes were assayed for the ability to hydrolyse glycans resembling those found in fungal and oomycete cell walls. Both pustulan and YBG induced high levels of overall activity, with far lower levels seen for curdlan cultures (Fig. 1C). The high protein yield from curdlan cultures suggests an abundant secretion of proteins not related to the hydrolysis of glycans tested here, but all secretomes could hydrolyse both β-1,3- and β-1,6- linked glucans to different extents. Interestingly, while the pustulan- and YBG-induced secretomes showed some chitinase activity, this was not the case for the curdlan secretomes and may indicate that the Glc-β-1,6-Glc

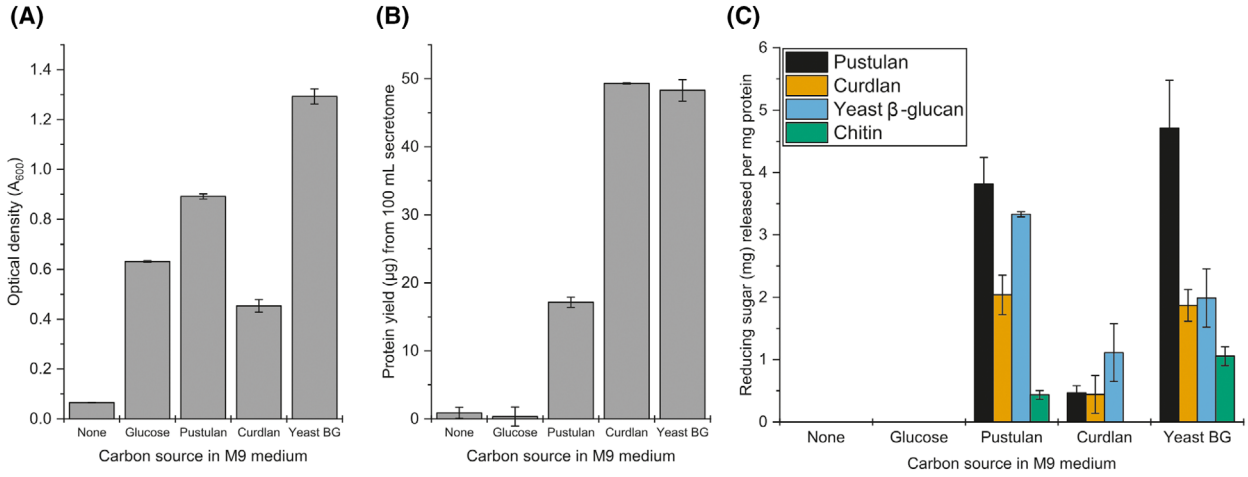

Fig. 1. Secretome analysis of *Chitinophaga pinensis* grown on fungal/oomycete type of cell wall components. (A) The optical density of cultures was measured after 3 days of incubation at 25 °C. (B) Protein concentrations in secretomes were measured using a Bradford assay. (C) Secretomes were assayed for the ability to hydrolyse polysaccharides representing some of the major glycan components of fungal and oomycete cell walls, using a DNSA-reducing sugar assay. Yeast BG = yeast β-glucan. All analyses were performed in triplicate: Error bars indicate SEM.

linkage induces chitinase secretion. This would allow the bacterium to target intact cell walls and may be mediated via Polysaccharide Utilisation Loci [10,19].

Characterisation of enzyme activities

As part of an ongoing effort to screen for novel enzyme activities, and based on an analysis of the CAZy-annotated genome of *C. pinensis* alongside our previous proteomic work [17,18], three candidate β-glucanases were identified, cloned and produced in recombinant form. The first panels in Figs 2–4 show the results of protein production and purification experiments. The protein we refer to as *Cp*Glu30A-CBM92A is encoded by the gene Cpin_4356 and includes a C-terminal CBM92 domain and a catalytic GH30_3 module. Due to its membership in CAZy subfamily GH30_3, this enzyme was identified as a candidate β-1,6-glucanase in our previous proteomic study, where it was detected in secretomes from cultures grown on polysaccharides [17]. The *Cp*Glu30A-CBM92A protein was produced in an intact two-domain form. We cloned variants lacking the CBM92 domain, but these could not be produced in soluble form. An N-terminal catalytic domain was cloned from the gene Cpin_2580 and is referred to as *Cp*Glu5$_N$. We previously proposed that the GH5 module of the Cpin_2580 protein could be a mannanase, as it was found at low levels in a mannan-induced secretome [17]. However, this enzyme is a member of the relatively under-explored GH subfamily 5_46, which is now known to include a bifunctional cellulase-xylanase [37] and a cellulase identified in a rumen metagenome [38], suggesting the potential for β-glucanase activities. The *Cp*Glu5$_N$ module proved to be insoluble unless produced as part of the full-length multi-modular Cpin_2580 protein. Finally, the gene Cpin_3536 encodes a bi-modular CAZyme comprising a GH64 putative β-glucanase and a CBM6 domain. This protein was detected in all secretomes analysed in our previous proteomic work, a study that indicated there may be constitutive production of certain enzymes involved in fungal cell wall deconstruction [17]. As well as these domains that we, respectively, refer to as *Cp*Glu64A and *Cp*CBM6A, which were produced and purified as separate recombinant proteins, the full-length Cpin_3536 protein contains βγ-crystallin regions, a common feature of CAZymes and thought to be involved in Ca^{2+} binding [39]. The modular recombinant protein *Cp*Glu64A-CBM6A analysed in this work also retains the C-terminal domain used by Bacteroidota for targeting the phylum-specific type 9 secretion system [40]. All of the produced enzymes were subjected to preliminary activity screens against a range of β-glucans (Table 1).

CpGlu30A is an endo-β-1,6-glucanase appended to a pustulan-binding CBM92 domain

The enzyme encoded by Cpin_4356 belongs to subfamily GH30_3 and so was expected to function as a β-1,6-glucanase. Bacterial enzymes from this subfamily

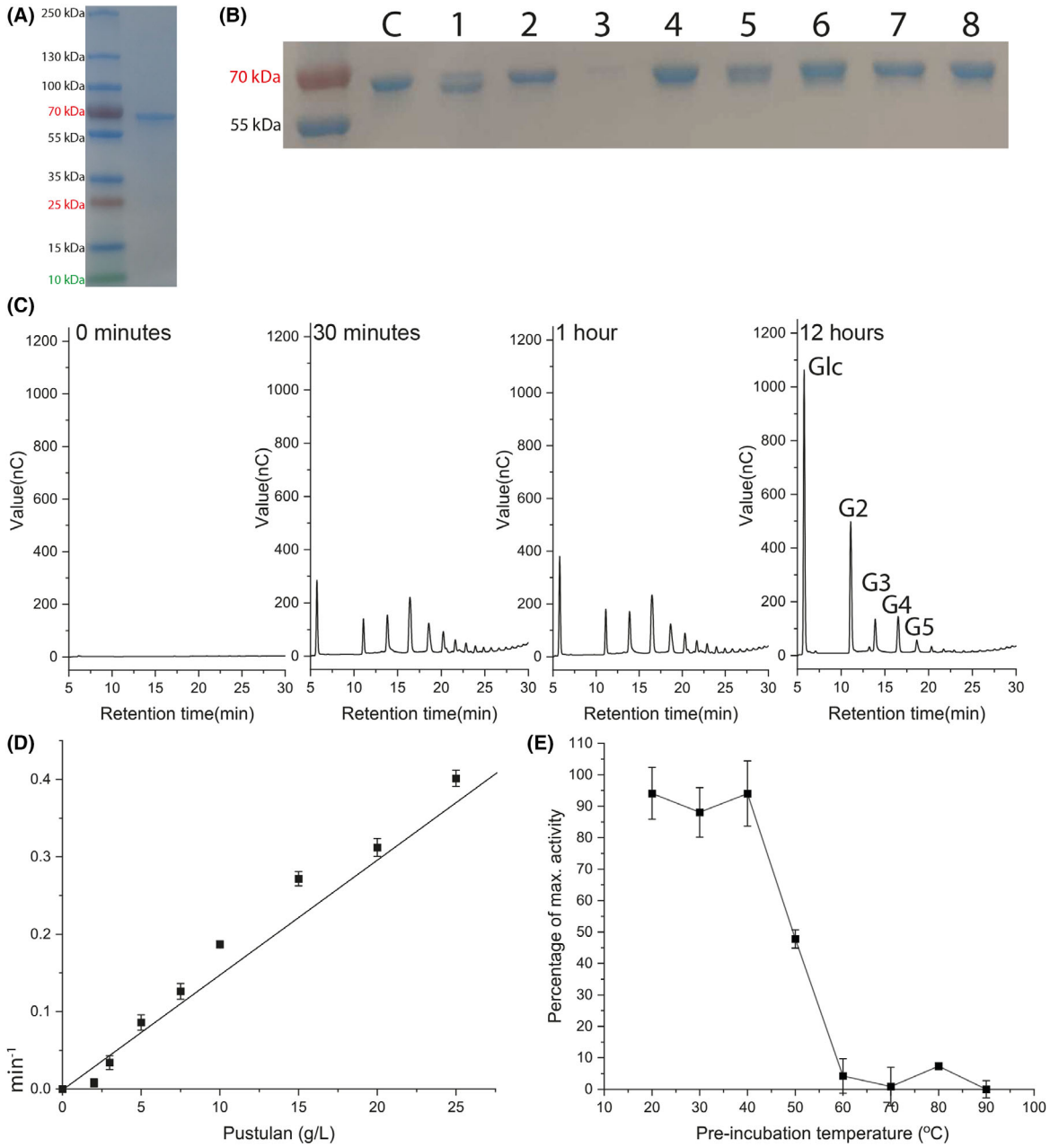

Fig. 2. *Endo*-β-1,6-glucanase activity of *Cp*Glu30A-CBM92A. (A) SDS/PAGE analysis of *Cp*Glu30A-CBM92A recombinant production. (B) Polysaccharide binding profile of *Cp*Glu30A-CBM92A. If a protein is absent or the intensity of the corresponding band is reduced in the SDS/PAGE gel, this indicates binding to the tested polysaccharides. The ligands used in this experiment are as follows: C, water (negative control); 1, scleroglucan; 2, lichenan; 3, pustulan; 4, barley β-glucan; 5, curdlan; 6, α-chitin; 7, β-chitin; 8, shrimp shell chitin. These gels show representative results of experiments that were repeated at least three times. The protein showed binding only to pustulan (a linear β-1,6-glucan). (C) *Cp*Glu30A-CBM92A is able to cleave pustulan into a series of β-1,6-linked gluco-oligosaccharides. Enzyme at 100 nM was incubated with the substrate at 2 g·L^{-1}. Time-course assays show reaction products becoming increasingly shorter over time, confirming the *endo* mode of action of the enzyme. Only two of the final reaction products (glucose and gentiobiose) could be definitively identified on HPAEC-PAD, as standards are not commercially available for longer β-1,6-linked gluco-oligosaccharides. The identities of other labelled oligosaccharides are surmised based on the retention time of laminarin-oligosaccharides. (D) Kinetic analysis of *Cp*Glu30A-CBM92A activity on pustulan. (E) Thermostability of *Cp*Glu30A-CBM92A activity against pustulan. Kinetic and thermostability experiments were performed in triplicate: Error bars indicate SEM.

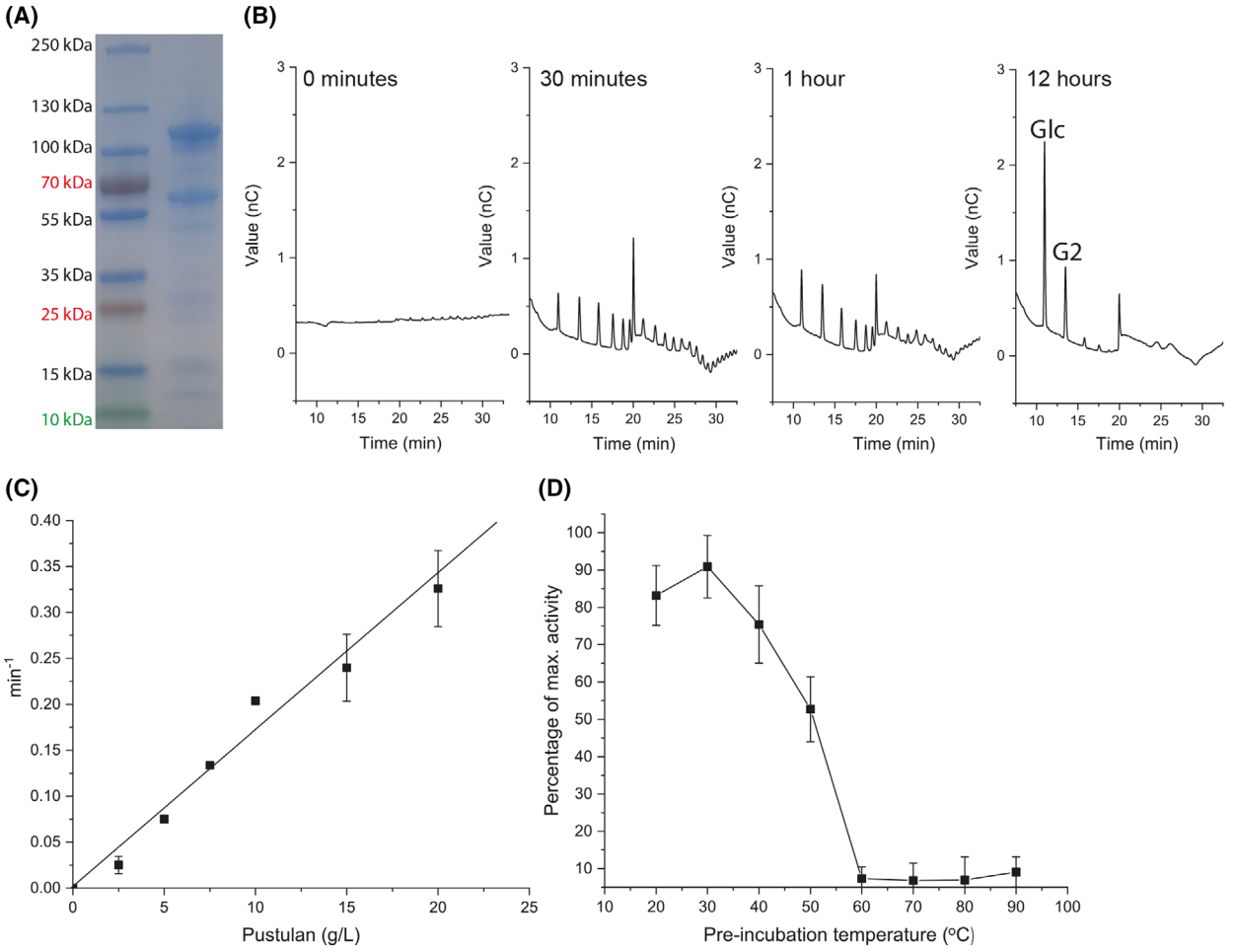

Fig. 3. *Endo*-β-1,6-glucanase activity of *Cp*Glu5$_N$. (A) SDS/PAGE analysis of *Cp*Glu5$_N$ recombinant production. Note that this domain could only be solubly and stably produced as part of the larger Cpin_2580 protein. The second band visible is a cleavage product lacking the *Cp*Glu5$_N$ domain and was verified not to possess any glucanase activity. (B) *Cp*Glu5$_N$ is able to cleave pustulan into a series of β-1,6-linked gluco-oligosaccharides. Enzyme at 100 nM was incubated with the substrate at 2 g·L^{-1}. Time-course assays show reaction products becoming increasingly shorter over time, confirming the *endo* mode of action of the enzyme. (C) Kinetic analysis of *Cp*Glu5$_N$ activity on pustulan. (D) Thermostability of *Cp*Glu5$_N$ activity against pustulan. Kinetic and thermostability experiments were performed in triplicate: Error bars indicate SEM.

have been discovered in marine [31] and gut [30] environments, and a structural investigation revealed an adaptation in the active site to accommodate the 'hook-like' structure of the pustulan substrate [30]. Indeed, our activity screens found that *Cp*Glu30A-CBM92A shows specificity for pustulan, with about ~ 10% relative activity on YBG, which has a β-1,3-glucan main chain with β-1,6-linked glucosyl branches. An assay revealed that the *Cp*Glu30A-CBM92A protein binds to pustulan exclusively (Fig. 2B). We propose that this binding derives from the CBM92 domain. A major biological function of CBMs is to increase the reaction rate by increasing the length of time an appended enzyme spends in proximity with its substrate [41], which typically occurs when a CBM binds to the same polysaccharide as its partner enzyme can hydrolyse. To date, only one characterised example of a CBM92 has been published: it displayed binding to carrageenan and was found natively appended to a κ-carrageenase enzyme named Cgk16A [35]. As the domains interact with the same polysaccharide, the CBM92 domain attached to Cgk16A may thereby improve the efficiency of the enzyme to which it is attached, and this is likely also the case for *Cp*Glu30A-CBM92A, perhaps suggesting a general role for CBM92 domains in substrate-tethering to promote enzyme activity. The linker between the enzyme and binding domains in *Cp*Glu30A-CBM92A is only

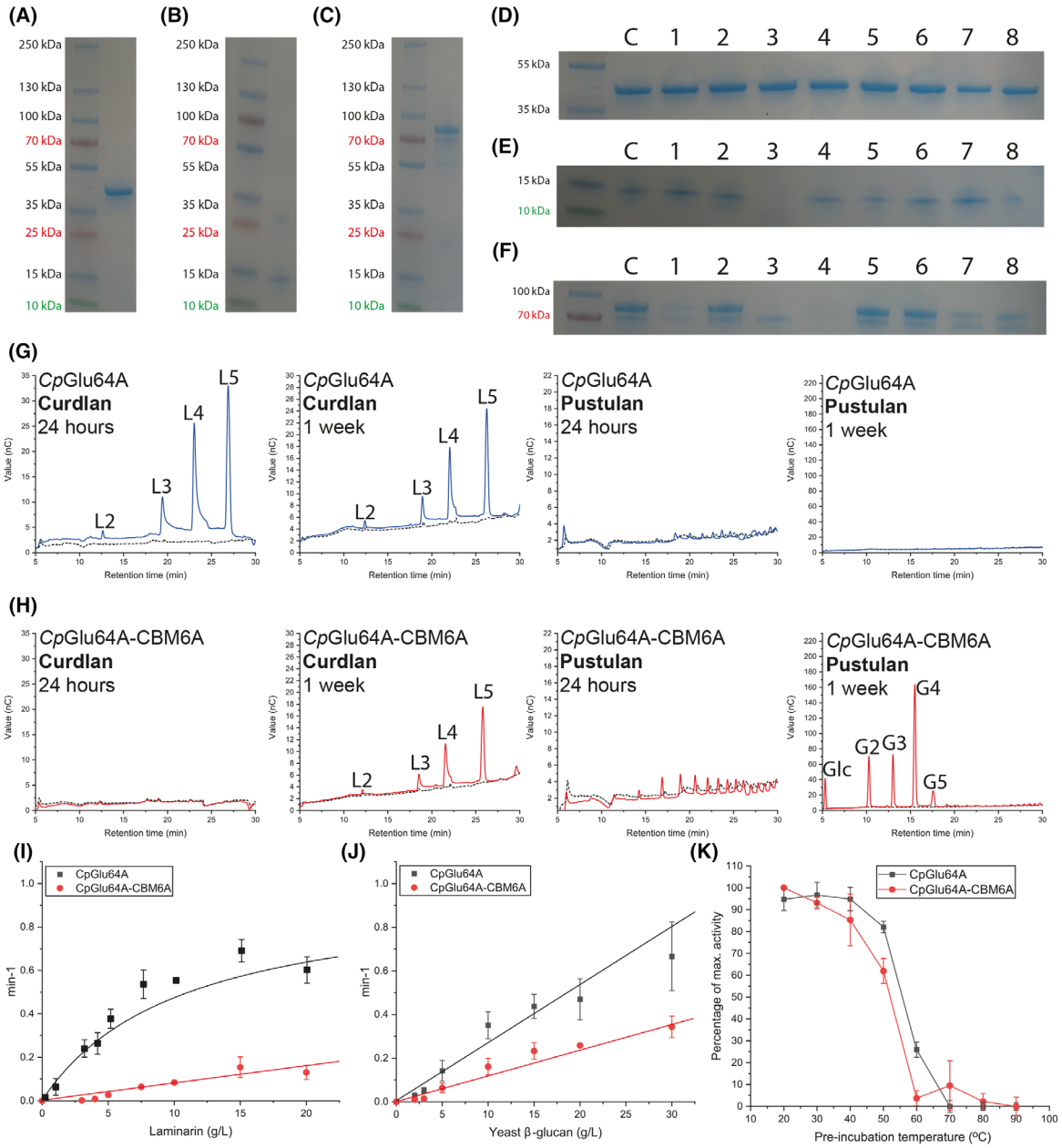

Fig. 4. *Endo*-β-glucanase activity of *Cp*Glu64A. (A–C) SDS/PAGE analysis showing recombinant production of *Cp*Glu64A, *Cp*CBM6A and *Cp*Glu64A-CBM6A, respectively. (D–F) Polysaccharide binding profiles of *Cp*Glu64A, *Cp*CBM6A and *Cp*Glu64A-CBM6A, respectively. The ligands used in this experiment are as follows: C, water (negative control); 1, pustulan; 2, Avicel cellulose; 3, curdlan; 4, lichenan; 5, chitin; 6, chitosan; 7, birchwood xylan; 8, ivory nut mannan. These gels show representative results of experiments that were repeated at least three times. *Cp*Glu64A showed no binding to any polysaccharide tested. *Cp*CBM6A showed binding to curdlan (possible weak binding to mannan). *Cp*Glu64A-CBM6A showed binding to pustulan, curdlan and lichenan (possible weak binding to xylan and mannan). (G) *Cp*Glu64A is an efficient β-1,3-glucanase, readily generating oligosaccharides from curdlan but shows no activity on pustulan. (H) *Cp*Glu64A-CBM6A requires several days of incubation to generate a detectable profile of reaction products from curdlan but has gained a β-1,6-glucanase function, allowing it to generate a defined profile of oligosaccharides from pustulan. In panels G and H, the chromatograms depicted in black dotted lines represent an enzyme-free incubation of substrate in buffer (i.e. control assays). For all experiments shown in HPAEC chromatograms, enzyme at 100 nM was incubated with the substrate at 2 $g \cdot L^{-1}$ for the indicated time. (I) Kinetic analysis of *Cp*Glu64A and *Cp*Glu64A-CBM6A activity on laminarin. (J) Kinetic analysis of *Cp*Glu64A and *Cp*Glu64A-CBM6A activity on yeast β-glucan. (K) Thermostability of activity against laminarin. Kinetic and thermostability experiments were performed in triplicate: Error bars indicate SEM.

Table 1. Summary of the specificities of enzymes investigated. In addition to the β-glucan substrates indicated in the table, *Cp*Glu5$_N$ was assayed against glucomannan, galactomannan, arabinoxylan and chitin, displaying no measurable activity on these substrates. The values indicate the percentage of maximum reducing sugar released from each substrate after a 20 h incubation. The abbreviation n.d. denotes that no activity was detected. 'Trace' indicates that some activity was observed, but it was less than 1% of the maximum observed for that enzyme.

Substrate	Substrate structure	Substrate origin	*Cp*Glu30A	*Cp*Glu5$_N$	*Cp*Glu64A	*Cp*Glu64A-CBM6A[a]
Pustulan	Linear β-1,6-glucan	Lichenous fungi	100%	100%	n.d.	Trace
YBG	β-1,3-glucan with some β-1,6-branching	Fungi	10%	n.d.	43%	86%
Laminarin	β-1,3-glucan with some β-1,6-branching	Brown algae	n.d.	n.d.	100%	100%
Curdlan	Linear β-1,3-glucan	Bacteria	n.d.	n.d.	5%	5%
Avicel	Crystalline cellulose (β-1,4-glucan)	Plants	n.d.	n.d.	n.d.	n.d.
Carboxymethylcellulose	Solubilised cellulose	Plants	n.d.	n.d.	n.d.	n.d.

[a]Note that *Cp*Glu64A-CBM6A is able to release hydrolysis products from pustulan after a longer incubation period (discussed above).

15 amino acids in length, suggesting that they could lie in close proximity on the same substrate chain. Analysis of pustulan reaction products by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) showed a regular series of oligosaccharides, confirming that the enzyme has an *endo* mode of action (Fig. 2C), with cleavage occurring randomly along the length of the pustulan chain. Michaelis–Menten parameters of pustulan hydrolysis by *Cp*Glu30A-CBM92A could not be fully determined due to the apparently high K_M of the reaction, but linear slope analysis was used to determine the k_{cat}/K_M of the reaction (Fig. 2D, Table 2). When stored for 1 h at different temperatures prior to being assayed, *Cp*Glu30A began to lose function if pre-incubated at 40 °C (Fig. 2E).

CpGlu5$_N$ is a weakly acting endo-β-1,6-glucanase

The modular protein encoded by gene Cpin_2580 includes the GH5_46 enzyme *Cp*Glu5$_N$, as well as two CBM92 domains and a GH18 enzyme. *Cp*Glu5$_N$ was assayed against a range of polysaccharides and showed hydrolytic activity only on pustulan. The GH18 domain was produced and tested separately and found not to display any β-glucanase activity (data not shown), so we presume that the β-1,6-glucanase activity of *Cp*Glu5$_N$ indeed derives from the GH5_46 module. Analysis of reaction products by HPAEC-PAD revealed that *Cp*Glu5$_N$ is able to hydrolyse pustulan into a complex mixture of long oligosaccharides, with the product profile becoming simpler as the reaction progresses and shorter oligosaccharide products are generated (Fig. 3B). The apparent concentration of reaction products for *Cp*Glu5$_N$ was significantly lower than that obtained from *Cp*Glu30A (Fig. 2C), perhaps indicating a lower catalytic efficiency, but the data suggest a similar *endo* mode of activity. *Cp*Glu5$_N$ therefore represents the first bacterial *endo*-β-1,6-glucanase discovered in family GH5_46. Again, full Michaelis–Menten parameters of pustulan hydrolysis could not be obtained due to an apparently high K_M, but the k_{cat}/K_M of the reaction was determined by linear slope analysis (Fig. 3D, Table 2). These parameters are highly similar to those for *Cp*Glu30A, which contrasts with the differences in catalytic efficiency that are suggested by the HPAEC-PAD analysis. We propose that *Cp*Glu5$_N$ may release a large amount of oligosaccharides that are too long to be resolved by HPAEC-PAD but which are measurable by DNSA reducing

Table 2. Kinetic parameters of enzymes investigated in this study. n.d., not determined.

Enzyme	Substrate	k_{cat} (min^{-1})	K_M ($g{\cdot}L^{-1}$)	k_{cat}/K_M
*Cp*Glu30A	Pustulan	n.d.	n.d.	0.017 ± (3.5 × 10^{-4})
*Cp*Glu5$_N$	Pustulan	n.d.	n.d.	0.014 ± 0.0016
*Cp*Glu64A	Laminarin	0.99 ± 0.12	7.02 ± 1.3	0.14
*Cp*Glu64A	YBG	n.d.	n.d.	0.025 ± 0.0047
*Cp*Glu64A-CBM6	Laminarin	n.d.	n.d.	0.0084 ± 0.0012
*Cp*Glu64A-CBM6	YBG	n.d.	n.d.	0.012 ± 0.0012

sugar assay, thus contributing to the kinetic assessment of activity. The β-1,6-glucanase is relatively stable when pre-incubated at up to ~40–50 °C (Fig. 3E).

CpGlu64A is a β-1,3-glucanase that shows a gain of endo-β-1,6-glucanase function when appended to CpCBM6A

All members of family GH64 characterised to date show β-1,3-glucanase activity [42–46], hydrolysing laminarin (a marine plant glycan comprising linear β-1,3-glucan with β-1,6-linked glucosyl branches [47]) and curdlan. Our activity screens confirmed that both *Cp*Glu64A-CBM6A and *Cp*Glu64A hydrolyse curdlan, laminarin and YBG to different extents, with some indication of additional weak activity against pustulan for *Cp*Glu64A-CBM6A only (Table 1). A pull-down assay was performed to determine the carbohydrate-binding capacity of the protein. Members of family CBM6 [48] have been shown to bind β-1,3-glucan, β-1,3-1,4-glucan, β-1,4-glucans [49–51] and xylan [52]. The GH64 domain alone showed no binding, as expected. *Cp*CBM6A when produced alone could bind only to curdlan, indicating specificity for Glc-β-1,3-Glc linkages. By contrast, the full-length *Cp*Glu64A-CBM6A protein showed clear binding to pustulan, curdlan and lichenan (Fig. 4D–F). To further probe the activities detected, the enzymes were incubated for 24 h or up to 1 week with curdlan and pustulan, and the reaction products analysed by HPAEC-PAD (Fig. 4G,H).

Produced as a single domain, *Cp*Glu64A is a strict β-1,3-glucanase that generates long oligosaccharides from curdlan. Reaction products were generated within the first minute (data not shown), accumulated over time and were not hydrolysed to shorter oligosaccharides even after a long incubation (Fig. 4G). This conforms to the atypical laminarin degradation pattern performed by other GH64 enzymes, producing long oligosaccharides that are not perfectly consistent with classical *endo* or *exo* modes of catalysis, such as the laminaripentaose-producing Bgl64A from *Paenibacillus barengoltzii* [43] and LPHase from *Streptomyces matensis* [42]. Despite showing the same level of reducing sugar formation as *Cp*Glu64A in the initial activity screen (Table 1), the bi-modular *Cp*Glu64A-CBM6A required several days of incubation to generate a similar abundance of detectable oligosaccharides from curdlan, but the same product profile was ultimately generated. Produced as a two-domain protein, *Cp*Glu64A-CBM6A can additionally function weakly as a β-1,6-glucanase, generating low amounts of short oligosaccharides from pustulan within 24 h, and a well-defined series of products with a degree of polymerisation (d.p.) from 1 to 5 after a longer incubation (Fig. 4H).

Kinetic rate analyses were performed for *Cp*Glu64A and *Cp*Glu64A-CBM6A using laminarin and YBG (Fig. 4I,J). Accurate measurements using curdlan and pustulan were not practical to achieve due to slow reaction rates and the low solubility of curdlan. Michaelis–Menten parameters of laminarin hydrolysis by *Cp*Glu64A were as follows: k_{cat} 0.99 ± 0.12 min^{-1}, K_M 7.02 ± 1.36 $g{\cdot}L^{-1}$, k_{cat}/K_M 0.14 $g^{-1}{\cdot}L{\cdot}min^{-1}$ (Table 2). For all other reactions, the apparent values of K_M were too high for full kinetic parameters to be determined (Table 2). The impact of the CBM6 domain is clearest in the laminarin assay, which shows a severe catalytic penalty for *Cp*Glu64A-CBM6A compared with *Cp*Glu64A alone. The CBM6 module had no measurable impact on the thermostability of the enzyme, which was similar to that of the other enzymes investigated here, dropping off at 40–50 °C (Fig. 4K).

The paradigmatic function of CBM6 proteins is to bind to the polysaccharide substrate of an appended GH [53], which can boost reaction rates in certain conditions [54]. In the case of *Cp*Glu64A and *Cp*Glu64A-CBM6A, kinetic analysis of the hydrolysis of laminarin and YBG showed that the CBM6 module in fact slowed down catalysis, suggesting that the CBM does not bind to these substrates in a manner that is productive for the enzyme. Instead, we see an alteration in enzyme specificity, which may be influenced by the binding preferences of the CBM. There are examples where a CBM binds a different carbohydrate than the substrate of its GH partner, and this can have complicated impacts on enzyme function [55–57], but it is rare for a CBM to significantly alter an enzyme's substrate specificity. In one example, the preference of a bacterial fructosidase for either branched or linear fructan polysaccharides could be modulated by the inclusion of a CBM66 domain that binds terminal fructofuranose residues, facilitating enzyme targeting of branching structures [58]. Few examples in the literature describe a CBM that altered the specificity of a partner GH to such an extent that a different linkage would be preferentially cleaved, although changing the binding specificity of the CBM appended to *endo*-glucanases and acetyl esterases can result in altered activity on complex biomass substrates [55,59,60].

Nonetheless, we speculated that the observed gain-of-function could, at least in part, be explained by the carbohydrate binding of *Cp*CBM6A. We surmise that the β-1,6-glucanase activity demonstrated by *Cp*Glu64A-CBM6A arises from the binding of the

modular protein to polysaccharides containing Glc-β-1,6-Glc linkages, with which the GH64 domain does not otherwise interact in a productive way. This includes the fungal polysaccharide scleroglucan, which has β-1,6-Glc substitutions on a main chain of β-1,3-glucan [61]. Indeed, Wu *et al* have previously shown that the *S. matensis* LPHase can be most properly characterised as an *exo*-acting enzyme despite the open groove topology of the active site [42]. This may suggest a role for *Cp*Glu64A-CBM6A as an *exo*-acting enzyme capable of debranching a fungal substrate such as scleroglucan, exposing the main chain for more efficient hydrolysis by the same or a similar enzyme acting on complex cell wall substrates where both linkages are present.

The characterisation of *Cp*Glu64A-CBM6A likely represents the first description of β-1,6-glucanase activity in family GH64 and underscores the role of CBMs in modulating enzyme function. The mechanism by which the CBM6 domain can expand the specificity of the GH64 module is not clear from this study. In the full-length protein, there is a stretch of 114 amino acids (including a beta/gamma crystalline-like Greek key structural motif) that may allow for flexible movement of the GH64 and CBM6 domains relative to each other, perhaps bringing the active site and binding site into close proximity in certain conditions. A future structural assessment of the bi-modular *Cp*Glu64A-CBM6A protein, including the structure of the inter-domain linker sequence, could shed light on whether new binding sites are formed at the domain interface.

Implications for biotechnology and biocontrol

The enzymes we describe here enrich the database of characterised bacterial β-1,6-glucanases, aiding future enzyme discovery efforts. Although it is clear from our data that enzymes from subfamily GH30_3 are the best adapted to release short metabolisable oligosaccharides from pustulan, all of the enzymes described here contribute to a robust metabolic system supporting the consumption of this recalcitrant polysaccharide by *C. pinensis*. Indeed, they may have biotechnological value in the production of medically relevant β-1,6-gluco-oligosaccharides (GOs). If used in synergy with chitinases, β-1,3-glucanases, and others, they may be able to liberate immunomodulating GOs directly from fungal biomass in a sustainable biorefinery-type process. These enzymes may also suggest a potential use of *C. pinensis* as a biocontrol species, as there are several mycoparasitic microbes that employ β-1,6-glucan deconstruction in their antifungal behaviours. Further study is required to draw firm conclusions in this regard. Although the potential for the species to control pathogens has been demonstrated in the characterisation of the antifungal pinensin lantibiotics described by Mohr *et al*. [62], it remains to be seen whether cell wall degrading enzymes are part of the antifungal and antioomycete arsenal of *C. pinensis*.

Materials and methods

Carbohydrate polymers utilised

A range of carbohydrate polymers was used for testing enzyme activity and protein binding. Chitin from shrimp shells was purchased from Sigma-Aldrich, USA. Chitosan, α-chitin and β-chitin were obtained from Maharani Chitosan PTV, Ltd (Gujarat, India) while scleroglucan (Actigum) was from Carbosynth, UK. Pustulan was purchased from Carbosynth, UK, while microcrystalline cellulose (Avicel®), starch, YBG, carboxymethylcellulose and birchwood xylan were from Sigma-Aldrich, Germany. Barley β-glucan, konjac glucomannan, curdlan, laminarin and lichenan were purchased from Megazyme, Ireland.

Analysis of secreted proteins

Strain growth

All reagents used in bacterial growth were purchased from Sigma-Aldrich, Germany, unless otherwise stated, and were of microbiological grade. Samples (50 μL) from starter cultures (10 mL) of *C. pinensis* grown in LB were inoculated into M9 minimal medium (10 mL) containing either glucose or a semi-soluble β-glucan polysaccharide (pustulan, curdlan, YBG) at a concentration of 5 $g{\cdot}L^{-1}$. Minimal medium not supplemented with carbon source served as a control and supported no growth. Two biological replicates were produced for each carbon source. Cultures were incubated at 25 °C with rotary shaking at 180 rpm. After 3 days of incubation, secretomes were harvested by centrifugation to pellet cells (4000 ***g*** for 15 min at 4 °C). Protein concentration was measured using a Bradford assay with a BSA standard curve [63]. Secretomes were tested for enzymatic activity using the enzyme activity assays described below.

Production and analysis of recombinant proteins

Gene cloning

Genes encoding the proteins under investigation were synthesised in a proprietary vector by ThermoFisher GeneArt. Genes were then sub-cloned into the expression vector pET21a (ThermoFisher, Stockholm, Sweden), which carries a C-terminal His_6 tag and confers ampicillin resistance.

Gene expression and protein purification

Plasmids containing a gene of interest were transformed into *Escherichia coli* BL21 (DE3) (Life Technologies) by heat shock at 42 °C for 30 s. Cells were grown at 37 °C with shaking in a selective LB medium containing 50 μg·mL^{-1} ampicillin for 2–3 h until an approximate OD_{600} was reached. At this point, gene expression was induced by the addition of 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and the temperature was lowered to 20 °C. Protein production proceeded for ~16 h. Cells were then collected by centrifugation at 6000 ***g*** for 10 min, resuspended in TALON Buffer A (50 mM sodium phosphate pH 6.5 with 300 mM sodium chloride) and lysed by sonication, then finally pelleted by centrifugation at 35 000 ***g*** for 30 min. The resulting supernatant liquid was collected and filtered using a 0.45-μm filter. Recombinant His_6-tagged proteins were purified using the TALON resin for immobilised metal ion affinity chromatography (IMAC), according to the manufacturer's instructions. Unbound or loosely bound nontarget proteins were washed from the TALON resin using TALON Buffer B (buffer A with 7.5 mM imidazole) and eluted using TALON Buffer C (buffer A containing increasing concentrations of imidazole, namely 37.5, 75 and 150 mM). Eluted proteins were concentrated and the buffer was exchanged into 50 mM sodium phosphate pH 6.0 using Amicon Ultra centrifugal filters with a molecular weight cut-off of 3 kDa (Millipore, Millipore/Merck, Darmstadt, Germany). The production and purification of recombinant proteins, as well as the apparent molecular weight, was confirmed by SDS/PAGE analysis.

Enzyme activity assays

Reducing sugar assays after polysaccharide hydrolysis

Hydrolysis of various polysaccharides was studied using the 3,5-dinitrosalicylic acid (DNSA) reducing sugar assay [64]. See the section 'Carbohydrate polymers utilised' for a full list of substrates and their sources. Briefly, substrates at 0–30 g·L^{-1} were incubated with the hydrolytic enzymes (either purified recombinant protein or *C. pinensis* secretome) at 10 nM–2 μM, in 50 mM sodium phosphate buffer. Secretomes were used at ~ 40 μg protein·mL^{-1}. Hydrolysis of the substrates was measured as an increase in reducing sugars detected by the DNSA assay, versus a standard curve of glucose monosaccharide. To this end samples of enzymatic reactions were added to an equal volume of DNSA reagent [1% (w/v) DNSA, 0.2% (v/v) phenol, 1% (w/v) NaOH, 0.01% (w/v) glucose and 0.05% (w/v) $NaSO_3$] to terminate the reactions, and the colour was developed by boiling the mixtures for 20 min and cooling on ice for 5 min, prior to measuring the absorbance at 575 nm on a Cary 50 spectrophotometer.

The thermostability of enzyme activities was assessed by pre-incubating the enzymes for 20 min at 20–90 °C, before briefly cooling the enzymes by placing them on ice. The enzymes were then added to a mixture of substrate and buffer and incubated for 15 min at 30 °C prior to the addition of an equal volume of DNSA to stop the reactions. After boiling, absorbance was measured at 575 nm as described above.

Reaction product analysis by high-performance anion-exchange chromatography with pulsed amperometric detection

High-performance anion-exchange chromatography with pulsed amperometric detection was used to analyse oligosaccharides, and was performed using a Dionex ICS-3000 high-performance liquid chromatography system operated by CHROMELEON software version 6.80 (Dionex, Stockholm, Sweden) using a Dionex CarboPac PA1 column. Solvent A was water, solvent B was 1 M sodium hydroxide, solvent C was 200 mM sodium hydroxide containing 170 mM sodium acetate, and solvent D was 1 M sodium acetate. Depending on the analytes, different gradients were employed. For the detection of gluco-oligosaccharides, the following gradient was used: prewash and column calibration, −5 to 0 min 15% B (0.5 mL·min^{-1}); sample injection, 0 to 16 min 15% B (0.5 mL·min^{-1}); gradient elution, 15 to 30 min 33% B (0.5 mL·min^{-1}), 30 to 31 min 33% B and 50% D (0.5 mL·min^{-1}); and column wash and final elution, 31 to 35 min 15% B (0.5 mL·min^{-1}). Carbohydrates were identified and quantified by comparing their retention times and peak areas to those of standards of known concentrations.

Carbohydrate-binding assays

Pull-down assays

Proteins were screened for the capacity to bind insoluble or semi-soluble polysaccharides using a pull-down assay [65]. Briefly, 900 μL of polysaccharide at 5 g·L^{-1} was incubated with protein at ~ 0.5–3 g·L^{-1} in 50 mM sodium phosphate buffer pH 6.0 for 3 h at room temperature. The reactions were incubated on a Stuart™ Rotator Disk turning at 30 rpm. The reactions were then centrifuged at 10 000 ***g*** for 5 min and the supernatant was collected without disturbing the pellets. Samples from the supernatants were analysed by SDS/PAGE. The absence of protein in the supernatant indicates binding to the insoluble polysaccharide, which formed a pellet during centrifugation.

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Conflict of interest

Our funding organisations played no part in the design or implementation of this study and had no influence on the production or submission of this article. The authors declare no conflict of interest.

Author contributions

LSM designed and coordinated the project. ZL, IE, CR and LSM performed most experimental work, with additional experiments performed by HH, HA, LS, ARI, MH and KK. Supervision was performed by LSM and VB. LSM and ZL wrote the manuscript, with input from other authors.

Data availability statement

The data that support the findings of this study are available in the manuscript figures and tables. Additional supporting raw data from replicate experiments are available from the corresponding author (mckee@kth.se) upon reasonable request.

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