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Glucose-Mediated Repression of Plant Biomass Utilization in the White-Rot Fungus *Dichomitus squalens*

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ABSTRACT The extent of carbon catabolite repression (CCR) at a global level is unknown in wood-rotting fungi, which are critical to the carbon cycle and are a source of biotechnological enzymes. CCR occurs in the presence of sufficient concentrations of easily metabolizable carbon sources (e.g., glucose) and involves downregulation of the expression of genes encoding enzymes involved in the breakdown of complex carbon sources. We investigated this phenomenon in the white-rot fungus *Dichomitus squalens* using transcriptomics and exoproteomics. In *D. squalens* cultures, approximately 7% of genes were repressed in the presence of glucose compared to Avicel or xylan alone. The glucose-repressed genes included the essential components for utilization of plant biomass—carbohydrate-active enzyme (CAZyme) and carbon catabolic genes. The majority of polysaccharide-degrading CAZyme genes were repressed and included activities toward all major carbohydrate polymers present in plant cell walls, while repression of ligninolytic genes also occurred. The transcriptome-level repression of the CAZyme genes observed on the Avicel cultures was strongly supported by exoproteomics. Protease-encoding genes were generally not glucose repressed, indicating their likely dominant role in scavenging for nitrogen rather than carbon. The extent of CCR is surprising, given that *D. squalens* rarely experiences high free sugar concentrations in its woody environment, and it indicates that biotechnological use of *D. squalens* for modification of plant biomass would benefit from derepressed or constitutively CAZyme-expressing strains.

IMPORTANCE White-rot fungi are critical to the carbon cycle because they can mineralize all wood components using enzymes that also have biotechnological potential. The occurrence of carbon catabolite repression (CCR) in white-rot fungi is poorly understood. Previously, CCR in wood-rotting fungi has only been demonstrated for a small number of genes. We demonstrated widespread glucose-mediated CCR of plant biomass utilization in the white-rot fungus *Dichomitus squalens*. This indicates that the CCR mechanism has been largely retained even though wood-rotting fungi rarely experience commonly considered CCR conditions in their woody environment. The general lack of repression of genes encoding proteases along with the reduction in secreted CAZymes during CCR suggested that the retention of CCR may be connected with the need to conserve nitrogen use during growth on nitrogen-scarce wood. The widespread repression indicates that derepressed strains could be beneficial for enzyme production.

KEYWORDS *Dichomitus*, carbon catabolite repression, CAZymes, regulation

White-rot fungi break down all wood components, are critical to the carbon cycle, and are a source of biotechnologically relevant enzymes (1). However, the extent of carbon catabolite repression (CCR) on plant biomass degradation in wood-rotting

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fungi is currently unknown. CCR occurs in the presence of sufficient concentrations of easily metabolizable carbon sources (e.g., glucose) and involves downregulation of the expression of genes encoding enzymes involved in the breakdown of complex carbon sources. CCR has been studied mainly in ascomycete fungi (2) and has been examined at a global level in various major groups of fungi, such as recently in anaerobic gut fungi (3), but not in wood-rotting fungi such as *Dichomitus squalens*.

The analysis of glucose-mediated CCR in wood-rotting fungi is limited to small sets of transcripts and enzymatic activities. In the white-rot fungus *Phanerochaete chrysosporium*, glucose-mediated repression of two cellulase-encoding genes has been reported (4), while in the brown-rot fungus *Postia placenta*, glucose-mediated repression was observed for four hemicellulase-encoding genes but not for four cellulase-encoding genes (5). In selected *Polyporales* sp. white-rot fungi, cellulase and xylanase activities were significantly reduced when the cultures were supplemented with glucose (6).

Plant biomass utilization by fungi requires the secretion of extracellular enzymes to degrade the polymeric components, transporters for the sugars released from the plant biomass, and intracellular catabolic enzymes. There is evidence for catabolite repression of each of these functional categories in various ascomycete fungi (2) but not in wood-rotting basidiomycete fungi. Genes encoding other enzymes, such as proteases, that can scavenge for the scarce nitrogen in wood by hydrolyzing proteins (7) have been shown to be carbon catabolite repressed in ascomycete fungi (8–10), but there are reports indicating that protease activities in basidiomycete leaf-litter degrading fungi lack CCR (11). A transcriptional regulator of CCR, CRE1 (also named CreA), has been extensively studied in several ascomycete fungi (12), while other proteins, such as kinases, can mediate CCR independently of CRE1/CreA (13). Recently, *cre1* was deleted in the white-rot fungus *Pleurotus ostreatus*, and exoproteomics detected increased abundance of a subset of *c*arbohydrate-*a*ctive en*zyme*s (CAZymes) under apparently nonrepressing conditions (14). Analysis of the extent of glucose-mediated CCR can demonstrate if there is a requirement to delete transcriptional or posttranscriptional regulators of CCR in wood-rotting fungi for improved enzyme yields and/or biomass degradation.

In *D. squalens*, sugar inducers of polysaccharide-degrading CAZyme-encoding genes have been identified (15), demonstrating an inducible (i.e., not constitutive) system for these enzymes, which provides it with an ability to partially tailor its molecular responses to wood composition (16). *D. squalens* has biotechnological potential both as a source of enzymes and for bioremediation and can be genetically modified (17), making it a highly suitable species to analyze the effects of CCR. The global effect of high glucose concentrations on the utilization of plant biomass components is not known in any wood-rotting fungus. Occurrence of glucose-mediated repression could be a tool to indicate whether, e.g., particular CAZyme families or expansin-like domain-containing proteins in wood-rotting fungi are involved in plant biomass degradation. In this study, we investigated the occurrence and extent of glucose-mediated repression in the white-rot basidiomycete *D. squalens* using two CAZyme-inducing polysaccharide substrates, each at two time points.

RESULTS

Identification of carbon catabolite-repressing conditions in *D. squalens*. Whether excess glucose could repress the secreted protein pattern from *D. squalens* cultures growing on ring plates containing either Avicel or xylan was examined (Fig. 1A). The secreted proteins after 2 days, visualized using SDS-PAGE, showed a clear repression pattern on each substrate, albeit a stronger one on Avicel, and were analyzed using exoproteomics (Fig. 1B). Hierarchical clustering of the exoproteins showed that the biological replicates from a condition generally clustered together (Fig. S1A). To investigate whether this repression extended beyond secreted proteins, the transcriptomes after 2 days as well as after 5 h were analyzed. Hierarchical clustering of all expressed genes showed two major time-dependent clusters (Fig. S1B). At the earlier time point, the genes clustered by polysaccharide substrate and by

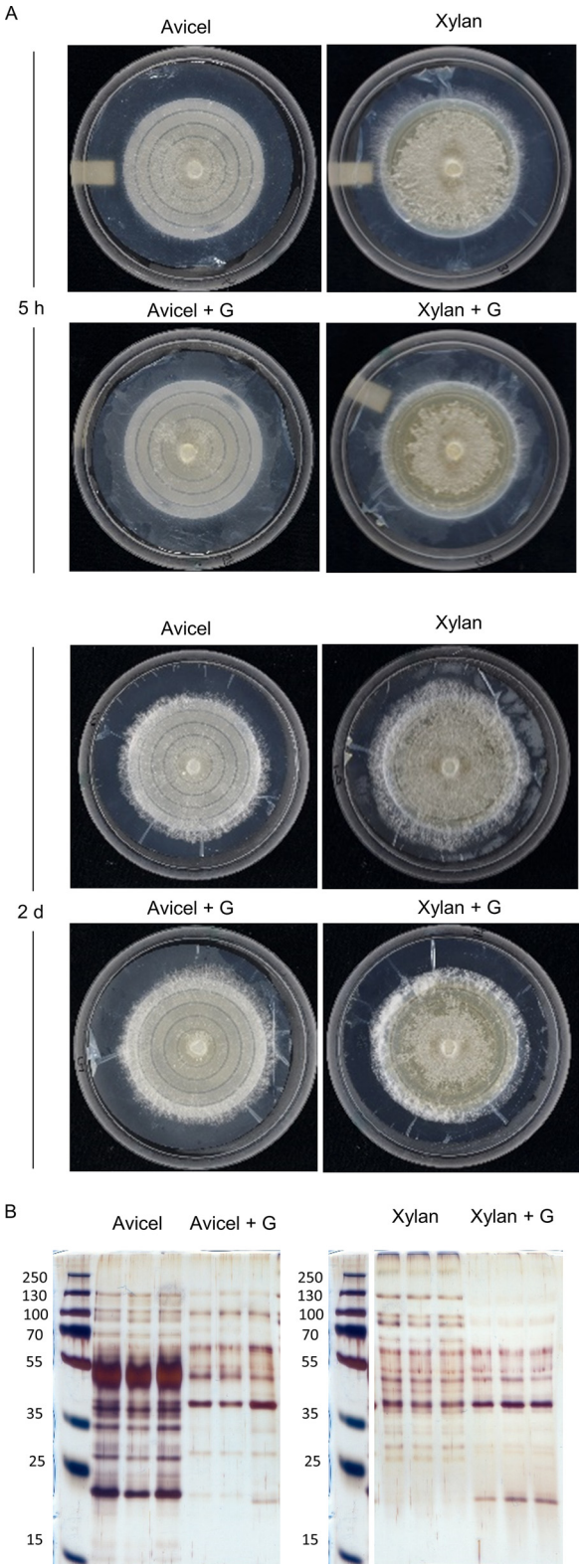

FIG 1 Carbon catabolite repression phenotypes of *D. squalens* during growth on Avicel or xylan in the absence and presence of glucose. (A) Representative images of the *D. squalens* cultures on ring-plates containing either Avicel cellulose or beech wood xylan with or without supplementation with 3% (wt/vol) D-glucose (G) from either 5 h or 2 days after transfer of the preculture, and (B) SDS-PAGE showing the banding pattern 2 days after transfer of preculture from protein samples used for proteomic analysis.

presence and absence of glucose. In contrast, at 2 days, transcripts from the Avicel-only cultures clustered separately compared to the other cultures from 2 days. Time, not the type of polysaccharide or the presence of glucose, appeared to be the most dominant factor in the clustering, suggesting that gene induction and/or repression changed substantially over time.

Extensive glucose-mediated repression was observed at a global level. Glucose-mediated repression of *D. squalens* genes was extensive, with approximately 7% of genes (1,042/15,295) repressed in one or more conditions (Table S1). A gene was considered glucose repressed if the expression was >2-fold lower in the presence of glucose, the adjusted *P* value (P_{adj}) was <0.05, and the fragments per kilobase per million (FPKM) value was >10 in the absence of glucose. A large majority of the glucose-repressed genes were only repressed in one condition (Fig. 2A). Not all genes were expressed in all these conditions, and a lack of induction on one of the polysaccharides, preventing detection of repression, could explain why approximately a quarter of the genes were found to be repressed on only one polysaccharide (Fig. 2B). The remainder of the genes that were repressed on only one polysaccharide were expressed on both, suggesting differences in how glucose-mediated repression was functioning on each polysaccharide. In the genes repressed under all four of the repression conditions, there was enrichment of Gene Ontology (GO) terms representing processes directly related to plant biomass utilization, but also to other processes (Fig. 2C and Table S2). The GO term "hydrolase activity, hydrolyzing *O*-glycosyl compounds" was enriched in glucose-repressed genes on both polysaccharides supplemented with glucose compared to the polysaccharides without glucose. In addition, "sugar transmembrane transporter activity" was enriched in the genes repressed on Avicel supplemented with glucose compared to Avicel alone. Transport of the sugars released from the polysaccharides is critical to growth on plant biomass, and enrichment in the glucose-repressed genes of GO terms for degradation as well as transport demonstrates that repression of these two processes is coordinated. On day 2, the GO terms enriched among the genes repressed on Avicel supplemented with glucose suggested repression of production from the GO terms "cellular amino acid biosynthetic process" and "tRNA aminoacylation for protein translation." Other GO terms were enriched in a subset of the conditions and displayed repression of biological functions beyond those directly related to plant biomass degradation (Table S2). These included GO terms for lipase activity repressed on xylan at 5 h, alcohol metabolic process on xylan at 2 days, and organic acid biosynthetic process on Avicel at 2 days.

Glucose repressed a broad range of lignocellulose-degrading activities. The *D. squalens* genome carries 233 putative plant biomass-degrading (PBD) CAZyme genes, and almost half (108) of these were glucose repressed in at least one condition. The majority of the repressed PBD CAZyme genes were repressed on only one of the two polysaccharides (Fig. 2D). This was due to either a lack of induction by released sugars on the other polysaccharide or differences in how the repression was being regulated, as more than half of these PBD CAZyme genes repressed on only one polysaccharide were expressed substantially on both substrates without glucose. The glucose-repressed genes on Avicel were less affected by the time point than those on xylan, on which 2.5 times more CAZyme genes were repressed at 2 days than at 5 h. In addition to genes encoding CAZymes acting on polysaccharides, half (5/10) of the manganese peroxidase (MnP)-encoding genes in *D. squalens* were repressed on xylan at 2 days. The total CAZyme gene expression was 5-fold higher on Avicel than that on xylan at 2 days, and this likely contributed to approximately 6-fold repression on Avicel compared to the less than 2-fold repression detected on xylan (Fig. S2). Hierarchical clustering also illustrated higher CAZyme gene expression on Avicel (Fig. 3 and Fig. S3). The most striking example of glucose-mediated repression was in cluster 3, which contained mainly genes encoding cellulose-acting CAZymes that were highly expressed on Avicel at 2 days and strongly repressed with average 100-fold repression by glucose (Fig. 3). On xylan, the repressed genes mainly encoded xylan-acting enzymes that were side

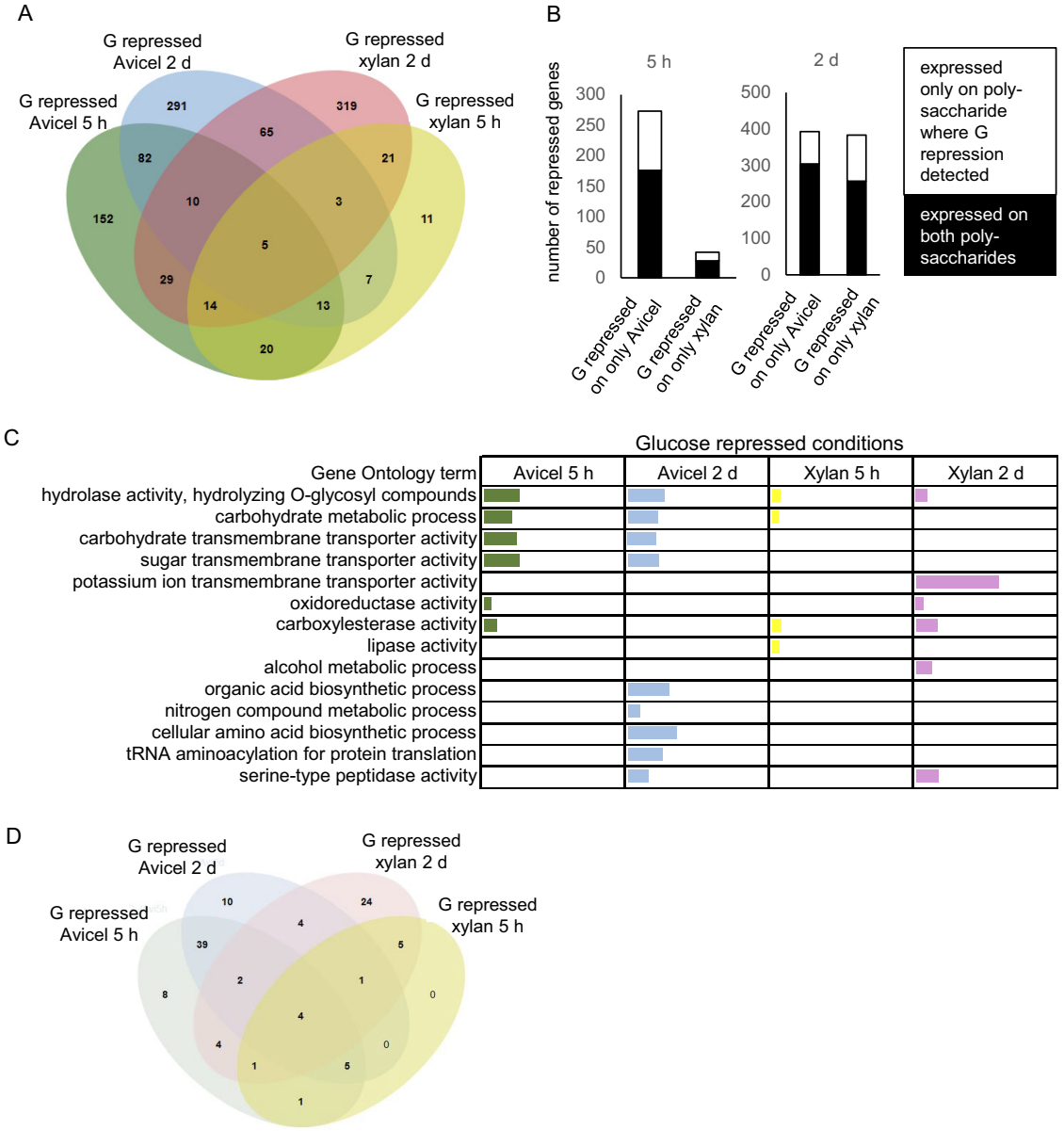

FIG 2 Widespread glucose-mediated repression affecting a broad range of biological functions in *D. squalens*. (A) Comparison of the number of transcripts repressed by glucose (G) on Avicel or xylan after 5 h or 2 days of transfer from preculture. (B) Number of genes that were glucose repressed on only one polysaccharide at a certain time point. Each bar is divided into the proportions of these genes that were substantially expressed on both polysaccharides or on only the polysaccharide where glucose repression was detected. (C) Selection of enriched Gene Ontology (GO) terms showing broad effects of glucose-mediated repression. The size of the colored bar indicates the percentage (for 100%, the bar would fill the entire cell) of the total number of genes annotated with a GO term that were present in the repressed genes. (D) Comparison of plant biomass-degrading CAZyme genes repressed by glucose on Avicel or xylan after 5 h or 2 days of transfer of preculture.

chain rather than backbone acting, although in the exoproteome, there was one endoxylanase protein with lower production in the presence of glucose at 2 days (Table S3). From the transcriptome analysis, it was clear that genes encoding a broad range of activities were repressed, including hemicellulolytic and pectinolytic activities.

Exoproteomics identified 57 PBD CAZymes in at least one of the 2-day conditions (Table S3). Strikingly, the five most abundant proteins secreted into the Avicel ring plates made up almost 80% of the total protein abundance, whereas in the ring plates on which Avicel was supplemented with glucose, these same proteins only made up approximately 10% of the total protein abundance. A protein was considered to have

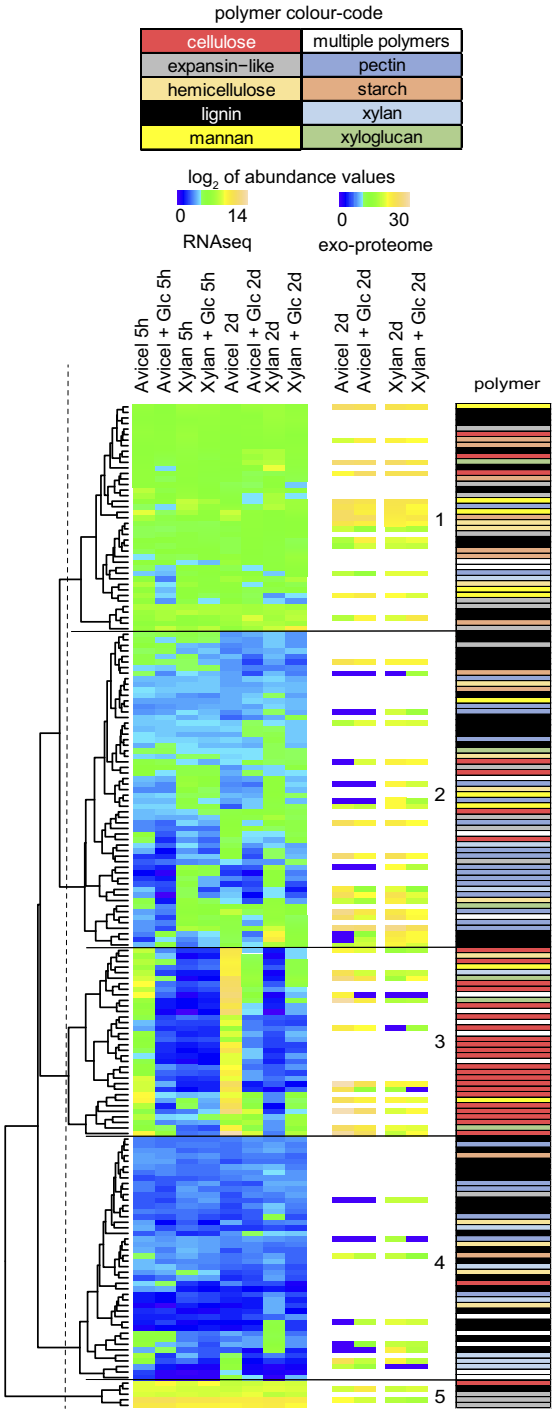

FIG 3 Repression of CAZymes affects a broad range of activities with strong support for the repressing pattern in the exoproteomics. Hierarchical clustering, using Euclidian distance, of transcript levels of plant biomass degrading CAZyme-encoding genes from *D. squalens* mycelia grown on either Avicel or xylan with or without supplementation with 3% (wt/vol) D-glucose (G). The CAZymes are color coded according to substrate groups on which they putatively act (enzymes that act on multiple polymers are colored white). The protein abundance from the exoproteome is shown adjacent to the transcript. See Fig. S3 for an image of the heat map displaying information about the gene to which each row corresponds.

significantly lower production in the presence of glucose if the abundance was >2-fold lower and the *P* value was <0.05 or, alternatively, if it was only identified in the absence of glucose. There was a better positive correlation for Avicel than xylan when the proteins that were produced at a significantly lower level in the presence of glucose

were compared to the repressed genes. On Avicel at 2 days, almost all of the CAZymes that were produced at a lower rate in the presence of glucose also had the encoding gene repressed (18/19). In contrast, less than half of the CAZymes that were produced at a lower rate in the presence of glucose on xylan at 2 days had the encoding gene repressed (11/24).

Repression of catabolism of plant-derived sugars. Sugars are catabolized via several carbon catabolic pathways, and intermediate metabolites can be inducers of genes encoding catabolic enzymes and CAZymes (18). Putative carbon catabolic enzymes in *D. squalens* were identified by homology to characterized enzymes from ascomycete fungi. Genes encoding 21 putative catabolic enzymes were glucose repressed, and half of these are involved in catabolism of hemicellulose or pectin sugars, such as pentoses, mannose, or galacturonic acid (Table S1). Only a subset of the expressed genes from catabolic pathways related to hemicellulose or pectin-derived sugars (pentose catabolic pathway, Leloir pathway, D-mannose pathway, D-galacturonic acid pathway, and L-rhamnose pathway) were glucose repressed at a time point on Avicel or xylan. Repression of the entry point to these pathways could repress the flux through the pathway, obviating the need to repress genes encoding subsequent enzymes, but the putative entry point to these pathways was not preferentially repressed. Some carbon catabolic genes were more highly expressed in the presence of glucose, but these were not shared across the repressed conditions. Glycolysis and the tricarboxylic acid (TCA) cycle catabolize glucose or its derivatives in the main energy-generating reactions of the cell. Surprisingly, few glycolytic or TCA cycle genes were more highly expressed in the presence of high glucose concentrations and none were more highly expressed at 5 h. In *Aspergillus oryzae*, many of the glycolytic and TCA cycle genes are more induced in glucose-rich than in glucose-depleted conditions (19). The general trend for glycolytic or TCA cycle genes was to be neither induced nor repressed in the presence of excess glucose but instead to remain constitutively expressed. The same trend was observed for genes from the pentose phosphate pathway (PPP), which catabolizes glucose in parallel to glycolysis. This was surprising because there could be a higher requirement for the ribose 5-phosphate generated by the PPP for use in synthesizing nucleotides in DNA replication if replication was occurring more frequently at higher growth rates, such as those in the presence of excess glucose. In contrast to the repression of *D. squalens* CAZyme genes, the magnitude of the repression was generally not greater than 2-fold (Fig. S4), and the proportion of the carbon catabolic enzyme-encoding genes that were repressed (27%) was smaller than that for CAZyme genes (46%).

Limited glucose-mediated repression of proteases. Proteases function in scavenging for nitrogen during fungal wood degradation, as the nitrogen content of wood is very low (7). The GO term for "serine-type peptidase activity" was enriched in the genes that were glucose repressed on each of the polysaccharides at 2 days (Fig. 2C). However, only a small proportion of protease-encoding genes (23/215) were repressed in at least one condition, mainly at the later time point, while the expression of three-quarters of protease-encoding genes was not significantly different (Table S1). This was reflected in the total abundance of transcripts encoding proteases, where the levels were not lower in the presence of excess glucose (Fig. 4). Exoproteomics identified 26 proteases in at least one of the 2-day conditions (Table 1). There were only three proteases produced at a lower rate in the presence of glucose in the cultures growing on each polysaccharide, whereas the majority of the proteases were not produced at a significantly lower rate in the presence of glucose. Indeed, the proteases were the other major functional category of secreted proteins, besides those degrading polysaccharides, that were secreted into the ring plates under all conditions.

Candidate transcriptional regulator of glucose repression. Direct transcriptional repression via transcription factors binding to the promoters of the glucose-repressed genes likely plays a role in *D. squalens*. The putative *D. squalens cre1* ortholog, identified as the reciprocal BLASTP match of *P. ostreatus cre1* (14), was glucose repressed on Avicel

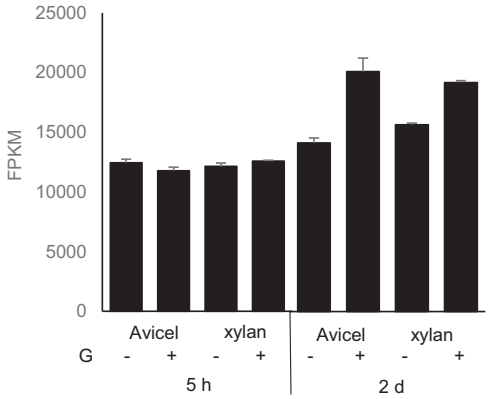

FIG 4 Total abundances of transcripts encoding proteases at 5 h and 2 days after transfer of *D. squalens* onto ring plates containing either Avicel or xylan with or without supplementation with 3% (wt/vol) D-glucose (G). Error bars represent standard errors ($n = 3$).

at 2 days but was not induced by the high glucose concentrations in the studied cultures. An alignment that included characterized CRE1/CreA proteins and putative CRE1 orthologs from three basidiomycetes, including *D. squalens*, showed conservation of the zinc binuclear cluster (Fig. S5). Surprisingly, there was little conservation between the ascomycete and basidiomycete proteins for the remainder of the sequence, suggesting that how the transcription factor functions could differ substantially between the phyla. However, there was substantial conservation within each phylum for the proteins for the remainder of the sequence suggesting conservation of function within the phyla. The conservation of the zinc binuclear cluster supported analysis of the presence of the binding motif of CRE1/CreA that has been identified in ascomycetes

TABLE 1 Summary of protease production status after exposure to Avicel or xylan with or without glucose supplementation

		Protease production status for[b]:	
Dicsqu464_1 protein ID[c]	Protease functional group[a]	Avicel vs Avicel + G	Xylan vs xylan + G
153959	Aspartyl protease	No sig. diff.	No sig. diff.
542799	Aspartyl protease	Higher in Avicel	Higher in xylan + G
812714	Aspartyl protease	Only in Avicel + G	Higher in xylan
813173	Aspartyl protease	No sig. diff.	No sig. diff.
941015	Aspartyl protease	Only in Avicel + G	No sig. diff.
976858	Aspartyl protease	Not present	No sig. diff.
1001160	Aspartyl protease	Higher in Avicel	No sig. diff.
367116	Metalloprotease	Higher in Avicel + G	Higher in xylan
830406	Metalloprotease	No sig. diff.	No sig. diff.
907349	Metalloprotease	Higher in Avicel + G	No sig. diff.
910760	Metalloprotease	No sig. diff.	No sig. diff.
636882	Serine protease	No sig. diff.	No sig. diff.
806666	Serine protease	No sig. diff.	No sig. diff.
917108	Serine protease	No sig. diff.	No sig. diff.
917629	Serine protease	No sig. diff.	Not present
921007	Serine protease	Higher in Avicel + G	No sig. diff.
926265	Serine protease	No sig. diff.	No sig. diff.
928513	Serine protease	Only in Avicel + G	No sig. diff.
929001	Serine protease	Higher in Avicel + G	Higher in xylan + G
977823	Serine protease	Higher in Avicel	Higher in xylan
1004022	Serine protease	No sig. diff.	No sig. diff.
917216	Other	No sig. diff.	No sig. diff.
944074	Other	No sig. diff.	No sig. diff.
953225	Other	No sig. diff.	No sig. diff.
969286	Other	No sig. diff.	No sig. diff.

[a]From Merops annotations; identified in the exoproteomics analysis.
[b]Differential production at 2 days after transfer of *D. squalens* onto ring plates containing either Avicel or xylan with or without supplementation with 3% (wt/vol) D-glucose (G). sig. diff., significant difference.
[c]ID, identifier.

TABLE 2 Summary of enrichment of CRE1/CreA binding motifs in the promoters of *D. squalens* genes that were repressed in the presence of glucose on either Avicel or xylan

Type(s) of motif	Motif sequence(s)	Mean no. of motifs per gene (all genes)	Enrichment of motifs in repressed genes (%)			
			Avicel, 5 h (325)[a]	Avicel, 2 days (476)[a]	Xylan, 5 h (94)[a]	Xylan, 2 days (466)[a]
Single direct	[GC][CT]GG[AG]G	1.48	+12.2	+14.3	+0.4	+16.1
Single reverse	C[CT]CC[AG][GC]	1.78	+32.4	+26.2	+18.7	+38.4
All singles	Both above-listed single motifs	3.21	+21.4	+20.2	+8.7	+28.3
Pair 1	[GC][CT]GG[AG]G[AGCT](1,100)[GC][CT]GG[AG]G	0.26	+19	+18.8	−9.5	+19.5
Pair 2	C[CT]CC[AG][GC][AGCT](1,100)C[CT]CC[AG][GC]	0.34	+43.9	+42	+8.8	+49.1
Pair 3	[GC][CT]GG[AG]G[AGCT](1,100)C[CT]CC[AG][GC]	0.25	+48.1	+24.9	+59	+44.1
Pair 4	C[CT]CC[AG][GC][AGCT](1,100)[GC][CT]GG[AG]G	0.21	+38.2	+29.8	−0.1	+47.2
All pairs	All above-listed paired motifs	0.8	+29.5	+26.6	+13.9	+37.2

[a]The total number of genes that were repressed in that condition is given in parentheses.

(20, 21) in the promoters of the *D. squalens* glucose-repressed genes. There was a trend for enrichment (~30%) of motifs in glucose-repressed *D. squalens* genes, as measured by the mean number of motifs per gene promoter (Table 2 and Table S4).

Repression of candidate proteins with potential roles in biomass degradation. A gene being subject to glucose-mediated carbon catabolite repression when cultured with Avicel or xylan makes the encoded protein a stronger candidate for involvement in plant biomass degradation. The previous section showed how genes encoding proteins with well-established involvement in plant biomass degradation, such as cellulases, were strongly glucose repressed, thus allowing inferences to be made about other repressed genes.

β-Glucuronidases from CAZyme family GH79 cleave β-linked glucuronic acid residues in arabinogalactan proteins (22), but these proteoglycans are a minor component of most lignocellulosic residues, and thus a role for β-glucuronidases in degradation of lignocellulose-rich residues is controversial. Recently, it was shown that β-glucuronidases can have a role in cellulose degradation, as polysaccharide monooxygenases catalyze oxidation of cellulose to glucuronic acid-containing oligosaccharides (23). Three *D. squalens* GH79 genes, two of which clustered closely with characterized β-glucuronidases from *Aspergillus niger* and *Neurospora crassa* (22) in phylogenetic analysis (Fig. S6), were repressed by glucose, further supporting a possible role for these proteins in lignocellulose degradation. The proteins from these three GH79 genes were detected with two of the three proteins produced at a lower rate in the presence of glucose (Table S3).

Previously, genes encoding expansin-like proteins were expressed when *D. squalens* was cultured with plant biomass substrates, suggesting a role in the degradation (24). Expansin-like proteins such as the *Trichoderma reesei* SWO1 can disrupt the cellulose structure (25) and can boost the sugar release in saccharification of a nonpretreated grass substrate (26). In *D. squalens*, there was limited repression of genes encoding expansin-like proteins, and their total expression was similar in absence or presence of glucose (Table S1). The only expansin-like protein detected in the exoproteins was not produced at a lower rate in the presence of glucose (Table S3); however, that does not exclude the possibility of a role for expansin-like proteins in plant biomass degradation, but just indicates a different regulatory control of the genes encoding them.

Apart from plant biomass degradation, the gene encoding a *D. squalens* protein, FIP-dsq2 (Dicsqu464_1_PID_921554), which was reported to have immunomodulatory and anticancer properties (27), was glucose repressed on Avicel at 5 h and 2 days, as well as on xylan at 2 days.

DISCUSSION

We have demonstrated using transcriptomics and exoproteomics that there is widespread glucose-mediated repression in a wood-rotting basidiomycete fungus. Although high concentrations of free sugars are naturally rare for a wood-rotting fungus, they were insightful in suggesting that the existence of CCR in *D. squalens* is partly to conserve use of the scarce nitrogen in its woody biotope. There was widespread glucose-mediated repres-

sion of CAZyme genes but not genes encoding nitrogen-scavenging proteases. Also, the pattern of repression provided support for direct and indirect mechanisms for how *D. squalens* reduced the expression of its CAZyme genes.

The strong repression by glucose supplementation of secreted CAZyme genes, but not of genes encoding proteases, suggests that CCR has been maintained by *D. squalens* to strongly conserve nitrogen use in its nitrogen-scarce woody biotope. It is possible that the sensing of increasing sugar concentrations signals successful breakdown of plant biomass and less of a need for high CAZyme secretion levels, which could be a drain on the limited nitrogen supply. Instead, the scarce nitrogen could be diverted to support fungal growth and achieve a lower mycelial C:N balance than would be possible if a higher protein secretion level was maintained. It did appear that there was more growth in the presence of excess glucose, as the mycelium was substantially thicker than that when cultured on either polysaccharide in the absence of excess glucose. Previously, in three leaf litter-degrading basidiomycetes, the total protease activity was not lowered by glucose supplementation (11). The lack of repression in *D. squalens* is partly in contrast to the CCR of proteases that can be found in ascomycete fungi (10, 28, 29), possibly due to differences in the biotope or lifestyle of the fungi. Regulation of proteases is a complex phenomenon and, in particular, the low nitrogen concentration in the culture medium, which is intended to replicate environmental conditions, may be a competing regulatory factor with any repressing effect of the glucose due to a physiological requirement of *D. squalens* to scavenge for nitrogen from proteins.

There were interesting temporal patterns to the induction and repression on both Avicel and xylan. On Avicel, there were higher CAZyme-encoding transcript levels at 2 days than at 5 h and a 3-times larger average fold change of repression at the later time point. The higher transcript levels at 2 days could be explained by the induction taking longer than 5 h to peak, but the possibility cannot be excluded that the low concentrations of glucose released from Avicel at 5 h were sufficient to repress. On xylan at 2 days, the correlation between the CAZymes produced at a lower rate in the presence of glucose and repressed genes was ~50%, whereas on Avicel the correlation was ~95%. The lack of repression of the genes encoding half of the CAZymes produced at a lower rate on xylan in the presence of glucose suggested that the repression of the genes occurred earlier than 2 days and had now ceased. Secondary (indirect) effects could also be contributing to the repression of these genes.

Whether the repression effect is primary (direct) or secondary (indirect) is difficult to ascertain. The presence of a *D. squalens* CRE1 ortholog and an enrichment of the CRE1/CreA binding motif in the promoters of the repressed genes suggest that there was a direct repression effect at the transcript level. In addition, at least two indirect repression effects were also suggested by the data sets. The enriched GO terms for "tRNA aminoacylation for protein translation" and "cellular amino acid biosynthetic process" in the Avicel cultures supplemented with glucose suggest an indirect effect leading to fewer exoproteins due to less protein synthesis and translation in the presence of glucose. The genes encoding β-glucosidases were glucose repressed mainly at 5 h on Avicel, and the three β-glucosidase proteins detected were not found to be produced at a lower rate in the presence of glucose. These nonrepressed β-glucosidases may indirectly contribute to an apparent repression effect by reducing the induction by means of hydrolysis of cellobiose, which was previously found to be a major inducer of *D. squalens* CAZymes (15).

Analysis of glucose repression in orthologs of *D. squalens* glucose-repressed genes of the white-rot fungus *P. chrysosporium* (4) and the brown-rot fungus *P. placenta* (5) revealed similar and contrasting repression trends. The *D. squalens* orthologs of the cellobiohydrolyase-encoding *Pc_cel6A* and cellobiose dehydrogenase-encoding *Pc_cdh* were also glucose repressed on Avicel at both time points, while the β-glucosidase-encoding *Pc_bgl3A* was not repressed in *P. chrysosporium*, but the *D. squalens* ortholog was repressed on Avicel at both time points (see Table S1 for the protein identifiers of the *D. squalens* orthologs). The 30-fold lower concentration of glucose used by Suzuki

et al. (4) may be a factor in the lack of repression compared to that in *D. squalens*, as well as the lack of an inducing carbon source in their study. In *P. placenta*, four cellulase genes analyzed by quantitative PCR were not found to be repressed by glucose compared to a control without a carbon source (5), while in our study, *D. squalens* orthologs for three of these genes (*Pp_bgl1*, encoding β-glucosidase, and *Pp_cel5B* and *Pp_cel12A*, encoding two respective endoglucanases) were repressed by glucose on Avicel at both time points. Four *P. placenta* hemicellulase-encoding genes were repressed by glucose (5), and the *D. squalens* orthologs of these were also repressed (*Pp_bxl1*, encoding β-xylosidase; *Pp_man5A*, encoding β-mannanase; and *Pp_xyn10A-1* and *Pp_xyn10A-2*, encoding endoxylanases). Both of these studies (4, 5) consider glucose repression compared to a control without a carbon source, whereas in our study, the control is an inducing polysaccharide.

It is somewhat surprising that high glucose concentrations had a widespread physiological effect in a wood-rotting fungus that would rarely experience such high concentrations in its woody environment, as recalcitrant lignocellulose degradation is a slow process occurring over several years (30). Glucose could potentially be sensed from catabolic intermediates that are also intermediates from catabolism of other sugars. *D. squalens* is predominantly found on softwoods (31), in which galactoglucomannan, containing a backbone of glucose and mannose, is the main hemicellulose. In ascomycetes, mannose can be catabolized in two steps to D-fructose-6-phosphate, which is the same intermediate to which glucose is catabolized (18). Thus, D-fructose-6-phosphate accumulation could more readily be the signal for excess mannose release than other plant biomass-derived sugars, such as xylose, that are catabolized by more complex pathways. There is evidence for cross talk between cellulose- and mannan-sensing pathways in *N. crassa* (32), although the levels of other sugars besides glucose, such as mannose, are also likely to be released slowly from the wood.

Testing lower glucose concentrations and investigating the repressing effect of other sugars were beyond the scope of this study. Also, investigating the repressing effect of high nitrogen concentrations on protease expression and production would be of interest, as our study used media with a low nitrogen concentration. The experimental conditions developed here facilitate screening based on protein secretion pattern or candidate CAZymes before more detailed transcriptome analysis. The conditions also facilitate analysis of deletions of candidate regulatory proteins, such as the *D. squalens cre1* ortholog. Moreover, other *D. squalens* strains could be tested, as differences in CAZyme gene induction between *D. squalens* monokaryotic and dikaryotic strains were reported previously (15).

In conclusion, for applications, derepressed strains would be of benefit for a higher yield of CAZymes from *D. squalens*, particularly for the strongly repressed genes encoding cellobiohydrolases. Wood-rotting fungi seem to maintain the CCR physiological response found in other major fungal lineages despite the rarity of high free sugar concentrations in their natural biotope. The scarcity of nitrogen in wood may be a contributing factor to the maintenance of CCR.

MATERIALS AND METHODS

Culturing conditions and biomolecule harvesting and extraction. *D. squalens* monokaryon CBS464.89 was maintained on malt extract agar (MEA) at 4°C and at −80°C in 30% glycerol. For precultures, 0.5-cm plugs from a culture of *D. squalens* growing on MEA were inoculated onto a 7.6-cm-diameter perforated (0.1 μM) polycarbonate membrane (Maine Manufacturing) on top of low–nitrogen asparagine–succinate (LN-AS) agar (1.5% [wt/vol]) adjusted to pH 4.5 (33) and containing either 1% (wt/vol) Avicel cellulose (Fluka) or beech wood xylan (Sigma) and incubated at 28°C for 3.5 days, after which the colony diameter was ~4.5 cm. The polycarbonate membrane containing the colony was then transferred to 9-cm ring plates (34) containing LN-AS liquid medium containing the same polysaccharide (1% [wt/vol]) as the preculture plate or that polysaccharide supplemented with 3% (wt/vol) D-glucose. The rings in each ring plate were numbered from 1 to 5 from the center to the outermost ring (see reference 34 for an image with numbering of the rings). The well of each ring had a depth and width of 0.5 cm and was separated from adjacent rings by a barrier of 0.1 cm. After 5 h or 2 days from the transfer, the mycelia growing above rings 2 to 5 were sampled for RNA extraction. Also, 2 days after transfer, the liquid in rings 2 to 5 was pooled (for SDS-PAGE and exoproteomics analysis), flash frozen in liquid nitrogen and stored at −20°C. LN-AS medium or agar containing Avicel or beech wood xylan was

autoclaved at 121°C for 15 min, and the filter-sterilized vitamin solution was added after autoclaving. A 20% (wt/vol) glucose stock solution was sterilized using a membrane filter.

Total RNA was extracted from mycelium, purified, and checked for quality as described previously (35). The mycelium was ground and mixed with TRIzol reagent (Invitrogen) after harvesting and stored at −80°C before proceeding with the remaining steps of RNA extraction.

Transcriptomic sequencing and analysis. Plate-based RNA sample preparation was performed on the PerkinElmer Sciclone next-generation sequencing (NGS) robotic liquid handling system using the Illumina TruSeq stranded mRNA high-throughput (HT) sample prep kit utilizing poly(A) selection of mRNA following the protocol outlined by Illumina (https://support.illumina.com/sequencing/sequencing_kits/truseq-stranded-mrna.html) and with the following conditions: total RNA starting material was 1 μg per sample and 8 cycles of PCR were used for library amplification. The prepared libraries were quantified using a Kapa Biosystems next-generation sequencing library quantitative PCR (qPCR) kit and run on a Roche LightCycler 480 real-time PCR instrument. The quantified libraries were then multiplexed with other libraries, and the pool of libraries was then prepared for sequencing on the Illumina NovaSeq sequencer using NovaSeq XP V1 reagent kits and anS4 flow cell, and following a 2 × 150-bp indexed run recipe.

Using BBDuk (the source code for BBDuk is available at the repository for the BBMap suite of tools, https://sourceforge.net/projects/bbmap), raw reads were evaluated for artifact sequence by kmer matching (kmer = 25), allowing one mismatch, and detected artifacts were trimmed from the 3′ end of the reads. RNA spike-in reads, PhiX reads, and reads containing any ambiguous bases (Ns) were removed. Quality trimming was performed using the Phred trimming method set at Q6. Finally, following trimming, reads under the length threshold were removed (minimum length of 25 bases or one third of the original read length, whichever was longer). Filtered reads from each library were aligned to the Dicsqu464_1 genome assembly (36) using HISAT2 version 2.1.0 (37). featureCounts (38) was used to generate the raw gene counts using gff3 annotations. Only primary hits assigned to the reverse strand were included in the raw gene counts (-s 2 -p -primary options).

Statistical analysis was performed using DESeq2 (39). Hierarchical clustering on the expressed PBD CAZyme genes using the $\log_2$ FPKM values (+1) was performed in the R statistical environment using the gplots package with the Euclidian distance and complete linkage options selected. Transcripts were considered differentially expressed if the DESeq2 fold change was >2 or <0.5 and P_{adj} was < 0.05, as well as the FPKM being >10, in at least one of the two conditions being compared. Transcripts with an FPKM of ≤10 were considered to have low (i.e., not substantial) expression. The plant biomass-degrading CAZyme annotations (40) were the same as those used previously (16). For protease annotations, the Merops (41) annotations from the Dicsqu464_1 JGI portal were used. The annotations for carbon catabolic enzymes in *D. squalens* were obtained by reciprocal BLASTP analysis using characterized carbon catabolic enzymes from *Aspergillus* species. Gene Ontology (GO) enrichment analysis was performed using BiNGO (42) using the default setting, with "GO_full" selected for the ontology.

SDS-PAGE and protein liquid chromatography-tandem mass spectrometry sample preparation and analysis. The liquid from rings 2 to 5 from each ring plate from the 2-day cultures was pooled before centrifugation at 3,200 × *g* for 1 h at 4°C to pellet any solid particles from the polysaccharide substrates. From Avicel-containing cultures, 4 ml was concentrated ~20-fold using Vivaspin 500 columns (5,000-kDa molecular weight cutoff; GE Life Sciences) by centrifugation at 15,000 × *g* and 4°C for 3 h. All of the liquid from the concentrated supernatants was then precipitated. For the xylan-containing cultures, 500 μl of the nonconcentrated supernatants was precipitated.

Proteins were precipitated on ice for 1 h using twice the sample volume of a solution of 20% trichloroacetic acid, 20 mM dithiothreitol (DTT), and 80% acetone, then centrifuged at 3,200 × *g* for 30 min at 4°C. The pellet was then mixed with a solution of 20 mM DTT and 80% acetone and incubated overnight at −20°C, then centrifuged at 3,200 × *g* for 30 min at 4°C. The air-dried pellet was resuspended in 150 μl 0.25% (wt/vol) anionic acid labile surfactant (AALS I; Protea Biosciences) solution (prepared in 200 mM ammonium bicarbonate, pH 7.8).

From the protein samples resuspended in the AALS solution, 7.5 μl was analyzed by SDS-PAGE. A 4× loading buffer (0.1 M Tris-HCl [pH 6.8], 42% glycerol, 4% [wt/vol] SDS, 0.02% [wt/vol] bromophenol blue, and 0.6 M β-mercaptoethanol) was used where samples were boiled for 2 min to denature the proteins, cooled on ice for 2 min, and centrifuged at ~10,000 × *g* for 2 min to remove insoluble material. The proteins were separated using a 12% (wt/vol) acrylamide SDS-containing running gel along with a PageRuler Plus prestained protein ladder with a 10- to 250-kDa size range (Thermo Fisher). The gels were silver-stained based on standard methods.

The objective of the proteomic analysis was to compare equivalent volumes of the culture supernatant. For the six samples from xylan-containing cultures, the same volume of each sample was digested with trypsin, followed by analysis of the same volume of cleaned-up digest by liquid chromatography-tandem mass spectrometry (LC-MS/MS). For the samples from Avicel-containing cultures, a three times larger volume was digested from the Avicel cultures supplemented with glucose, followed by analysis of the same volume of cleaned-up digests by LC-MS/MS. Protein samples were digested with trypsin for proteomic analysis as previously described (43). Dried peptide digest samples were solubilized in a solution of 5% acetonitrile, 0.1% formic acid, and 4 fmol/μl of trypsin-digested bovine serum albumin (BSA) (Michrom Bioresources) used as the internal standard. Aliquots (5 μl) from all 12 cleaned-up digest samples were analyzed by LC-MS/MS using an Easy-LC II nano-high-performance liquid chromatography (HPLC) system connected in-line with a Velos linear trap quadrupole (LTQ) Orbitrap mass spectrometer (Thermo Fisher). LC-MS/MS data peptide and protein identification was done using the *D. squalens* protein sequence databases obtained from the Joint Genome Institute Dicsqu464_1 generated gene models (36). Protein identification and quantification were performed

using the Proteome Discoverer 2.2 (Thermo Fisher) precursor ion quantitation workflow. Normalized individual protein area values were expressed as a fold value of the protein area value determined for the BSA internal standard.

The values for the samples from Avicel cultures supplemented with glucose, where a 3× larger volume was used for the trypsin digests, were divided by three. This correction for dilution was suitable, as the amounts of proteins analyzed were within the linear range of detection as validated by comparing by LC-MS/MS 3-fold dilutions of two of the samples. A minimum of two peptides matched to a protein with at least one peptide of unique sequence was considered sufficient for identification in the data set. For a protein to be considered present in a condition, the requirements were an abundance measurement in at least two of the replicates for that condition. For an identified protein to be considered to be produced at a significantly higher or lower rate, the requirements were a >2-fold difference in mean abundance values and a *P* value of <0.05 from a two-tailed heteroscedastic (assuming unequal variances) *t* test of the $\log_2$-transformed abundance values. Where it was not possible to calculate a *P* value, a protein was considered to be only present in one of the two conditions being compared if there were abundance measurements in ≥2 of the replicates for the condition where the protein was present and an abundance measurement in ≤1 replicate for the other condition. The remaining proteins in the data set were categorized as not significantly different or not present in that comparison.

Identification and alignment of CRE1/CreA protein sequences. The *P. ostreatus* CRE1 (14) sequence was used to identify putative CRE1/CreA orthologs by reciprocal BLASTp analysis with the MycoCosm web portal in *D. squalens* (Dicsqu464_1), *P. chrysosporium* (Phchr1), and *P. placenta* (PosplRSB12_1). The protein sequences of the putative CRE1/CreA orthologs and the characterized CRE1/CreA from *P. ostreatus* (14), *Fusarium oxysporum* (44), *Trichoderma reesei* (45), *N. crassa* (46), and *Aspergillus nidulans* (47) were aligned using Clustal Omega (48) with default parameters and visualized with Jalview (49). The conserved domain database (50) was used to identify the zinc binuclear cluster domains that were annotated on the alignment (Fig. S5).

Promoter analysis for CRE1/CreA binding motifs. Up to 1,000 bp of promoter sequence upstream of the coding region of the genes was obtained from the Dicsqu464_1 genome annotation (36). The reported CreA binding motif 5′-SYGGRG-3′ and its sub-motifs 5′-[GC][CT]GGGG-3′ and 5′-[GC][CT]GGAG-3′ were searched against both strands of promoter sequences as in a previous study (45) using an in-house Perl script.

Data availability. The reads from each of the transcriptome sequencing (RNA-seq) samples were deposited in the Sequence Read Archive at NCBI under the individual sample accession numbers SRP215076, SRP215080, SRP215081, SRP215085, SRP215089, SRP215091, SRP215092, SRP215095, SRP215099, SRP215112, SRP215118, SRP215119, SRP215122, SRP215123, SRP215127, SRP215138, SRP215142, SRP215150 to SRP215155, and SRP215157. The mass spectrometry data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE (51) partner repository under the data set identifiers PXD014774 and 10.6019/PXD014774.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01828-19.

SUPPLEMENTAL FILE 1, PDF file, 3.2 MB.
SUPPLEMENTAL FILE 2, XLSX file, 5.5 MB.
SUPPLEMENTAL FILE 3, XLSX file, 0.04 MB.
SUPPLEMENTAL FILE 4, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 5, XLSX file, 0.5 MB.

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We declare no competing interests.

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