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Systems analysis in *Cellvibrio japonicus* resolves predicted redundancy of β-glucosidases and determines essential physiological functions

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SUMMARY

Degradation of polysaccharides forms an essential arc in the carbon cycle, provides a percentage of our daily caloric intake, and is a major driver in the renewable chemical industry. Microorganisms proficient at degrading insoluble polysaccharides possess large numbers of carbohydrate active enzymes, many of which have been categorized as functionally redundant. Here we present data that suggests that carbohydrate active enzymes that have overlapping enzymatic activities can have unique, non-overlapping biological functions in the cell. Our comprehensive study to understand cellodextrin utilization in the soil saprophyte *Cellvibrio japonicus* found that only one of four predicted β-glucosidases is required in a physiological context. Gene deletion analysis indicated that only the *cel3B* gene product is essential for efficient

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AUTHOR CONTRIBUTIONS

CEN generated *C. japonicus* mutants and performed growth analysis experiments, generated heterologous expression plasmids for experiments in *E. coli*, performed HPAEC-PAD analysis, and contributed to writing the manuscript.
AR cloned and characterized the enzymes used in this study.
CM cloned and characterized the enzymes used in this study.
JAW assisted with HPAEC-PAD method development and data analysis.
HJG supervised the recombinant enzyme component of the study and contributed to writing the manuscript.
JGG designed the study, generated and analyzed the RNAseq data, supervised the remaining work, and contributed to writing the manuscript.
All authors read and approved the final submitted version of the manuscript.

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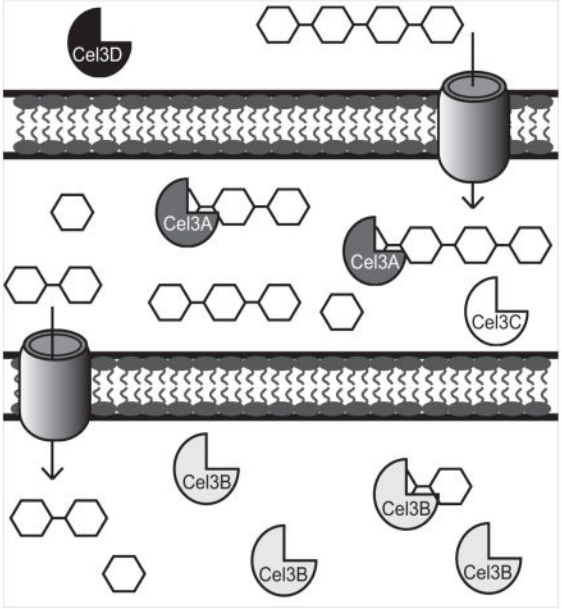
cellodextrin utilization in *C. japonicus* and is constitutively expressed at high levels. Interestingly, expression of individual β-glucosidases in *Escherichia coli* K-12 enabled this non-cellulolytic bacterium to be fully capable of using cellobiose as a sole carbon source. Furthermore, enzyme kinetic studies indicated that the Cel3A enzyme is significantly more active than the Cel3B enzyme on the oligosaccharides but not disaccharides. Our approach for parsing related carbohydrate active enzymes to determine actual physiological roles in the cell can be applied to other polysaccharide-degradation systems.

Graphical Abstract

Bacteria that can degrade polysaccharides in the environment often encode hundreds of enzymes for this process. It is unclear, however, if all of these enzymes are essential for polysaccharide degradation or have overlapping functions. Our study of the four β-glucosidases from the saprophytic bacterium *Cellvibrio japonicus* indicate these enzymes share a few biochemical properties, but have little physiological functional overlap.



Keywords

β-glucosidase; cellodextrin; cellulose; *Cellvibrio japonicus*; functional redundancy

INTRODUCTION

Degradation of insoluble polysaccharides, particularly lignocellulose, is a critical biological process that substantially impacts life on a scale that ranges from individuals to ecosystems. A calculated 10^{11} tons of lignocellulose is annually turned over as part of global nutrient cycles (Lynd *et al.*, 2002, Shin, 2010). Furthermore, an estimated 10% of the daily caloric intake of humans is derived from short chain fatty acids produced from lignocellulose degradation by gut microbiota (Lynd *et al.*, 2002, Martens *et al.*, 2014), the composition of

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which changes significantly depending on the type of plant biomass in the diet (Hehemann *et al.*, 2010). The economics of polysaccharide degradation have also been of intense interest in the renewable fuel and chemical industry, as lignocellulose degradation is thought to be a sustainable alternative to petroleum (Bokinsky *et al.*, 2011).

From both biotechnological and environmental perspectives, the key challenge in harnessing the energy in lignocellulose, or any other insoluble polysaccharide, is overcoming the so-called recalcitrance of the polymers (Marriott *et al.*, 2014, Himmel *et al.*, 2007). For example, cellulose comprises 30–50% of plant cell walls (Jordan *et al.*, 2012), and while a single cellulose polymer is structurally simple (un-branched (1,4)-β-D-glucose), the extensive network of hydrogen bonding creates a crystalline matrix and renders this polysaccharide insoluble (Lei, 2014). To completely degrade cellulose, the activities of three Carbohydrate Active enZyme (CAZyme) families (Lombard *et al.*, 2014) are required: endo-1,4-β-glucanases, exo-1,4-β-glucanases, and β-glucosidases (Horn *et al.*, 2012, Jordan *et al.*, 2012, Lombard *et al.*, 2014). A fourth activity from lytic polysaccharide mono-oxygenases (LPMOs) has recently been shown to also be important for efficient cellulose depolymerization in aerobic systems (Vaaje-Kolsstad *et al.*, 2012).

The microbes that have overcome the recalcitrance of insoluble polysaccharides often have genomes that encode a hundred or more CAZymes. A common characteristic of these polysaccharide-degrading microorganisms is the presence of multiple genes encoding enzymes that are predicted to have the same enzymatic activity (Mba Medie *et al.*, 2012). In some model bacteria, several of these enzymes have been characterized and labeled as “redundant” (Couger *et al.*, 2015, Gibson *et al.*, 2011, Morgenstern *et al.*, 2014, Schellenberger *et al.*, 2011). However these assessments have been based exclusively on biochemical and structural data. This has proven problematic for industry efforts to find the most economical enzyme mixtures for biotechnological applications, or ecological studies to uncover the major factors driving lignocellulose degradation in a physiologically relevant manner. Several groups have expressed the necessity of *in vivo* studies because purely *in vitro* data have shown to be poor predictors of enzyme activities on physiologically relevant substrates (Zhang *et al.*, 2002, Cartmell *et al.*, 2011, Naas *et al.*, 2014).

To overcome this challenge, a recent report employed a systems approach using physiologically relevant substrates and the genetically tractable model bacterium *Cellvibrio japonicus* to examine cellulose degradation (DeBoy *et al.*, 2008, Gardner & Keating, 2012). The physiological relevance of biochemically important enzymes was shown, as well as the power of genetic analysis to uncover functions for “unknown” enzymes (Gardner *et al.*, 2014). In this study, we examine the latter stages of cellulose degradation, focusing on cellodextrin utilization and the four β-glucosidases (GH3 family; Cel3A, Cel3B, Cel3D, and Cel3D) found in *C. japonicus*. Previous bioinformatics analysis of *C. japonicus* CAZymes predicted that Cel3D was secreted, Cel3A and Cel3C were located in the periplasm, and Cel3B was located in the cytoplasmic (DeBoy *et al.*, 2008). The GH3 family of β-glucosidases was the focus of this study because *C. japonicus* does not have any GH1 β-glucosidases, which are common in other cellodextrin utilizing microorganisms (Ketudat Cairns & Esen, 2010). Our data indicate that these enzymes play unique roles within the cell based on differences in their predicted localizations, expression patterns, and specific

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activities despite the capacity of all these β-glucosidases to confer the ability to utilize cellobiose in a non-cellulolytic bacterium. In addition, we discuss the differences between functional redundancy and biochemical redundancy in the context of polysaccharide degradation. We argue that it is likely that most enzymes labeled as "functionally redundant" in polysaccharide degrading bacteria are merely biochemically redundant, and the true physiological function of these enzymes has yet to be uncovered.

RESULTS

Cel3B is the β-glucosidase that makes the most significant contribution to the utilization of cellobiose by *C. japonicus*

The four β-glucosidase genes in *C. japonicus* (*cel3A*, *cel3B*, *cel3C*, and *cel3D*) were deleted individually and in combination and the mutants were grown on glucose, cellobiose, or insoluble cellulose to assess growth fitness (Fig. 1). When grown on glucose, the growth rates for the single, double, triple, and quadruple β-glucosidase mutants were all similar to that of wild type as was the maximum growth attained (Fig. S1). When the single deletion mutants were grown using cellobiose as the sole carbon source, the Δ*cel3B* mutant had a decreased growth rate compared to wild type, but was able to attain the same maximum optical density (OD) as the wild type bacterium. When grown with insoluble cellulose as the sole carbon source, the Δ*cel3B* mutant had a decreased growth rate compared to wild type and also a decreased maximum OD (Table S2). In addition, the lag time of the *cel3B* mutant increased from three to four hours (Table S1). Single deletions of *cel3A, cel3C,* and *cel3D* resulted in no significant growth defect when grown with cellobiose or insoluble cellulose (Fig. 1A and D). A Δ*gsp* mutant (defect in protein secretion) was used as a negative control for the insoluble cellulose experiments as it has previously been shown to be unable to utilize cellulose (Gardner & Keating, 2010).

Double deletion mutants were constructed combinatorially and tested as above (Fig. 1B and E). The Δ*cel3A*Δ*cel3C,* Δ*cel3A*Δ*cel3D,* and Δ*cel3C*Δ*cel3D* double mutants displayed no growth defect on cellobiose or insoluble cellulose. The Δ*cel3B*Δ*cel3C* and Δ*cel3B*Δ*cel3D* double deletion strains had growth rates similar to the Δ*cel3B* single mutant on cellobiose and insoluble cellulose (Table S1 and Table S2). The Δ*cel3A*Δ*cel3B* double deletion had a more exaggerated growth defect than the Δ*cel3B* single mutant, primarily due to a considerably longer lag phase compared to the Δ*cel3B* single mutant (Fig. 1B). On insoluble cellulose the Δ*cel3A*Δ*cel3B* double mutant grew more slowly and reached a lower maximum OD than the Δ*cel3B* single mutant (Table S2).

The combinatorial generation of triple deletion mutant strains were a powerful analytical tool as only one predicted β-glucosidase remained in these strains (Fig. 1C and F). The Δ*cel3A*Δ*cel3C*Δ*cel3D* mutant displayed no growth defect on either cellobiose or insoluble cellulose. The growth of Δ*cel3B*Δ*cel3C*Δ*cel3D* on cellobiose and insoluble cellulose was similar to the Δ*cel3B* single mutant (Table S1 and Table S2). The Δ*cel3A*Δ*cel3B*Δ*cel3D* mutant grew similar to the Δ*cel3A*Δ*cel3B* double mutant on both cellobiose and insoluble cellulose (Table S1 and Table S2) On cellobiose the Δ*cel3A*Δ*cel3B*Δ*cel3C* mutant grew more slowly and had a longer lag phase than the wild type or the other triple mutant strains

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(Table S1 and Table S2). On insoluble cellulose however, the Δ*cel3A*Δ*cel3B*Δ*cel3C* mutant grew similarly to the Δ*cel3A*Δ*cel3B* and Δ*cel3A*Δ*cel3B*Δ*cel3D* mutant strains.

A strain with all four of the β-glucosidases deleted was constructed and grown in either cellobiose or insoluble cellulose containing media (Fig. 1C and F). When cultured on cellobiose as the sole carbon source, the Δ*cel3A*Δ*cel3B*Δ*cel3C*Δ*cel3D* (Δ4βG) strain displayed a similar phenotype to Δ*cel3A*Δ*cel3B*Δ*cel3C* strain, both in terms of growth rate and maximum OD attained (Table S1). In addition, the Δ4βG strain had a very slow growth rate on insoluble cellulose and long lag phase on both cellobiose and insoluble cellulose. When using insoluble cellulose, the Δ4βG strain did not attain wild type levels of growth (Table S2). Overall, the Δ4βG strain behaved very similarly to the Δ*cel3A*Δ*cel3B*Δ*cel3C* triple mutant strain.

β-glucosidase mutants exhibit decreased cellobiose consumption

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was performed to analyze glucose and cellobiose consumption rate changes of the Δ*cel3B,* Δ*cel3A*Δ*cel3B*, and Δ4βG mutant strains compared to wild type. These strains were chosen for analysis based on the growth defect phenotypes observed (*vide supra*). As expected, the consumption of glucose was nearly identical between all mutant strains and wild type. All strains began to enter stationary phase nine hours after inoculation (Fig. 2A). By 15 hours after inoculation all of the strains had consumed nearly all of the glucose in the medium, and after 24 hours inoculation there was no detectable glucose in any of the samples (Fig. 2B).

When grown on cellobiose, the wild type strain had a short one-hour lag and then grew rapidly before entry into stationary phase after nine hours. Upon entry of stationary phase there was still 7.49±0.17 mM left of the original 18.24±0.20 mM of cellobiose in the wild type samples. However, the cellobiose concentration continued to decrease through stationary phase in the wild type samples, dropping below 1 μM by 24 hours after inoculation. The Δ*cel3B* mutant had a three-hour lag before reaching exponential growth and entered stationary phase 12 hours after inoculation, at which point ~40% of the cellobiose remained (Fig. 2C). After 24 hours the disaccharide was fully consumed by the Δ*cel3B* mutant. The amount of cellobiose remaining in the medium of the Δ*cel3A*Δ*cel3B* and Δ4βG flasks changed little nine-hours post inoculation, with only a 0.33mM reduction for Δ*cel3A*Δ*cel3B* flasks and no measurable change in the Δ4βG flasks. Upon entering stationary phase, 18 hours after inoculation, there was ~28% cellobiose remaining in the Δ*cel3A*Δ*cel3B* flasks and ~30% in the media of the Δ4βG flasks (Fig. 2D). Unlike wild type and the Δ*cel3B* strain, there was detectable cellobiose remaining in the media of Δ*cel3A*Δ*cel3B* and the Δ4βG flasks (0.098±0.11 mM and 0.43±0.1 mM, respectively), 24 hours post inoculation, indicating these mutants were unable to consume all of the cellobiose in the same timeframe as a wild type strain.

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Individual β-glucosidases heterologously expressed in *E. coli* are sufficient for cellobiose utilization

Our genetic analysis suggested that only *cel3B* is required for efficient cellobiose utilization as indicated by the Δ*cel3A*Δ*cel3C*Δ*cel3D* mutant (Fig. 1C), however a previous enzymatic study showed that Cel3A is a highly active β-glucosidase (Rixon *et al.*, 1992). This led us to hypothesize that perhaps all four of the predicted β-glucosidases in *C. japonicus* do indeed have sufficient β-glucosidase activity to support growth on cellobiose. To examine this hypothesis, the four β-glucosidases were cloned individually into the pBBRMCS-5 vector (Elzer *et al.*, 1994) and expressed in *E. coli* K-12. As expected, the expression of these genes did not affect growth on glucose (Fig. 3A) and a strain containing an empty pBBRMCS-5 vector was unable to grow on cellobiose (Fig. 3B). When tested on cellobiose, three distinct phenotypes were displayed by the *E. coli* strains expressing the *C. japonicus* β-glucosidases. Expression of *cel3A* resulted in rapid growth, with a short lag of four hours and a growth rate of 0.34±0.01 generations per hour (gen hr^{-1}). Strains expressing *cel3C* or *cel3D* grew similarly, with growth rates of 0.10±0.002 and 0.11±0.01 gen hr^{-1}, respectively, and a lag of eight to ten hours. The strain expressing *cel3B* had a growth rate of 0.11±0.004 gen hr^{-1} and a lag of 24 hours before starting to grow. Despite the differences in growth rate, expression of the four β-glucosidase genes resulted in the same maximum OD attained for the *E. coli* strains. This suggests that while in *C. japonicus* the GH3 genes are not functionally equivalent, when expressed in *E. coli*, which removes much of the physiological and regulatory context, the GH3 genes all do have similar enzymatic action on the disaccharide cellobiose.

RNAseq analysis reveals *cel3B* is constitutively expressed at high levels

To examine the physiological extent for gene expression, we performed RNAseq analysis. Gene expression profiles for *C. japonicus* when using cellodextrins were collected with either glucose or cellobiose as the sole carbon source (Fig. S2). Samples were taken from either exponentially growing or stationary phase cells and subjected to RNAseq analysis. Using two carbon sources and two sampling points, there are four pair-wise comparative analyses possible: exponential (glucose) vs. exponential (cellobiose) (Fig. S2), exponential vs. stationary phase for glucose or cellobiose, or stationary (glucose) vs. stationary (cellobiose) (Fig. S3). The comparative analyses from the exponential vs. stationary phase for glucose or cellobiose are useful as control experiments to discover the genes that are up-regulated in response to changes in growth rate; none of the GH3 genes were regulated by growth rate.

Analysis of the exponential (glucose) vs. exponential (cellobiose) comparison (Fig. S2) revealed that only ten genes annotated as CAZymes were up-regulated and only one was a β-glucosidase (*cel3A*) (Table S3). The other up-regulated CAZymes were predicted to be active on a diverse range of substrates such as xylan, starch, and mannan. Bioinformatic analyses of these genes indicate they are transcribed alone (DeBoy *et al.*, 2008), suggesting their up-regulation on cellobiose is not driven by co-expression in an operon. To test if these up-regulated CAZymes were playing a role in cellobiose utilization, we generated mutant strains for these genes and assessed the fitness of the strains using media containing cellobiose as the sole carbon source. All of these strains grew like wild type (Fig. S2). This

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result indicated that these genes were part of a regulatory program, rather than having a direct role in cellobiose utilization.

Comparative analysis is often useful to find differentially expressed genes; however, a drawback of this type of analysis is that it will not uncover constitutively expressed genes. An examination of the RNAseq data in the absence of comparison, as determined by the reads per kilobase per million mapped reads (RPKM), we found that *cel3B* is highly constitutively expressed (Table 1). The *cel3A* gene, while inducible by cellobiose, had an expression level that was 2.7-fold lower than *cel3B* expression. The *cel3C* and *cel3D* genes had low levels of expression under all growth conditions and were 27-fold and 15-fold lower than *cel3B* on cellobiose, respectively.

The enzymatic activites of *C. japonicus* β-glucosidases indicates preference for different cellodextrins

The data described above indicated that of the four β-glucosidases Cel3B plays the most important role in cellobiose utilization by *C. japonicus,* although Cel3A also contributes to the degradation of the disaccharide. These results prompted detailed analysis of the kinetic properties of all four GH3 β-glucosidases. Cel3A, Cel3B, Cel3C, and Cel3D were produced in recombinant form from *E. coli* and purified to electrophoretic homogeneity. The kinetics of the four enzymes were evaluated against a series of β-1,4-glucooligosaccharides. The data are summarized in Table 2 with example Michaelis-Menten plots for Cel3A and Cel3B displayed in Fig. S4. Cel3B was ~50-fold more active than Cel3A and Cel3C against cellobiose, with Cel3D displaying very low activity against the disaccharide. Against substrates with a degree of polymerization ≥3, the activity of Cel3A and Cel3C increased while Cel3B decreased. Indeed, Cel3A and Cel3C appear to contain three glucose-binding subsites, extending from −1 to +2, as the activity of the enzyme against cellotriose, cellotetraose and cellopentaose were similar. Cel3D displayed only modest, catalytic efficiency against cellotetraose. It should be noted that the difference in activity against these substrates likely reflects their cellular location and contribution to cellulose degradation. The periplasmic location of Cel3A is consistent with exposure to cello-oligosaccharides derived from the extracellular degradation of β-1,4-glucans (Rixon *et al.*, 1992, Gardner, 2016). The predicted cytoplasmic location of Cel3B (DeBoy *et al.*, 2008) and its strong preference for cellobiose suggests that the inner membrane of *C. japonicus* contains a transporter that specifically imports the disaccharide into the cytoplasm.

DISCUSSION

Cellulose degradation is biochemically simple, requiring three to four enzymatic activities (Horn *et al.*, 2012, Jordan *et al.*, 2012, Vaaje-Kolsstad *et al.*, 2012). However, the most effective cellulose degraders in the environment often possess dozens of CAZymes for cellulose degradation (Mba Medie *et al.*, 2012). This has led to many statements in the literature about “functional redundancy” in regards to environmentally or biotechnologically relevant polysaccharide degradation (Couger *et al.*, 2015, Gibson *et al.*, 2011, Morgenstern *et al.*, 2014, Schellenberger *et al.*, 2011). However, this sentiment is formulated on almost exclusively biochemical and structural data and does not factor in physiologically relevant

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parameters. In this study, we use a systems approach to examine cellodextrin utilization, the final stages of cellulose degradation, in a saprophytic bacterium. Our data indicate that the four β-glucosidases present in *C. japonicus* are not "functionally redundant," but share attributes that can be classified as "biochemically redundant". This study may provide an example how to probe CAZymes in polysaccharide degrading microorganisms in a comprehensive manner to better classify, model, and predict CAZyme functions.

RNAseq analysis corroborated the mutational data and demonstrates the utility of both comparative and absolute expression analyses. When using a comparative approach with glucose as the reference condition, the *cel3A* gene was the only up-regulated β-glucosidase on cellobiose (~8-fold) during exponential growth. Several additional CAZyme genes were up-regulated on cellobiose, but this likely reflects a suite of genes under similar regulatory control, which is responsive to cellobiose in a manner similar to the cellulose regulon of *Neurospora crassa* (Znameroski *et al.*, 2012). Deletion of these up-regulated CAZyme genes in *C. japonicus* did not influence cellobiose utilization (Table S2), again suggesting that these genes are part of a complex regulatory response, where cellodextrin utilization is only one part. The fact that more cellulases were not up-regulated was not surprising, as previous studies in *N. crassa* have shown that the lignocellulose degrading response was induced by cellobiose but was masked by the effects of carbon catabolite repression in the presence of β-glucosidases generating glucose (Znameroski *et al.*, 2012). By examining the absolute level of expression (RPKM), it was revealed that the *cel3B* gene was highly expressed across all times and substrates, and nearly 2.7-fold higher than the induced expression of the *cel3A* gene in cellobiose. Expression of *cel3C* or *cel3D* genes was low and not induced to significant levels on cellobiose.

The mutational analyses allowed us to determine a hierarchy of the β-glucosidases in *C. japonicus* in a physiological context, with *cel3B* being critical, *cel3A* having a supporting role, *cel3C* having a minor role, and *cel3D* having a negligible role in regards to cellobiose utilization. We observed a strong, reproducible growth defect with the single *cel3B* mutant strain when grown on cellobiose and insoluble cellulose (Fig. 1A and D). The HPAEC-PAD analysis of media during growth on cellobiose also indicated a decreased rate of cellobiose consumption directly correlated with the decreased growth rate observed for Δ*cel3B* mutant strains (Fig. 2). The physiological roles of these genes were entirely consistent with their expression levels in response to cellobiose. Severe growth defects resulting from a single gene inactivation were also observed in our previous work on cellulose and xyloglucan degradation in *C. japonicus* (Gardner *et al.*, 2014, Larsbrink *et al.*, 2014). Single-gene mutants have been shown to elicit strong effects in other microbes; for example, the inactivation of a single GH9 endo-processive endo-glucanase gene in *Clostridium phytofermentens* was enough to abolish growth on cellulose in this anaerobe (Tolonen *et al.*, 2009). However, the power of a combinatorial mutagenesis approach became evident with the creation of the double and triple β-glucosidase mutant strains. The analyses of the double mutant strains revealed a contribution to cellobiose utilization by *cel3A* but not *cel3C* or *cel3D* (Fig. 1B and E). Analysis of triple deletion strains, specifically the Δ*cel3A*Δ*cel3C*Δ*cel3D* mutant that was able to grow like wild type on cellobiose and insoluble cellulose (Fig. 1C and F), gave strong evidence that *cel3B* is the dominant β-glucosidase in *C. japonicus*. The addition of the *cel3C* deletion to the Δ*cel3A*Δ*cel3B*

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background elicited another third growth phenotype that was absent from the Δ*cel3A*Δ*cel3B*Δ*cel3D* triple mutant. Finally the quadruple mutant behaved nearly identical to the Δ*cel3A*Δ*cel3B*Δ*cel3C* mutant, providing further evidence that the *cel3D* gene product was the least important for cellodextrin utilization in *C. japonicus* (Fig. 1C and F). Therefore, from a physiological perspective, the hierarchy for cellodextrin utilization in *C. japonicus* is *cel3B* > *cel3A* > *cel3C* ≫> *cel3D*.

Interestingly, the Δ4βG strain was still able to grow despite lacking all predicted β-glucosidases. This may be a result of other glucan 1,4-β-glucosidase activity from other cellulases. Glucan 1,4-β-glucosidase activity (EC 3.2.1.74) is found in GH3, GH5, and GH9 families, and *C. japonicus* has genes encoding 15 GH5 and 3 GH9 enzymes (Lombard *et al.*, 2014). The GH5 family is large with enzymes that target a diverse range of substrates including cellulose, mannan, and xyloglucan. Of the GH5 genes encoded by *C. japonicus* there are nine enzymes that are predicted to play a role in cellulose degradation (Lombard *et al.*, 2014). The GH5 family is further divided into subfamilies, many of which are poly-substrate specific, with more than one enzyme activity experimentally determined. The poly-substrate specificity could indicate that although many of the GH5 cellulases are not predicted to have the β-glucosidase activity, it is possible they have this activity in addition to, or instead of, their predicted activity. Aspeborg *et. al.* point out that annotated CAZyme activities could be absent in GH subgroups due to limitations in experimentally tested substrates (Aspeborg *et al.*, 2012). This may hold true for the predicted cellulolytic GH5 and GH9 gene products in *C. japonicus* and will need to be further explored in the future.

Heterologous expression experiments in the non-cellulolytic *E. coli* indicated that all four of the GH3 genes of *C. japonicus* were sufficient to act as β-glucosidases. The expression of *cel3A* yielded rapid growth on cellobiose (Fig. 3B), which was not surprising as previous work indicated that Cel3A was a highly active enzyme (Rixon *et al.*, 1992). The growth rate differences between the *E. coli* strains are likely consequences of a combination of 1) the GH3 genes being heterologously over-expressed, 2) catalytic parameters of the GH3 enzymes, and 3) poor transport of cellobiose into *E. coli* (Hall & Faunce, 1987). For example, Cel3A was determined to have low activity against cellobiose but substantially higher activity against cellotetrose and cellopentose (Table 2). When tested kinetically, Cel3B was determined to have high activity on cellobiose, although almost ~2-fold lower than the efficiency of Cel3A on cellotetrose and cellopentose. In addition, Cel3B has low activity on cellotriose, cellotetrose, and cellopentose. The differences in enzyme efficiencies may indicate specialization of the enzymes for the cellodextrins they encounter in the environment. Cel3A is membrane associated so it is positioned close to the cell and able to cleave glucose off of the larger cellodextrins, while Cel3B is cytoplasmic and most likely encounters cellobiose. Interestingly, *E. coli* cells expressing *cel3C* and *cel3D* also grew when provided cellobiose, suggesting that *cel3C* and *cel3D* can have β-glucosidase activity *in vivo*. However, it should be noted that growth observed in a heterologous system may not necessarily be biologically relevant because these experiments force expression higher than what is found in the native bacterium. For example, the enzymatic analysis of Cel3D found cellobiose to be a very poor substrate, which reinforces the *C. japoincus* mutant data that indicate that Cel3D does not play a role in cellobiose metabolism.

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The synthesis of these data support the hypothesis that the β-glucosidases of *C. japonicus* are in fact playing different roles within the cell and are not functionally redundant (Fig. 4). This model is built from a previous bioinformatics study of *C. japoncus* CAZymes, which placed the GH3 enzymes in three distinct cellular spaces (DeBoy *et al.*, 2008), however to date only one of these (Cel3A) has been experimentally confirmed (Rixon *et al.*, 1992). This study adds to the model strong physiological data, including gene expression analysis, enzyme kinetics, and growth phenotypes of β-glucosidase mutant strains, and we argue that the four β-glucosidases of *C. japonicus* are playing unique roles within the cell despite their predicted redundancy. For this particular study, given that the activities of the GH3 enzymes are similar, the Cel3B β-glucosidase is dominant because of its high constitutive expression level and cellular location in the cytoplasm. We argue that to be considered "functionally redundant," four physiologically relevant parameters must be considered: time (when the enzyme is expressed), space (cellular location), substrate specificity (including side activity), and kinetics (including enzyme synergy). Cel3B, the cytoplasmic constitutively expressed enzyme, appears to be responsible for degrading cellobiose that is transported into the cytoplasm. Cel3A while induced by cellobiose appears to be more specific for degrading cellotetrose and cellopentose. The lack of growth defects caused by the deletion of the *cel3C* and *cel3D* genes in *C. japonicus*, despite being able confer the ability to utilize cellobiose in *E. coli*, indicate these enzymes are biochemically able to act as β-glucosidases and we would term this "biochemical redundancy" not "functional redundancy." The severe growth defects of the double deletions retaining only Cel3C and Cel3D indicate that these enzymes are unable to compensate for the lack of Cel3A and Cel3B when utilizing cellobiose. We argue that CelD is not functionally redundant because biochemical analysis indicates very low activity on cellobiose, and *cel3D* mutants are phenotypically wild type. Stated another way, Cel3D is not functionally redundant because it does not function physiologically (or enzymatically in a robust way) as a β-glucosidase. Therefore current investigations are looking for a biological role for Cel3D in the degradation of non-cellulose oligosaccharides.

Extrapolating beyond the utilization of cellodextrins, we believe that a "biochemical redundancy" and "functional redundancy" debate in regards to other CAZymes would have a similar outcome as suggested by this study. By calling into question if a set of CAZymes are truly "functional redundant" and striving to determine the physiological relevance of a gene product, we will better inform CAZyme capabilities and therefore better position them for environmental, biotechnological, and human health applications, as this approach will tease out which "redundant" enzymes are important in the degradation of a substrate they can all use.

EXPERIMENTAL PROCEDURES

Genetic techniques

Generation and verification of *C. japonicus* knockout mutants was done as previously described (Gardner & Keating, 2010). Deletion mutants were generated as previously described (Nelson & Gardner, 2015) with the following modifications. Briefly, the 500 bp upstream and downstream region of the targeted gene was amplified by PCR rather than being synthesized by GeneWIZ (South Plainfield, NJ) and assembled into the pK18*mobsacB*

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vector (Schafer *et al.*, 1994) via the assembly method of Gibson *et al.* (Gibson, 2009). Primers used for the construction of vectors and PCR verification of *C. japonicus* mutants are listed in Table S4. For heterologous expression studies the *cel3A, cel3B, cel3C,* and *cel3D* genes were synthesized by GeneWIZ and cloned into the pBBRMCS-5 vector (Elzer *et al.*, 1994). The pBBRMCS-5 vectors containing the individual β-glucosidases were transformed into chemically competent *E. coli* K-12 using standard protocols (Green & Rogers, 2013).

Growth analysis

C. japonicus strains were grown in MOPS defined medium (Neidhardt *et al.*, 1974) containing glucose, cellobiose, or filter paper as the sole carbon source. *E. coli* strains were grown in lysogenic broth (LB) (Bertani, 1951) or M9 (Neidhardt *et al.*, 1974) minimal media containing glucose or cellobiose. Cultures were grown as described by Nelson & Gardner (Nelson & Gardner, 2015). Briefly, strains were inoculated from a MOPS-glucose plate into 5 mL of MOPS-glucose broth and grown for 24 hours, then sub-cultured (1:100 dilution) into fresh medium used for growth analysis (*e.g.* MOPS-cellobiose). Growth was measured by optical density at 600 nm (OD_{600}) using a Spec20D+ spectrophotometer (Thermo Scientific) or a Tecan M200Pro microtiter plate reader (TECAN Trading AG, Switzerland). Cultures were grown at 30°C with high aeration (220 rpm) in 18 mm culture tubes or in a 96 well flat bottom polystyrene assay plate (Corning Inc, Corning, NY). *C. japonicus* strains were grown in MOPS defined medium containing glucose (0.25% w:v), cellobiose (0.5% w:v), or filter paper (1% w:v) as the sole carbon source (Nelson & Gardner, 2015). All growth experiments were performed in biological triplicate with statistical significance calculated when appropriate using the GraphPad Prism 6 software package (La Jolla, CA). Exit from lag phase is a complex process and while many methods and models have been proposed (Zwietering *et al.*, 1992), for this study it was defined simply as a >25% increase in OD from the starting OD. When required, kanamycin was used at a concentration of 50 μg mL^{-1}, gentamycin at a concentration of 15 μg mL^{-1} and ampicillin at a concentration of 100 μg mL^{-1}.

Quantification of cellobiose consumption

C. japonicus wild type and deletion strains Δ*cel3B,* Δ*cel3A*Δ*cel3B,* Δ4βG were grown in 250 mL baffled flasks as described above. Cultures were grown for 24 hours with OD_{600} read every hour for the first 15 hours and a final time point after 24 hours for wild type and *Δcel3B.* For Δcel3AΔ*cel3B* and Δ4βG an initial OD_{600} reading taken after inoculation then OD_{600} was read every hour from nine hours post inoculation to 24 hours post inoculation. Samples were collected right after inoculation and 24 hours post inoculation for all samples. Additionally, samples for wild type and Δ*cel3B* were taken every three hours from three hours post inoculation until 15 hours post inoculation and for Δ*cel3A*Δ*cel3B* and Δ4βG samples were taken every three hours from nine hours post inoculation until 21 hours post inoculation. Sampling was as follows: 5 mL was aseptically removed and transferred to 18 mm culture tubes for OD_{600} readings in a Spec20D+ spectrophotometer (Thermo Scientific) and returned to the original flask. A 1 mL sample was removed after OD_{600} readings and pelleted. The supernatant was filtered using 0.45 μM Spin-X column (Corning Inc., Corning,

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NY), aliquotted (200 μL), and stored at −20 °C. All experiments were performed in biological triplicate.

Samples were analyzed by HPAEC-PAD using a Dionex ICS-5000$^+$ system (Thermo Scientific) equipped with a 4 × 250 mm SA-10 anion-exchange column (Thermo Scientific) and a 4 × 50 mm SA-10 guard column (Thermo Scientific). A flow rate of 1mL/min was used and the temperature of the column was kept at 30 °C. The 25 μL samples were injected and run isocratically with 20 mM NaOH. Data analysis was performed with the Dionex Chromeleon 7 (Thermo Scientific) software package.

RNAseq sampling and analysis

Gene expression analysis was performed similarly as that previously described (Gardner *et al.*, 2014). Briefly, *C. japonicus* cultures were grown in 500 mL flasks and growth was monitored with OD_{600} readings every hour for the first 12 hours of growth and again at 24 hours. A 35 mL sample was taken during exponential growth phase and again during stationary phase as determined by OD_{600}. Metabolism of the sample was stopped with 5ml of a phenol/ethanol solution (5%/95%) within 2 minutes of sampling. The samples were then immediately pelleted by centrifugation at 8000 × *g* for five minutes, the supernatant decanted, and the cell pellets flash frozen in a dry ice/ethanol bath for five minutes, then stored at −80°C. RNA extraction, library preparation, multiplexing, and sequencing was performed by GeneWIZ (South Plainfield, NJ). Illumina HiSeq2500 was performed in 50bp single-reads with at least 10 million reads generated per sample. The raw sequence data generated was converted into FASTQ files using Illumina CASSAVA 1.8.2, which were then imported into CLC Genomics Workbench 7.5.1. Low quality base pairs were trimmed from the ends and the sequence reads were mapped to the *C. japonicus* reference genome with RPKM values calculated for genes. Log_2 transformation and quantile normalization was performed for the RPKM values and a Student's T-test conducted to compare gene expression between various groups of samples. Additional analysis and visualization of the data was performed using the ArrayStar (DNASTAR, Madison, WI) software package. The raw data have been submitted to GEO (GSE90955).

Recombinant expression, purification, and kinetic analysis of Cel3A and Cel3B

C. japonicus genomic DNA was used for the PCR amplification of full length Cel3B and Cel3A lacking its signal peptide. The primers also contained sequence for the *Nde*I and *Xho*I restriction sites, and the PCR products were cloned into the *E. coli* expression vector pET28b. *E. coli* expression of Cel3A and Cel3B and the subsequent purification of the two enzymes to electrophoretic homogeneity by immobilized metal affinity chromatography using a Talon (immobilized cobalt) column was as described previously (Cuskin *et al.*, 2015). The enzymes were assayed against β-1,4-cello-oligosaccharides obtained from Megazyme International using the Glucose Detection Kit supplied by the same company. The assays were carried out in 50 mM sodium phosphate buffer, pH 7.5 at 37°C. When measuring both K_m and k_{cat} the initial rate was determined at five or more concentrations of substrate. When determining only catalytic efficiency, k_{cat}/K_m, the initial rate at a substrate concentration $\ll K_m$ was determined.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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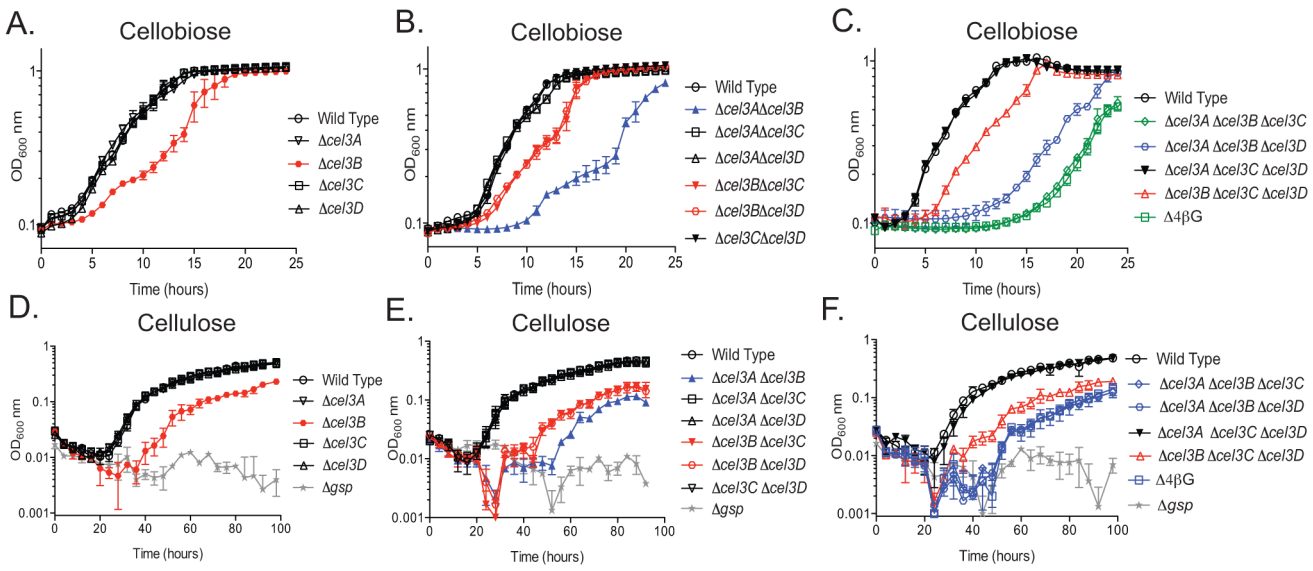

Fig. 1. Deletion of multiple β-glucosidases enzymes shows varying contribution to cellobiose and filter paper utilization

Growth analysis of all *C. japonicus* mutants grown using, 0.5% cellobiose or 1% cellulose as the sole carbon source. **(A-C)** Growth analysis of single **(A)**, double **(B)**, triple and quadruple **(C)** deletion mutants grown using cellobiose as the sole carbon source. **(D-F)** Growth analysis of single **(D),** double **(E)**, triple and quadruple **(F)** deletion mutants grown using cellulose as the sole carbon source. All experiments were performed in biological triplicate where error bars represent standard deviation and are present for all data points, although sometimes too small to be observed. All strains grew similarly to wild type when glucose was the carbon source used, which is shown in Supplemental Figure S1.

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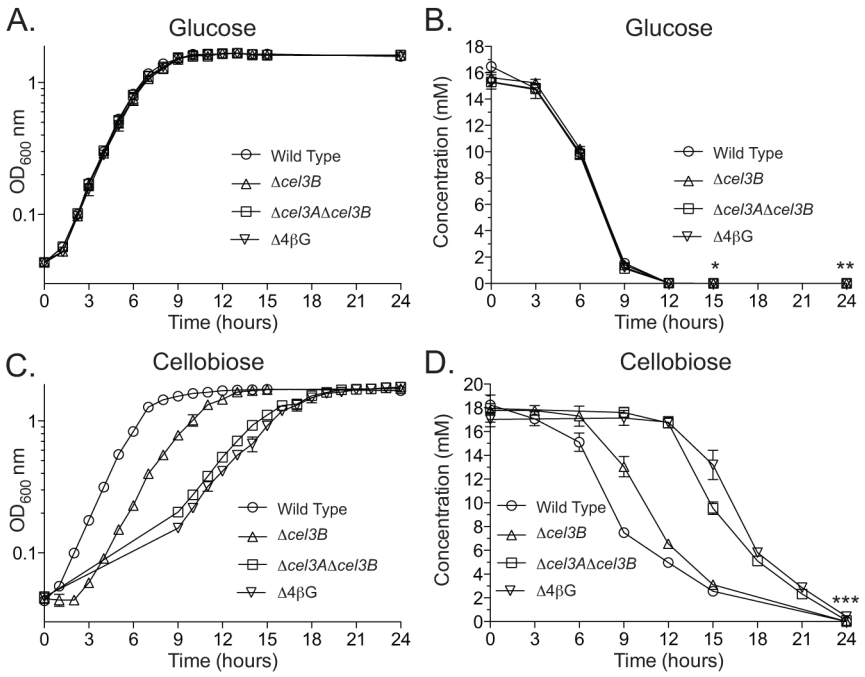

Fig. 2. Decrease in rate of cellobiose consumption mirrors growth defects of β-glucosidase deletion mutants

Quantification of 0.25% glucose and 0.5% cellobiose consumption during growth of wild type *C. japonicus* and Δ*cel3B*, Δ*cel3A*Δ*cel3B*, and Δ4βG mutants. **(A & B)** All four strains were grown using glucose as the sole carbon source **(A)**. During growth, samples were taken every three hours and the concentration of remaining glucose was quantified using HPAEC-PAD **(B). (C & D)** All four strains were grown using cellobiose as the sole carbon source **(C).** Samples were taken every three hours and quantified using HPAEC-PAD **(D)**. The glucose and cellobiose experiments were both performed in biological triplicate. Error bars represent the standard deviation and are present for all data points. The limit of quantitation for glucose and cellobiose using the HPAEC-PAD parameters described in the Materials and Methods was 1 μM. The "*" symbol indicates Δ*cel3A*Δ*cel3B* was below the level of quantitation, "**" indicates all four strains were below the level of quantitation, and "***" indicates wild type and Δ*cel3B* were below the level of quantitation.

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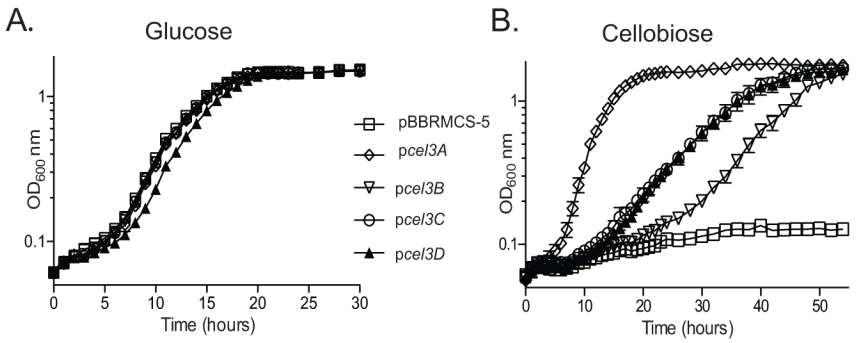

Fig. 3. All four β-glucosidases from *C. japonicus* are individually sufficient to confer cellobiose utilization to a non-cellulolytic organism

The four β-glucosidases were cloned individually into the pBBRMCS-5 vector and transformed into *E. coli* K-12 and grown on 0.25% glucose **(A)** and 0.5% cellobiose **(B)**. The empty pBBRMCS-5 vector was also transformed into K-12 as a control strain. Experiments were performed in biological triplicate and the error bars, in most cases too small to be seen, represent the standard deviation.

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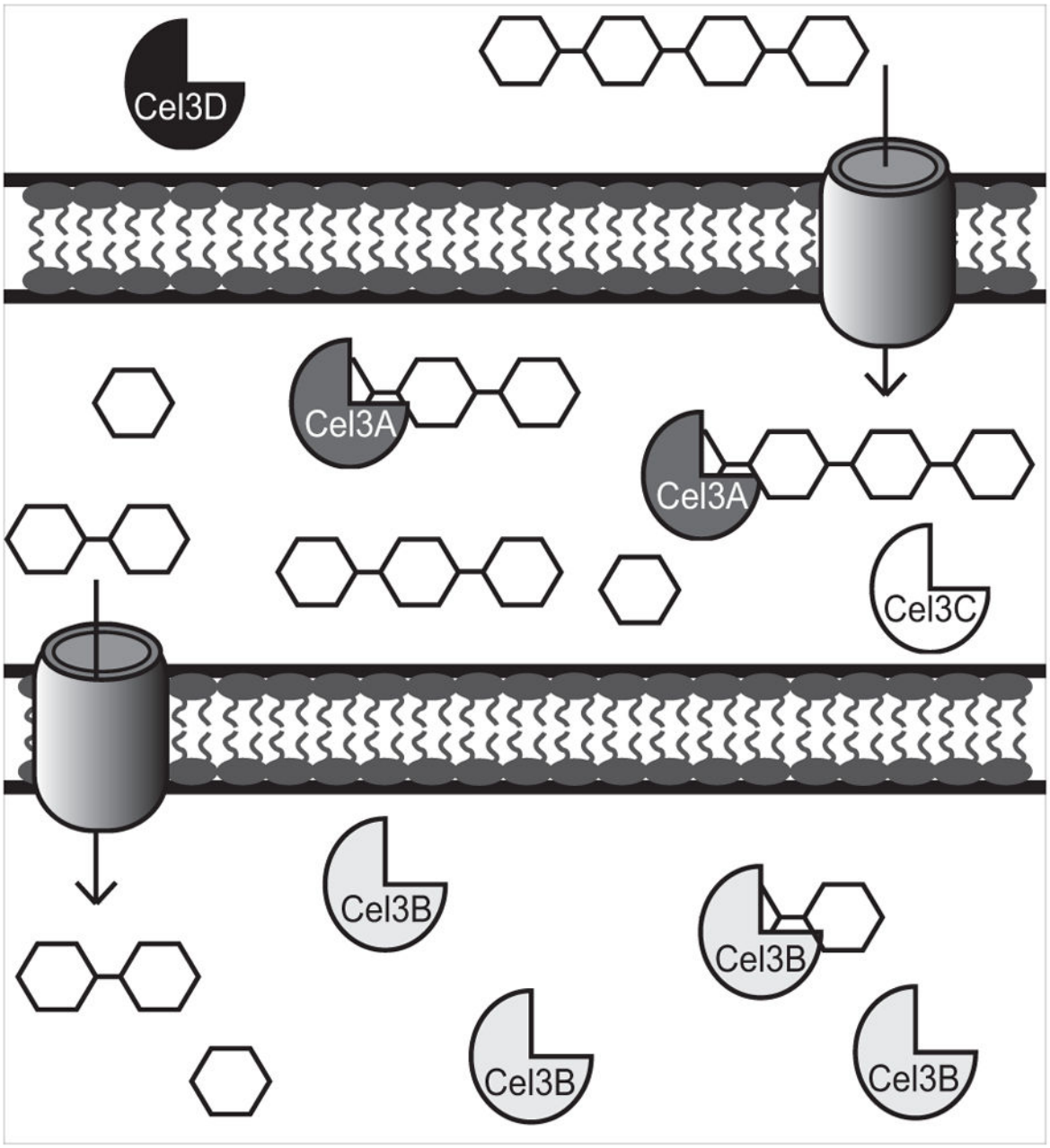

Fig. 4. Proposed model for soluble cellodextrin utilization in *C. japonicus*

Cel3D, represented with black icons, is predicted to be located extracellularly. Cel3A, dark gray icons, and Cel3C, white icons, are predicted to be membrane associated which has been confirmed for Cel3A by Rixon *et. al.* (Rixon *et al.*, 1992). Based off of enzyme activities, large cello-oligosaccharides are transported into the periplasm where Cel3A degrades them. Smaller cello-oligosaccharides like cellobiose are transported into the cytoplasm where Cel3B, which is represented with light gray icons, breaks them down into glucose. When

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cellobiose is transported into the cell, *cel3A* expression is induced while *cel3B* is constitutively expressed as indicated by RNAseq experiments.

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Table 1

Expression of the GH3 genes during exponential growth in defined minimal media[a]

Gene	Glucose (RPKM[b])	Cellobiose (RPKM)
cel3A	183±17.9	522±34.1
cel3B	1420±70.7	1410±42.3
cel3C	42.1±21.7	52.2±5.64
cel3D	58.7±17.8	93.8±3.38

[a] Experiment was performed in biological triplicate

[b] Reads per kilobase per million mapped reads

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Table 2

Kinetics of *C. japonicus* GH3 β-glucosidases

Enzyme	Substrate	k_{cat} (min^{-1})	K_m (mM)	k_{cat} / K_m (min^{-1} mM^{-1})[a]
Cel3A	Cellobiose	117±2.5	1.8±0.094	65
Cel3B	Cellobiose	5385±175	1.5±0.13	3590
Cel3C	Cellobiose	168±6	1.9±0.14	89
Cel3D	Cellobiose	10±0.7	11.5±1.5	0.9
Cel3A	Cellotriose	1240±37	0.23±0.02	5391
Cel3B	Cellotriose	731±42	1.5±0.16	487
Cel3C	Cellotriose	846±28	1.1±0.07	769
Cel3D	Cellotriose	227±18	6.2±0.94	37
Cel3A	Cellotetraose	ND	ND	5306±105
Cel3B	Cellotetraose	ND	ND	522±12
Cel3C	Cellotetraose	ND	ND	856±22
Cel3D	Cellotetraose	ND	ND	91±2.5
Cel3A	Cellopentaose	ND	ND	5126±133
Cel3B	Cellopentaose	ND	ND	464±16
Cel3C	Cellopentaose	ND	ND	865±14
Cel3D	Cellopentaose	ND	ND	44±1.7

[a] In experiments where only k_{cat}/K_m was determined, substrate concentrations $\ll K_m$ were used as the initial rate provides a direct readout of k_{cat}/K_m and the rate is directly proportional to substrate concentration. To ensure the assays were in this linear region of Michaelis-Menten kinetics (enabling direct determination of k_{cat}/K_m), assays were carried out at three substrate concentrations to verify there was a linear relationship between rate and substrate concentration. For Cel3A, Cel3B, Cel3C and Cel3D the substrate ranges used to determine k_{cat}/K_m were 0.02 to 0.08 mM, 0.1 to 0.4 mM, 0.05 to 0.2 mM and 0.25 to 2 mM, respectively. The assays used to determine k_{cat} and K_m had three technical replicates. When only k_{cat}/K_m was determined nine technical replicates were used. Values are reported ± the standard error of the mean.

[b] Not determined