Understanding the strategies used by bacteria to degrade polysaccharides constitutes an invaluable tool for biotechnological applications. Bacteria are major mediators of polysaccharide degradation in nature; however, the complex mechanisms used to detect, degrade, and consume these substrates are not well-understood, especially for recalcitrant polysaccharides such as chitin. It has been previously shown that the model bacterial saprophyte *Cellvibrio japonicus* is able to catabolize chitin, but little is known about the enzymatic machinery underlying this capability. Previous analyses of the *C. japonicus* genome and proteome indicated the presence of four glycoside hydrolase family 18 (GH18) enzymes, and studies of the proteome indicated that all are involved in chitin utilization. Using a combination of *in vitro* and *in vivo* approaches, we have studied the roles of these four chitinases in chitin bioconversion. Genetic analyses showed that only the *chi18D* gene product is essential for the degradation of chitin substrates. Biochemical characterization of the four enzymes showed functional differences and synergistic effects during chitin degradation, indicating non-redundant roles in the cell. Transcriptomic studies revealed complex regulation of the chitin degradation machinery of *C. japonicus* and confirmed the importance of CjChi18D and CjLPMO10A, a previously characterized chitin-active enzyme. With this systems biology approach, we deciphered the physiological relevance of the glycoside hydrolase family 18 enzymes for chitin degradation in *C. japonicus*, and the combination of *in vitro* and *in vivo* approaches provided a comprehensive understanding of the initial stages of chitin degradation by this bacterium.

Chitin, a linear polymer of β(1→4)-linked N-acetylglucosamine, is the second most abundant polysaccharide on earth after cellulose and a major source of fixed carbon and nitrogen, especially in the oceans (1). The rate of chitin degradation has been calculated to match the rate of synthesis, resulting in little net accumulation of chitin in the environment, suggesting that the microbial communities that derive nutrition from this polysaccharide are able to degrade it with high efficiency (2). Moreover, chitin is increasingly being recognized as an important feedstock for the production of renewable chemicals, and chito-oligosaccharides are being investigated for several biomedical applications (3–6). The strategies used by microbes to efficiently chitin bioconversion are gaining interest, because current chemical methods used for the industrial processing of chitin are inefficient and wasteful (7, 8). Therefore, the combination of environmental importance and industrial/medical relevance has renewed interest in the bioconversion of chitin (9, 10).

Depolymerization of chitin-rich substrates has been studied predominately from biochemical and structural perspectives, and as a consequence there is considerable understanding on certain mechanistic aspects of enzymatic degradation (11). Chitin deconstruction shares many features with cellulose degradation, which is not surprising given that chitin has a crystalline structure similar to that of cellulose and differs chemically via an N-acetyl substitution at the C2 carbon (12). Specifically, chitin depolymerization is achieved through a concerted effort of endo- and exo-acting enzymes to reduce polymer length to short oligosaccharides that are then converted to N-acetylglucosamine monomers by hexosaminidases for entry into cellular metabolism (Fig. 1) (6). Additionally, the action of lytic polysaccharide monooxygenases (LPMO) in aerobic microbes promotes chitin degradation by acting on crystalline regions of the substrate (13–15).

Physiological and genetic aspects of chitin degradation, especially for terrestrial bacteria, are not well-characterized. One reason for this lack of knowledge is the multiplicity of chitinolytic enzymes in many, but not all, chitin-degrading bacteria, which makes functional analysis of the individual enzymes challenging. Although several model organisms have been used to study chitin degradation (16–18), one that is emerging as a powerful model, because of the available systems biology tools,
is the saprophytic Gram-negative bacterium *Cellvibrio japonicus* (19, 20). This bacterium is a potent chitin degrader (13) and has a suite of nine genes encoding enzymes with predicted functions in chitin degradation (21). Specifically, the *C. japonicus* genome is predicted to encode four chitinases from the glycoside hydrolase family 18 (GH18), one GH19 chitinase, two hexosaminidases (GH20), one chitosanase (GH46), and one chitin-specific LPMO (auxiliary activity, AA10) (21). Secretome analysis (22) has shown that the four GH18s, and to a much lesser extent the GH19, are abundant during growth on chitin. Generally, the GH18s and LPMOs are considered key enzymes for bioconversion of crystalline chitin. The physiological role of GH19 enzymes in chitin degradation is less clear (23, 24).

The possession of large numbers of carbohydrate active enzymes (CAZymes) (25) is a hallmark feature of *C. japonicus*. There is increasing evidence that the CAZymes of *C. japonicus* that belong to the same GH family are not functionally redundant but have unique physiological functions (14, 26). In the current study, the physiological roles of the four *C. japonicus* GH18 chitinases were determined focusing on the initial stages of chitin degradation. Sequence analysis indicates that CjChi18B and CjChi18C are secreted enzymes containing at least one chitin-specific carbohydrate-binding module (CBM), whereas CjChi18A is a single domain protein that likely is membrane-anchored (Fig. 2). Through RNAseq analysis, we determined that these four chitinases were highly up-regulated while chitin was being used as the sole carbon source. Combinatorial mutational analyses determined that only the *chi18D* gene product is essential for chitin degradation. Biochemical characterization of the catalytic domains of these four GH18 chitinases indicated that CjChi18D is substantially more active toward insoluble chitin than the other GH18 chitinases, underpinning its importance in chitin degradation. The functional insights into the initial stages of chitin degradation by saprophytic bacteria that are provided in this study have the potential to accelerate the development of industrial chitin bioconversion strategies.

**Results**

*CjChi18D is essential for the degradation of α-chitin*

According to previous sequence analysis (21), *C. japonicus* possesses four GH18 chitinases (CjChi18A, CjChi18B, CjChi18C, and CjChi18D). A recent proteomic study (22) detected the four enzymes in the secretome of *C. japonicus* growing on α- and β-chitin, which suggested the importance of these enzymes for efficient chitin degradation. To elucidate the physiological relevance of these enzymes, we generated a suite of GH18 deletion mutants and assessed their fitness on insoluble chitin substrates, including an environmentally relevant substrate, crab shell.

Wildtype and GH18 deletion mutant strains all grew well in defined media with either glucose (Glc) or GlcNAc as the sole source of carbon (Fig. S1). A Δgps mutant, which lacks the entire 9.4 kb *gps* operon encoding the type II secretion system that is needed for secretion of most enzymes, was also able to grow on these monosaccharides, as previously demonstrated (13, 27). When the GH18 single deletion mutants were grown using insoluble α-chitin or crab shell as the sole carbon source, distinct phenotypes emerged (Fig. 3). When α-chitin was the sole source of carbon, the Δ*chi18D* mutant was unable to grow. The other three single deletion strains (Δ*chi18A*, Δ*chi18B*, and Δ*chi18C*) all had similar growth rates and maximum optical densities (OD) as the wildtype (Table S1A). Interestingly, the Δ*chi18B* and Δ*chi18C* single mutant strains had more protracted lag phases than the wildtype strain when using α-chitin as carbon source (Fig. 3A). When using crab shells as the only source of carbon, the Δ*chi18D* mutant was unable to grow, whereas the single mutants of Δ*chi18A*, Δ*chi18B*, and Δ*chi18C* displayed growth similar to wildtype (Fig. 3C and Table S1B).

To test for functional redundancies between the GH18 chitinases, we generated all combinations of double deletion mutants and then assessed growth using α-chitin or crab shells as the sole carbon source. The double mutant Δ*chi18BΔchi18C* resulted in a slower growth rate and a longer lag phase when grown on α-chitin in comparison to either of the single deletion mutants or the wildtype (Fig. 3B). The growth rate of the Δ*chi18BΔchi18C* double mutant was reduced 37% compared with wildtype (Table S1A). The Δ*chi18AΔchi18B* double mutant grew like the Δ*chi18B* single mutant, whereas the Δ*chi18AΔchi18C* double mutant grew like the Δ*chi18C* single mutant. The Δ*chi18AΔchi18BΔchi18C* triple mutant recapitulated the growth defect observed in the Δ*chi18BΔchi18C* double mutant in terms of the growth rate, lag phase, and the maximum OD (Fig. 3B and Table S1A). These results suggest that the *chi18B* and *chi18C* gene products have non-redundant
functions during degradation of α-chitin, whereas the chi18A gene product does not play a rate-limiting role. Interestingly, the double mutants exhibited wildtype-like phenotypes when grown using crab shells as the sole carbon source (Fig. 3D). The growth rates of the /H9004 chi18B and /H9004 chi18C single mutants and the /H9004 chi18B /H9004 chi18C double mutant were similar to the wildtype strain when grown using crab shells. For all tested strains, the growth rates when using crab shells as the sole carbon source were substantially reduced compared with α-chitin. The results of experiments using β-chitin (Fig. S2) were very similar to the results obtained with α-chitin.

**CjChi18D is a potent secreted chitinase**

Sequence alignment of the catalytic domains of the four chitinase showed large sequence variation. CjChi18A and CjChi18D share higher sequence identity (32%), whereas CjChi18C and CjChi18D showed the lowest identity (19%), as determined by BLAST alignment (28). As predicted by LipoP (29) and SignalP (30) software tools, all four of the C. japonicus GH18 chitinases have a signal sequence; however, CjChi18A has a SPaseII-cleaveable sequence and is predicted to be an outer membrane associated lipoprotein. The CjChi18B, CjChi18C, and CjChi18D enzymes have a SPaseI-cleaved sequence (29) and are predicted to be secreted. The domain structure of the four GH18 chitinases is summarized in Fig. 2. The LipoP/SignalP software predictions are in agreement with a proteomics study that showed the occurrence of all four GH18 chitinases in the secretome (22).

To further assess the contribution of individual GH18 enzymes as effectors of chitin degradation, we used our suite of GH18 mutants to assess secreted activity using colloidal chitin plate assays (Fig. 4 and Table S2). A wildtype strain generated a robust zone of clearing, whereas a Δgsp secretion-deficient mutant generated no zone of clearing. We observed a similarly
Chitin degradation in C. japonicus

striking phenotype with the Δchi18D single mutant strain, which also generated no zone of clearing. The remaining GH18 single mutants displayed approximately wildtype zones of clearing. Interestingly, relative to the wildtype, the Δchi18B Δchi18C double mutant showed a clear reduction in the size of the clearing zone. The Δchi18A Δchi18B Δchi18C triple mutant displayed a zone of clearing similar to the Δchi18B Δchi18C double mutant. The reduced chitin-degrading capacities of the triple mutant are in agreement with the observed growth defects of these strains when grown on α-chitin.

The GH18 chitinases have different activities toward chitin and (GlcNAc)_n

To further investigate the features of the four GH18 chitinases, they were cloned, expressed, and purified for biochemical characterization. Despite substantial efforts, soluble full-length multidomain CjChi18B, CjChi18C, and CjChi18D proteins could not be obtained. Therefore comparative biochemical analysis was conducted with overexpressed catalytic domains. All four chitinases were able to degrade α-chitin, although with greatly varying efficiency, but were clearly highest for CjChi18D (Fig. 5). As expected (31), versions of CjChi18B and CjChi18D containing their CBM5 domain gave higher yields compared with their respective solitary catalytic domains. The catalytic domain of CjChi18C gave higher yields than the catalytic domains of CjChi18A and CjChi18B, but all were poor in chitin degradation compared with CjChi18D.

Analysis of products generated during degradation of α-chitin showed that CjChi18Acat initially produced both GlcNAc and (GlcNAc)_2. The product profiles for the other variants (CjChi18Bcat, CjChi18Bcat+CBM5, CjChi18Ccat, CjChi18Dcat, and CjChi18Dcat+CBM5) showed mainly (GlcNAc)_2 and smaller amounts of GlcNAc (Fig. S3), as is usual for GH18 chitinases. It is noteworthy that the product profile of CjChi18Bcat was different from CjChi18Bcat+CBM5 after 48 h, where GlcNAc is the dominating product for CjChi18Bcat, whereas (GlcNAc)_2 is the dominating product for CjChi18Bcat+CBM5 (Fig. S3). For CjChi18Acat, only GlcNAc was detected after 48 h, indicating an N-acetylhexosaminidase activity for this enzyme.

Synergy experiments (Fig. 6) confirmed the dominating role of CjChi18D in chitin bioconversion and revealed synergistic effects for various enzyme combinations, such as when mixing
amounts of the monomer were detected for the other three chitinases (results not shown).

**C. japonicus GH18 chitinase genes are highly expressed during chitin utilization**

To obtain further insight into the regulation of chitinase expression and to complement the previous secretome study (22), we used RNAseq to determine changes in gene expression during growth using α-chitin relative to growth using glucose. Analysis of samples from the exponential growth phase revealed significant up-regulation of 73 CAZyme genes (Table S3A). The seven most strongly up-regulated genes were all related to chitin bioconversion, including the LPMO (6.7-fold, log₂ scale), the hex20B hexosaminidase (4.1-fold), the nag9A gene (3.5-fold) that encodes a putative N-acetylhexosamine 6-phosphate deacetylase, and the four GH18 chitinases in the following order chi18D > chi18C > chi18A > chi18B, the fold change (log₂ scale) being 6.7-, 5.1-, 3.9-, and 3.6-fold, respectively (Fig. 8 and Table S3A). Several other genes putatively involved in chitin bioconversion were also up-regulated, albeit to a lesser extent, including the GH19 chitinase (2.2-fold, log₂ scale), the other GH20 hexosaminidase (hex20A; 2.7-fold), and the GH46 chitosanase and two polysaccharide deacetylases (2.6-, 1.6-, and 1.4-fold, respectively).

The comparison of gene expression during early stationary phase yielded similar results (Fig. S5A and Table S3B). This analysis revealed the up-regulation of 47 CAZyme genes, of which seven are implicated in chitin degradation (lpmo10A, chi18D, chi18C, chi18B, nag9A, hex20A, and chi19A). Although the expression data, in particular for the exponential comparison, showed a strong up-regulation of chitin-relevant genes, there was also up-regulation of a wide variety of CAZyme genes associated with the degradation of other polysaccharides such as starch, xylan, cellulose, pectin, arabinan, mannan, β-glucan, and xyloglucan. Therefore, the regulation of the chitin response resembles the general response previously observed for cellulose (14). When comparing the transcriptomes of the exponential and the stationary phase during growth on chitin, none of the significant changes in expression concerned CAZyme genes (Fig. S5B).

**Discussion**

A previous report demonstrated that *C. japonicus* can grow using both purified chitin and unrefined crab shells as a sole nutrient source (13). Additionally, a recent proteomic study found several secreted GH18 chitinases, suggesting a robust response to chitin (22). It has remained unknown, however, how these GH18 chitinases contribute to the degradation of chitin, and to what extent the four GH18 genes in the *C. japonicus* genome are equivalent. In this study, through an implementation of transcriptomic, genetic, and biochemical approaches, we assessed the physiological functions of the four GH18 enzymes. The synthesis of *in vitro* and *in vivo* data provides a comprehensive understanding of chitin degradation by *C. japonicus*.

**The architecture of the family GH18 chitinases indicates differential biological roles**

Analysis using LipoP (29) predicts that the *CjChi18A* enzyme is a lipoprotein with a signal sequence cleaved by signal peptidase II (SPII). The presence of a glutamine in position +2 after the cleavage site of SPII further suggests that *CjChi18A*
resides attached to the outer membrane (32–35). Interestingly, CjChi18A was found in high amounts in a recent secretome study (22); however, the precise location of this enzyme remains undefined. It is likely that CjChi18A functions in degrading short chito-oligosaccharides, because the absence of CBMs limits the association of this enzyme with polymeric chitin (31, 36). Such a function is supported by the biochemical data for CjChi18A showing high activity on soluble substrates and an ability to produce monomers.

Analysis of the CjChi18B, CjChi18C, and CjChi18D enzymes indicated a signal peptide that is cleaved by signal peptidase I (29). The growth defect on chitin observed in the Δgsp mutant suggests that these three enzymes are transported to the extracellular space by the type II secretion system (37). The CjChi18B, CjChi18C, and CjChi18D enzymes all possess a CBM5 and a CBM73 domain (38); however, the orientations of these domains differ between the enzymes (Fig. 2). As found with many CAZymes, the CjChi18B, CjChi18C, and CjChi18D chitinases contain serine-rich regions separating the individual domains, which likely act as flexible linkers (39).
Analysis of the domain organization of the *C. japonicus* chitinases, functional data, homology modeling of their 3-D structures (Fig. S6), and comparison with well-characterized GH18 chitinases from *Serratia marcescens* (18) all indicate that the *C. japonicus* chitinolytic system represents a suite of complementary activities. Available data from known processive chitinases show that there are hallmarks specific for processive enzymes, involving an α+β domain in the structure and conserved aromatic amino acids lining the catalytic cleft (40–43). Structural modeling of the *C. japonicus* GH18 chitinases and sequence alignment with known processive chitinases (Fig. S7) show that *Cj*Chi18B and *Cj*Chi18D have these specifications. *Cj*Chi18B has a large α+β domain (~110 amino acids) and a “tunnel-like” substrate-binding cleft, whereas *Cj*Chi18D has a much smaller α+β domain (~60 amino acids) and a more open active-site cleft. Compared with the chitinases from *S. marcescens* (Fig. S6), the structural model of *Cj*Chi18B shows a more closed active site than exo-processive *Sm*ChiA and *Sm*ChiB, whereas *Cj*Chi18D seems to have a more open active site cleft than these two *Serratia* enzymes. *Cj*Chi18A has the α+β domain (~65 amino acids) but lacks most of the aromatic residues; the most similar *S. marcescens* enzyme seems to be *Sm*ChiD, which, like *Cj*Chi18A, primarily produces monomers (44). *Cj*Chi18C does not have features characteristic for processivity and is more similar to *Sm*ChiC, which is a non-processive endo-chitinase, displaying an open substrate binding cleft that is characteristic for non-processive glycoside hydro-lases (36).

**The GH18 enzymes of *C. japonicus* have divergent functionalities but are reliant on *Cj*Chi18D**

The growth and chitinase secretion data strongly pointed to the chi18D gene product being essential for the degradation of chitinous substrates (Figs. 3 and 4 and Fig. S2). These results reinforce two features previously described for *C. japonicus*, specifically, that secreted enzymes are essential for recalcitrant polysaccharide degradation and that single CAZymes elicit major physiological effects (27, 45–47). Other individual enzymes did not seem essential for growth, but the Δchi18B Δchi18C double deletion mutant did show reduced growth and reduced chitin conversion efficiency (Figs. 3 and 4), indicating that these two chitinases have complementing functionalities and that they contribute to chitin conversion but are not essential. The absence of growth defects of the multiple mutants while growing using crab shells is likely a consequence of the slow overall growth rate.

Biochemical characterization of the catalytic domains revealed clear functional differences (Figs. 5 and 7, Fig. S4, and Table S4) and showed that the GH18 enzymes act synergistically during the degradation of chitin (Fig. 6). Enzyme synergy during the course of chitin degradation has been described previously for *S. marcescens* and is generally ascribed to collaboration between endo- and exo-acting enzymes, where endo-acting enzymes generate chain ends that are substrates for exo-acting enzymes that usually show processive action (48). The synergistic effects observed for the best combinations of *C. japonicus* chitinases (Fig. 6) are of similar size as the effects observed for the *S. marcescens* enzymes (49, 50). It is worth noting that the clearest synergy was observed when combining *Cj*Chi18B, for which there are strong indications of exo-processive action, and *Cj*Chi18C or *Cj*Chi18D, which both could be endo-acting enzymes (vide infra). Previous studies with β-chitin have shown that culture supernatants of chitin-grown *S. marcescens* and chitin-grown *C. japonicus* have similar chitinolytic power (22).

**The GH18 chitinases exhibit processivity during chitin hydrolysis**

Although it is difficult to determine the degree of processivity from standard substrate degradation experiments, such experiments do provide indications. During degradation of crystalline chitin, processive enzymes tend to generate high (GlcNAc)2/GlcNAc ratios (43, 51). Furthermore, the degradation of (GlcNAc)6 by processive enzymes will yield a high (GlcNAc)2/(GlcNAc)4 ratio because such enzymes will process the chitohexaoside into three chito-biose moieties rather than releasing the chitotetraose and chitobiose into solution after a single hydrolytic event (as would be expected for a non-processive enzyme). Of the GH18 chitinases of *C. japonicus*, only *Cj*Chi18B showed these high ratios (Table S4), indicating that this enzyme is the most processive, as also suggested by the structural model discussed above (Fig. S6). The other three enzymes did not show clear signs of processivity. *Cj*Chi18C displays all hallmarks of a non-processive enzyme, and *Cj*Chi18A is unique because it primarily produces monomers, which is not really compatible with processivity because the repeating unit in a chitin chain is a dimer. These latter two enzymes showed the highest specific activities toward the hexamer, suggesting that they primarily act on soluble substrates. On the basis of the experimental data, it is difficult to judge whether the four enzymes are endo- or exo-acting, and it should be noted that mixed endo-/exo-modes do occur (52). *Cj*Chi18B appears to be exo-processive, whereas *Cj*Chi18C looks to be endo-non-processive. The situation for *Cj*Chi18A and *Cj*Chi18D is less clear, because the deep but still relatively open active site clefts of these enzymes are compatible with both endo- and exo-action and because the biochemical data were not conclusive regarding processivity.

**The chitinolytic machinery of *C. japonicus* is highly responsive to the presence of chitin substrates**

There was significant up-regulation of the predicted *C. japonicus* genes encoding chitin-active proteins (GH18, GH19, GH20, GH46, and AA10 families) during both the exponential phase and the stationary phase (Table S3). The up-regulation of a number of other CAZyme genes not involved in chitin degradation was striking but not surprising. *C. japonicus* has been shown to have both substrate-dependent and growth rate-dependent control of CAZyme gene expression (14). In regards to the substrate-specific response, *C. japonicus* seems to have two variations: one that is general (where diverse CAZyme genes are up-regulated) and one that is specific (where only substrate-specific CAZymes genes are up-regulated). The response observed for cellulose is an example of the former (14), and the response for xylan is the latter (46). The regulatory network for the chitinolytic response appears to be
analogous to the cellulose response, which is not surprising given the similarities of the substrates.

The expression data also indicated that the most up-regulated chitin-relevant gene encodes the chitin-specific LPMO, CjLPMO10A. It has been shown that this enzyme will act synergistically with chitinases (13) but in the present study, the effects of adding the LPMO to the chitinases was limited (Fig. 6). This is likely an effect of the substrate used in this study, which was ball-milled into fine particles. It has been previously shown that such ball-milling reduces the crystallinity of the substrate, hence reducing the activity of the LPMO (53).

**Final remarks**

The proposed model (Fig. 1) for crystalline chitin degradation by *C. japonicus*, based on in vitro, in vivo, and in silico analyses is that the four GH18 chitinases and CjLPMO10A synergistically degrade chitin. CjLPMO10A plays an important role, as indicated by the transcriptomic data presented here and previously published biochemical data (13). CjChi18D, being the most powerful chitin degrader, likely also acts on the more recalcitrant parts of the substrate, releasing chitooligosaccharides. CjChi18B and CjChi18C act on the more accessible chitin fibers, converting them into smaller chitooligosaccharides, whereas CjChi18A converts chito-oligosaccharides to N-acetylglucosamine.

**Experimental procedures**

**Growing conditions**

*C. japonicus* strains were grown using MOPS minimal medium (54) containing 0.2% (w/v) glucose, 0.5% (w/v) N-acetylglucosamine, 0.25% (w/v) α-chitin from shrimp shells (Sigma–Aldrich), 0.2% (w/v) β-chitin from squid pen, or 4% (w/v) crab shells from *Callinectes sapidus* as the sole source of carbon. The following protocols were developed to prepare the chitin containing substrates. The coarse flakes of α-chitin were sieved through the top piece of a 130-mm Buchner polypropylene filter to homogenize the particle size (diameter of ~4 mm). β-Chitin from squid pen was manually ground in a ceramic mortar and pestle and then passed through a plastic filter with 4-mm diameter holes. For the crab shells, any membranous material was discarded, and the shells were then thoroughly washed and rinsed. Clean and air-dried shells were manually ground and then sieved through a 4-mm diameter filter. To remove any generated crab shell dust, the filtrate was rinsed through a Buchner polypropylene filter. The shell pieces were subjected to autoclave sterilization.

*Escherichia coli* strains were grown using lysogenic broth (55). For growth analysis studies, the strains were initially grown for 24 h in 5 ml of MOPS–glucose medium. Then an 18-mm test tube with 5 ml of MOPS–chitin broth was inoculated with a 1:100 dilution of the prepared overnight cultures. All cultures were incubated at 30 °C with an aeration of 225 RPM. For insoluble substrates, growth was measured as a function of the decrease of absorbance at 600 nm (A_{600}) (Tecan Trading AG, Switzerland). All growth experiments were performed in biological triplicate. Plate media were solidified with 1.5% agar. When required, kanamycin was used at a concentration of 50 μg/ml.

**Generation of deletion mutants**

Deletion mutants of the family GH18 chitinases of *C. japonicus* were made and verified using previously published protocols (27, 45). A suicide vector was generated by cloning ±500 bp up- and downstream from the gene to be deleted into the pK18mobsacB vector (57) at the EcoRI and Xba1 sites. The pK18mobsacB plasmids to make the deletions of genes chi18A, chi18B, and chi18C were synthetized by GeneWiz (South Plainfield, NJ), whereas the plasmid to make the deletion of chi18D was amplified by PCR and constructed via Gibson assembly (58). All vectors were electroporated into *E. coli* S17 λpir. Through tri-parental mating, the deletion plasmid was conjugated into *C. japonicus* using an *E. coli* helper strain containing the plasmid pKK2013 (59). Recombinant colonies were selected using kanamycin selection, with a counter selection carried out via sucrose toxicity. The deletion mutants were confirmed by PCR, and the primers used for the construction and verification of *C. japonicus* mutants are listed in Table S5.

**Visualization of colloidal chitin degradation**

Colloidal chitin was prepared as described in (60). Colloidal chitin plates were made using MOPS-defined medium supplemented with 2% (w/v) colloidal chitin and 0.2% (w/v) glucose. To assay for chitin degradation, 10 μl of overnight cultures of the *C. japonicus* strains to be analyzed were spotted onto the chitin plate and incubated for 4 days at 30 °C. Then the plates were stained with a 0.1% (w/v) Congo red solution for 10 min followed by 10 min destaining with a 1 M NaCl solution, as described previously for detection of degradation of carboxyl methyl cellulose (27, 45, 56).

**Transcriptomic analysis**

A transcriptomic analysis was conducted for *C. japonicus* grown using glucose or α-chitin. To prepare the samples, the protocol described by Gardner et al. (14) was followed. Briefly, 35 ml of cell culture were aliquoted into 50-ml conical tubes with 5 ml of a stopping solution made with ethanol and saturated phenol (19:1). The cells were pelleted by centrifugation at 8000 × g for 5 min at 4 °C. The supernatant was removed, and the cell pellets were immersed in a dry ice/ethanol bath for 5 min. The frozen cell pellets were then stored at −80 °C. For each carbon source, the samples were taken in biological triplicate and at two time points: the beginning of the exponential phase (0.1 > A_{600} > 0.2) and the beginning stationary phase. GeneWiz performed RNASEq using an Illumina HiSeq2500 (50-bp single-reads; >10 million reads/sample), and sequence data were analyzed using CLC Genomics Workbench (Qiagen). The log_{2} transformation and a Student’s *t* test were used for the comparative analysis of *C. japonicus* gene expression. An adjusted *p* value > 0.01 and a log_{2} fold change > 2 were selected as significance cutoff parameters. RNASEq data for glucose grown *C. japonicus* cells (GSE90955) was used as done previously (26). The chitin-specific RNASEq data that was generated...
for this study can be found in the NCBI GEO database (GSE108935).

**Bioinformatics analysis**

We determined the predicted CAZy domains presented using the Database for Automated Carbohydrate-Active Enzyme Annotation (38). Using LipoP (29) and SignalP (30), we determined the putative location of the chitinolytic enzymes. Three-dimensional models of the GH18 chitinases were generated using PyMod 2.0 (61).

**Cloning and expression of chitinases**

Synthetic genes of the four full-length chitinases, optimized for expression in *E. coli*, were purchased from GeneScript. These genes encoded the catalytic domain for CjChi18A and the full-length protein (without signal peptide) for CjChi18B, CjChi18C, and CjChi18D. Primers for amplification of the genes were designed so that a His tag followed by a TEV protease cleavage site was introduced at the N terminus of all proteins and a stop codon was introduced before the C-terminal His tag encoded in the vector. Using different primers for amplification, genes encoding different versions (Table S6) of the modular proteins were generated, which were cloned into the pNIC-CH vector (62) using ligation independent cloning (63). The DNA sequences of the genes were confirmed by Sanger sequencing. Plasmids were transformed into *E. coli* BL21 cells. Protein expression was tested by inoculating 50 ml of lysogenic broth + 50 μg/ml kanamycin with 500 μl of an overnight culture. The cultures were grown at 37 °C, and when the OD reached 0.6, the cells were induced by adding IPTG to a final concentration of 0.2 mM. After growth overnight at 30 °C, the cells were harvested by centrifugation (6164 × g, 12 min, 4 °C) and resuspended in 5 ml of 20 mM Tris-HCl, 150 mM NaCl, 5 mM imidazole, pH 8.0. DNasel and PMSF were added to final concentrations of 1.4 μg/ml and 0.1 mM, respectively, before the cells were lysed by sonication (pulse 3 s on, 3 s off for 3–5 min), using a VC750 VibraCell sonicator (Sonics & Materials, Inc.). Following cell disruption, the samples were centrifuged (11,814 × g, 12 min, 4 °C), after which the supernatant was collected and filtrated (0.22 μm). Analysis by SDS-PAGE showed that only CjChi18A was soluble. Except for the full-length enzymes, which were not produced in detectable levels, all other enzyme variants were produced in large amounts but were insoluble.

Production of CjChi18A was scaled up to a 500-ml culture following the same protocol as above for production and harvesting. The filtrated supernatant containing CjChi18A was used for protein purification by nickel affinity chromatography using a HisTrap HP 5 ml column (GE Healthcare Life Sciences) connected to an Äkta pure system (GE Healthcare Life Sciences) using a HisTrap HP 5 ml column (GE Healthcare Life Sciences) used for protein purification by nickel affinity chromatography as described above. After harvesting the cells (7025 × g, 12 min, 4 °C), the pellet was resuspended in 20 ml of 50 mM Tris, pH 8.0, 0.2 mM NaCl, and 50% of the cell suspension was used in the further steps. After another centrifugation (18,459 × g, 10 min, 4 °C), the pellet was resuspended in 10 ml of 50 mM Tris, pH 8.0, 0.2 mM NaCl and, after addition of lysozyme (final concentration, 200 μg/ml) and DNasel (final concentration 0.1 mM), the samples were incubated on ice for 30 min. The samples were subsequently sonicated (pulse 3 s on, 3 s off, 2–4 min), and inclusion bodies were harvested by centrifugation (18,459 × g, 10 min), after which the protein pellet was resuspended in 25 ml of washing buffer (20 mM Tris-HCl, 100 mM NaCl, 2% Triton X-100, pH 8.0). The centrifugation and subsequent resuspension in washing buffer steps were repeated twice, and after the final centrifugation, the pellet was dissolved in 5 ml of cold denaturing solution (50 mM Tris-HCl, pH 8.5, 100 mM NaCl, 5 mM guanidine HCl, 1 mM EDTA, and 20 mM DTT), vortexed for 1 min, and incubated overnight at 4 °C with slow rotation of the sample tubes. The samples were then centrifuged at 9000 × g for 10 min at 4 °C. The supernatant containing the denatured protein was then collected for refolding.

The sample containing the denatured protein (~5 ml) was added to 250 ml of cold refolding buffer (100 mM Tris-HCl, 0.4 mM L-arginine, 0.5 mM oxidized glutathione, 5 mM reduced glutathione) at a rate of 1 ml/h under intense stirring at 4 °C. After adding all protein, the solution was stirred overnight at 4 °C, before centrifugation (9000 × g, 10 min, 4 °C). The supernatant was collected and dialyzed (10-kDa molecular mass cutoff) against 2.5 liters of 50 mM Tris-HCl, 0.1 mM NaCl, pH 8.0, overnight. The dialysis solution was changed once, after ~6 h. After collecting the dialyzed sample, the protein was purified with nickel affinity chromatography as described above. All refolded proteins stayed soluble during these procedures, and the yields of recovered purified soluble protein were typically 1–5 mg/liter culture.

The presence and purity of the proteins was confirmed by SDS-PAGE, and relevant fractions were pooled before the protein solution was concentrated to 26.4 mg/ml for CjChi18A and 1–3.5 mg/ml for the unfolded/refolded chitinases, with concomitant buffer exchange to 50 mM Tris-HCl, 100 mM NaCl, pH 8.0, using Amicon Ultra-15 centrifugal filters with 10,000 Nominal Molecular Weight Limit (Merck Millipore). All resulting protein solutions were stable for weeks at 4 °C. Protein concentrations were determined by measuring the absorbance at 280 nm and using the theoretical extinction coefficient (calculated using the ExPASy ProtParam tool available from https://web.expasy.org/protparam). The catalytic domain of CjLPMO10A was expressed and purified as previously described by Forsberg et al. (13).

**Activity assays**

Standard reactions contained 15 g/liter α-chitin (extracted from *Pandanus boralis*; Seagarden, Karmsund, Norway) or 2 mM chito-oligosaccharides (Megazyme, Bray, Ireland), 0.1 mg/ml BSA and 20 mM BisTris, pH 6.5, unless stated otherwise. For chitin degradation 0.5 μM enzyme was used, whereas for chito-oligosaccharide degradation 50 nM enzyme was used, unless stated otherwise. The reaction mixtures were incubated in a thermomixer at 30 °C and 800 rpm, and enzyme activity was quenched by adding sulfuric acid to a final concentration of

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**Chitin degradation in *C. japonicus***

Standard reactions contained 15 g/liter α-chitin (extracted from *Pandanus boralis*; Seagarden, Karmsund, Norway) or 2 mM chito-oligosaccharides (Megazyme, Bray, Ireland), 0.1 mg/ml BSA and 20 mM BisTris, pH 6.5, unless stated otherwise. For chitin degradation 0.5 μM enzyme was used, whereas for chito-oligosaccharide degradation 50 nM enzyme was used, unless stated otherwise. The reaction mixtures were incubated in a thermomixer at 30 °C and 800 rpm, and enzyme activity was quenched by adding sulfuric acid to a final concentration of
Chitin degradation in C. japonicus

25 mm. A Rezex ROF fast acid H+ (8%) ion-exclusion column (Phenomenex) installed on a Dionex Ultimate 3000 HPLC with UV detection (194 nm) was used to analyze and quantify degradation products as described by Meek et al. (64). Conversion yields were calculated as percentages of the theoretical maximum after correction of the amount of chitin in the reaction for ash and water content. Reactions with (GlcNAc)₆ as substrate were analyzed using a Rezex ROA-organic acid H⁺ (8%) ion exclusion column (Phenomenex) installed on a Dionex Ultimate 3000 HPLC, using a column temperature of 65 °C. An 8-µl sample was injected on the column, and the mono/oligosaccharides were eluted isocratically at 0.6 ml/min with 5 mm sulfuric acid as mobile phase. The chito-oligosaccharides were monitored by measuring the absorbance at 194 nm. Standards with known concentrations of GlcNAc (Sigma) and (GlcNAc)₂₋₆ (MegaZyme) were used to determine the concentrations of (GlcNAc)₆ and the degradation products.

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