

Brief report

Improvement of homologous GH10 xylanase production by deletion of genes with predicted function in the *Aspergillus nidulans* secretion pathway

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Summary

Filamentous fungi are important cell factories for large-scale enzyme production. However, production levels are often low, and this limitation has stimulated research focusing on the manipulation of genes with predicted function in the protein secretory pathway. This pathway is the major route for the delivery of proteins to the cell exterior, and a positive relationship between the production of recombinant enzymes and the unfolded protein response (UPR) pathway has been observed. In this study, *Aspergillus nidulans* was exposed to UPR-inducing chemicals and differentially expressed genes were identified by RNA-seq. Twelve target genes were deleted in *A. nidulans* recombinant strains producing

homologous and heterologous GH10 xylanases. The knockout of *pbnA* (glycosyltransferase), *ydjA* (Hsp40 co-chaperone), *trxA* (thioredoxin) and *cypA* (cytrophilin) improved the production of the homologous xylanase by 78, 171, 105 and 125% respectively. Interestingly, these deletions decreased the overall protein secretion, suggesting that the production of the homologous xylanase was specifically altered. However, the production of the heterologous xylanase and the secretion of total proteins were not altered by deleting the same genes. Considering the results, this approach demonstrated the possibility of rationally increase the production of a homologous enzyme, indicating that *trxA*, *cypA*, *ydjA* and *pbnA* are involved in protein production by *A. nidulans*.

Introduction

Microbial enzymes find a myriad of applications in many fields such as chemical, fermentation, agricultural, textile, pharmaceuticals and food production (Singh *et al.*, 2019). Choosing a suitable expression system is critical for high-yield enzyme production, and many bacteria, filamentous fungi and yeasts have been commonly used to express recombinant enzymes (Demain and Vaishnav, 2009). In the bioenergy field, techno-economic analysis demonstrated that enzyme costs are much higher than generally considered. Therefore, the optimization of cell factories for large-scale processes can contribute to reducing the costs of enzyme production (Klein-Marcuschamer *et al.*, 2012).

Aspergillus is one of the most studied genera of filamentous fungi due to the medical, food spoilage and industrial relevance of some species (Gabielli *et al.*, 2014; Frisvad *et al.*, 2018; Taniwaki *et al.*, 2018). Several *Aspergilli* have a long history as producers of plant polysaccharide modifying and degrading enzymes, which allows broad utilization of carbon sources from different substrates. *Aspergillus nidulans* is a genetic model that has been extensively studied regarding metabolism,

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cellular development and regulation, contributing to the understanding of eukaryotic cell biology and molecular processes (Brandl and Andersen, 2017). The main advantage of filamentous fungi, in relation to other microbial cell factories, is the potential to produce high quantities of extracellular enzymes (Brandl and Andersen, 2017; de Vries *et al.*, 2017). Differently from prokaryotes, which have simpler pathways of protein secretion and is frequently essential for the organism fitness and pathogenicity (Maffei *et al.*, 2017), the secretion of proteins in eukaryotes, such as filamentous fungi, involves a more complex machinery in which N-glycosylation can influence considerably (Dai *et al.*, 2013).

Random mutagenesis generally used for strain breeding may introduce many mutations into genomic DNA, resulting in higher productivity, though mutations are mostly unknown. On the other hand, rational genetic manipulation of target genes is time-consuming (Nevalainen and Peterson, 2014). Strain engineering by deletion of extracellular protease genes (Zhang *et al.*, 2014), deletion of autophagic genes (Yoon *et al.*, 2013), overexpression or deletion of genes associated with the secretory pathway (Schalén *et al.*, 2016) and manipulation of unfolded protein response (UPR) pathway (Yu *et al.*, 2017) have been commonly explored as a rational approach to increase protein production in fungal systems. Furthermore, improvement of protein secretion has also been obtained by introducing multiple copies of heterologous genes (Liu *et al.*, 2014), by using native or artificial strong regulators (Zhang *et al.*, 2017), gene fusion to a well-secreted homologous protein, using native signal sequences and others (Schalén *et al.*, 2016).

An important drawback when using fungi as cell factory is that the expressed heterologous protein can be lost or stuck in the secretory pathway due to issues in processing, post-translational modifications (PTMs) or misfolding, thus inducing the endoplasmic reticulum (ER) stress. ER stress activates UPR to alleviate stress and restore homeostasis, promoting cell survival and adaptation (Heimel, 2015; Sun and Su, 2019). Under low levels of unfolded proteins, Ire1 is bound to BiP that is an ER-resident Hsp70 chaperone. However, in cases of high levels of unfolded proteins, BiP is dissociated from Ire1, resulting in the Ire1 oligomerization and trans-autophosphorylation. Consequently, Ire1 conformation is altered, which enables the ability to bind in cis-acting elements present in the promoters of UPR-target genes, known as UPRs. Ire1 homodimer activates the RNase domain that has *hac1u* (uninduced) as its substrate, removing an unconventional intron resulting in the *hac1i* (induced) mRNA. This results in the translation of Hac1p, the bZIP transcription factor responsible for the restoration of ER homeostasis (Krishnan and Askew, 2014). Sims *et al.*

(2005) showed that approximately 23% of genes upregulated in *A. nidulans* recombinant strain producing chymosin were also upregulated when UPR was chemically induced by dithiothreitol (DTT). In addition, transcriptional analysis of mutant yeast strains with improved secretion of a heterologous α -amylase showed a series of altered cellular processes, and the balancing of amino acid biosynthesis seemed to be particularly important (Huang *et al.*, 2017). Approximately 600 strains of *Neurospora crassa* carrying single deletion in genes with predicted functions in the secretory pathway were investigated for alterations in secretion, and seven strains hyperproducing cellulolytic enzymes were identified. Mutants implicated the loss of the Sterol Regulatory Element Binding Protein (SREBP) pathway (Reilly *et al.*, 2015; Qin *et al.*, 2017).

Here, a data bank of *A. nidulans* differentially expressed (DE) genes under chemically induced ER stress was generated by RNA-seq using DTT and tunicamycin (Tm). Twelve target DE genes with predicted function in the secretory pathway were selected for single-gene deletions in *A. nidulans* recombinant strains producing homologous and heterologous xylanases. After the single deletion of four genes with predicted function in different metabolic processes, the production of the homologous xylanase was increased, whereas the production of the heterologous xylanase remained unaltered. In addition, we investigated the presence of UPRs in the promoters of the deleted genes since those elements were described in *Aspergillus niger* (Muller *et al.*, 2006), but not in *A. nidulans*.

Results and discussion

Initially, two *A. nidulans* strains producing recombinant endo-xylanases (EC 3.2.1.8) were constructed to evaluate the expression and production of recombinant enzymes classified in the same glycoside hydrolase (GH) family but from different microbial sources. The gene encoding for the heterologous xylanase (*tpet_0854*) was isolated from the hyperthermophilic bacterium *Thermotoga petrophila*, while AN7401/*xlnE* was selected as a homologous model. These xylanases share 53% of amino acid sequence similarity and belong to the family GH10 (Fig. 1A), and both enzymes have two predicted N-glycosylation sites (Fig. S1).

Both genes were cloned into the pEXPYR expression vector and transformed into *A. nidulans* (Segato *et al.*, 2012). *xlnE* was more expressed (Fig. 1B), and XlnE was more secreted than Tpet_0854 (Fig. 1C and Fig. S2), despite the higher copy numbers of *tpet_0854* (5 copies) compared with the single copy of *xlnE* in the genomes of the recombinant strains (Fig. 1D) (Appendix S1). Although the native gene was present in the genome and

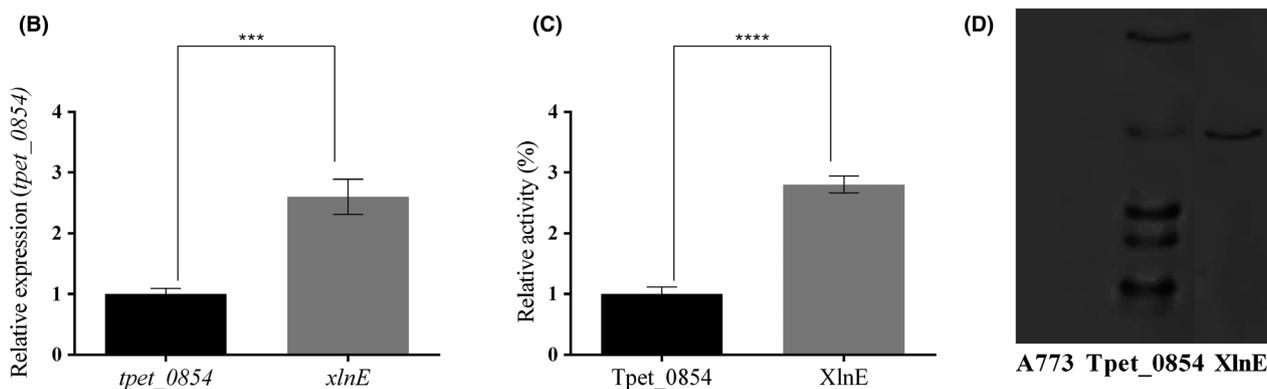


Fig. 1. Overview of homologous and heterologous xylanase sequence, expression and production in *A. nidulans*. The *A. nidulans* Tpet_0854 and XlnE strains were grown in liquid MM supplemented with 2% maltose for 36 h at 37°C.

A. *In silico* protein alignment of xylanase sequences. GH10 domains are highlighted in black.

B. Gene expression calculated by qPCR using the Relative Standard Curve Method. The *tubC* (tubulin) and *tpet_0854* genes were used as reference and calibrators respectively. (***) *P*-values between 0.001 and 0.0001.

C. Xylanase activity in the crude extracellular filtrate produced by the *A. nidulans* Tpet_0854 and XlnE strains. (***) *P*-values between 0.001 and 0.0001.

D. Southern analysis. Probes were obtained by PCR amplification of the xylanase gene from *Thermotoga petrophila* and the region between AN7401 and the *pyrG^{Af}* gene for the *A. nidulans* Tpet_0854 and XlnE strain, respectively. A773: wild-type.

the reinsertion of the *xlnE* in random locus was carried out, the transformed *xlnE* was not inserted into the native site (Fig. S3). Moreover, no native xylanase production was detected in all the enzyme production experiments carried out in minimal media supplemented with maltose. Only the recombinant XlnE was produced since it is under the control of the glucoamylase promoter.

To generate a database of genes induced by chemical stress, the expression of canonical UPR markers, such as *bipA* and *hacA* (*hacA^I* and *hacA^U*), was analysed by

quantitative PCR (qPCR) (Appendix S1). The expression levels of these genes were higher at early stages (2 h) than at later periods (8 h) after chemical stress induction (Fig. S4), and thus short exposition periods were selected to perform RNA-seq analysis (Appendix S1). Cellular stress was induced with Tm and DTT, which prevent N-glycosylation of newly synthesized proteins and the formation of disulphide bonds, respectively (Fan *et al.*, 2018; Yoo *et al.*, 2018). A total of 294 million reads were generated, more than 94% of the filtered

reads were mapped to the *A. nidulans* FGSC A4 reference genome, and a total of 10,774 genes were analysed (Table S1). For DTT, 1905 and 1172 genes were DE at 2 and 8 h, respectively, while 312 and 1862 genes were DE at 2 and 8 h of exposition to Tm respectively (Fig. S5A and B, Table S2).

Gene ontology (GO) enrichment of upregulated genes was performed for functional analysis (Appendix S1). The GO term oxidoreductase activity was enriched in the 2 h DTT treatment. Catalytic activity was found highly enriched, and lipid metabolic process, carboxylic acid metabolic process, organic acid metabolic process, cellular amino acid metabolic process and oxoacid metabolic process were moderately enriched in the 8 h DTT treatment. Regarding downregulated genes, the GO terms organonitrogen compound metabolic process, lipid metabolic process, catalytic activity and cytoplasm were enriched in 2 h DTT, whereas oxidoreductase activity and extracellular region were enriched in 8 h DTT. Additionally, the GO term secondary metabolic process was enriched in the upregulated genes in the 2 h Tm treatment, whereas intracellular was enriched in 8 h Tm (Fig. S5C).

The RNA-seq data demonstrated to be consistent with previous works (Travers *et al.*, 2000; Sims *et al.*, 2005; Guillemette *et al.*, 2007; Carvalho *et al.*, 2012). The presence of transcripts related to oxidoreductase activity, mainly at the 8 h chemical treatment, could be partially explained by the role of DTT as a reducing agent. In addition, sustained ER stress was reported to cause oxidative stress in yeast cells by the UPR-regulated oxidative folding machinery in the ER and mitochondria (Haynes *et al.*, 2004). This seems to be a 'side effect' of cellular response to ER stress, that is the oxidative protein folding intensified by loading of client proteins into the ER somehow results in ROS production. Lipid and cellular amino acid metabolic processes were also enriched, which could be explained by the ER expansion to accommodate higher influx of

newly synthesized proteins (Pineau and Ferreira, 2010) and by the supply of cells with amino acids to synthesize proteins involved in the protection against reactive species respectively (Harding *et al.*, 2003; Herzog *et al.*, 2013). Moreover, downregulation of extracellular proteins could be associated with the so-called process repression under secretion stress (RESS), which is a feedback mechanism activated in response to impairment in protein folding or transport (Pakula *et al.*, 2003).

Genes with predicted function in the secretory pathway were selected using a previously reported secretory model for *Aspergillus oryzae* (Liu *et al.*, 2014). A total of 375 homologous genes were found in *A. nidulans* by using the *Aspergillus* Genome Database (AspGD; Table S3). Among them, 114 were upregulated and 53 genes were downregulated under DTT treatment, whereas 27 genes were upregulated and 13 genes downregulated under Tm treatment (Fig. S6A). Target genes for single deletion in *A. nidulans* were selected based on three criteria: (i) genes up- or downregulated in both treatment periods (2 and 8 h); (ii) top 25 genes with the highest or lowest fold-change; and (iii) genes with no redundant function in the secretory pathway, covering a wide range of biological processes. These rankings resulted in the selection of 12 genes (Fig. S6B and Table S4).

The following step in our strategy was the deletion of the target genes in the *A. nidulans* strains producing XlnE and Tpet_0854 in order to evaluate their influence on the production of distinct recombinant xylanases. Initially, auxotrophy for uridine and uracil was regenerated by selecting XlnE and Tpet_0854 strains growing in MM with 5-fluorotic acid (5-FOA) (Appendix S1). A total of 20-30 colonies presenting 5-FOA-resistance were obtained after three days of cultivation. After monosporic purification, more than 95% of the isolates showed uridine and uracil auxotrophy (*pyrG*) while maintaining equivalent levels of xylanase secretion compared with their parental strains (data not shown). Gene deletions in XlnE and Tpet_0854

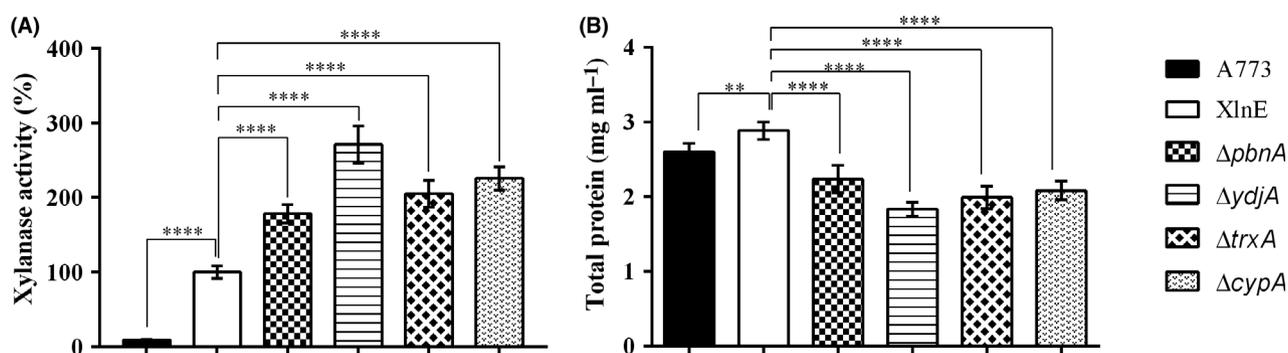


Fig. 2. Extracellular xylanase and total proteins secretion by the *A. nidulans* XlnE mutant strains. The *A. nidulans* XlnE strain and mutants were grown in liquid MM supplemented with 2% maltose for 36 h at 37°C.

A. Xylanase activity was assayed with Azo-Xylan as substrate, pH 5.5 at 50°C, and data regarding the XlnE strain are shown.

B. Protein quantification was assayed by the BCA method. (**) *P*-values between 0.01 and 0.001; (****) *P*-values <0.0001.

pyrG strains were confirmed by diagnostic PCR and Southern blot (Appendix S1) (Fig. S7).

XlnE, $\Delta pbnA$, $\Delta ydjA$, $\Delta trxA$ and $\Delta cypA$ strains showed 78, 171, 105 and 125% higher xylanase activity than the control strain at 36 h of induction (Fig. 2A), respectively, and even higher after 48 h (Fig. S8) (Appendix S1).

Additionally, we investigated whether the improvement observed in xylanolytic activity resulted because of a specific increase in XlnE production or an increase in total protein secretion. Interestingly, lower protein secretion was observed in the mutant strains, suggesting that single deletions specifically increased the XlnE production (Fig. 2B). On the other hand, no changes in xylanase production, nor in the levels of secreted proteins were observed in the Tpet_0854 mutant strains (Fig. S9).

In an attempt to address how the single deletions increased secretion of the homologous xylanase, we investigated the presence of UPREs in the promoters of *pbnA*, *cypA*, *ydjA* and *trxA* genes using the JASPAR

database (Khan *et al.*, 2018). Our analysis of *A. nidulans* promoters using the UPREs motifs found in *Aspergillus niger* was inconclusive. Thus, we used the *hac1* upstream consensus sequence from *S. cerevisiae* (profile MA0310.1) as a model and the highest relative score was found for the following motifs: GACACGTC (0.969), AACACGTC (0.937), GCCACGTA (0.955), AACACATA (0.802), ACCACGTT (0.900), CCCACGTT (0.854) and GACACGTA (0.984) in the promoters of *bipA*, *pdiA*, *hacA*, *pbnA*, *ydjA*, *trxA* and *cypA*, respectively, suggesting that these genes may be regulated by UPR. The CACGT motif was not found in the *pbnA* promoter, suggesting that this gene is probably independent of *hacA*-mediated UPR (Fig. 3A).

The XlnE mutant strains were cultivated on MM agar plates supplemented with glucose, CMC, xylose, starch or pectin to investigate growth phenotypes as well as in the presence of DTT to evaluate chemical cellular stress resistance. Deleted genes were not essential to carbohydrate metabolism nor to metabolize DTT since all four

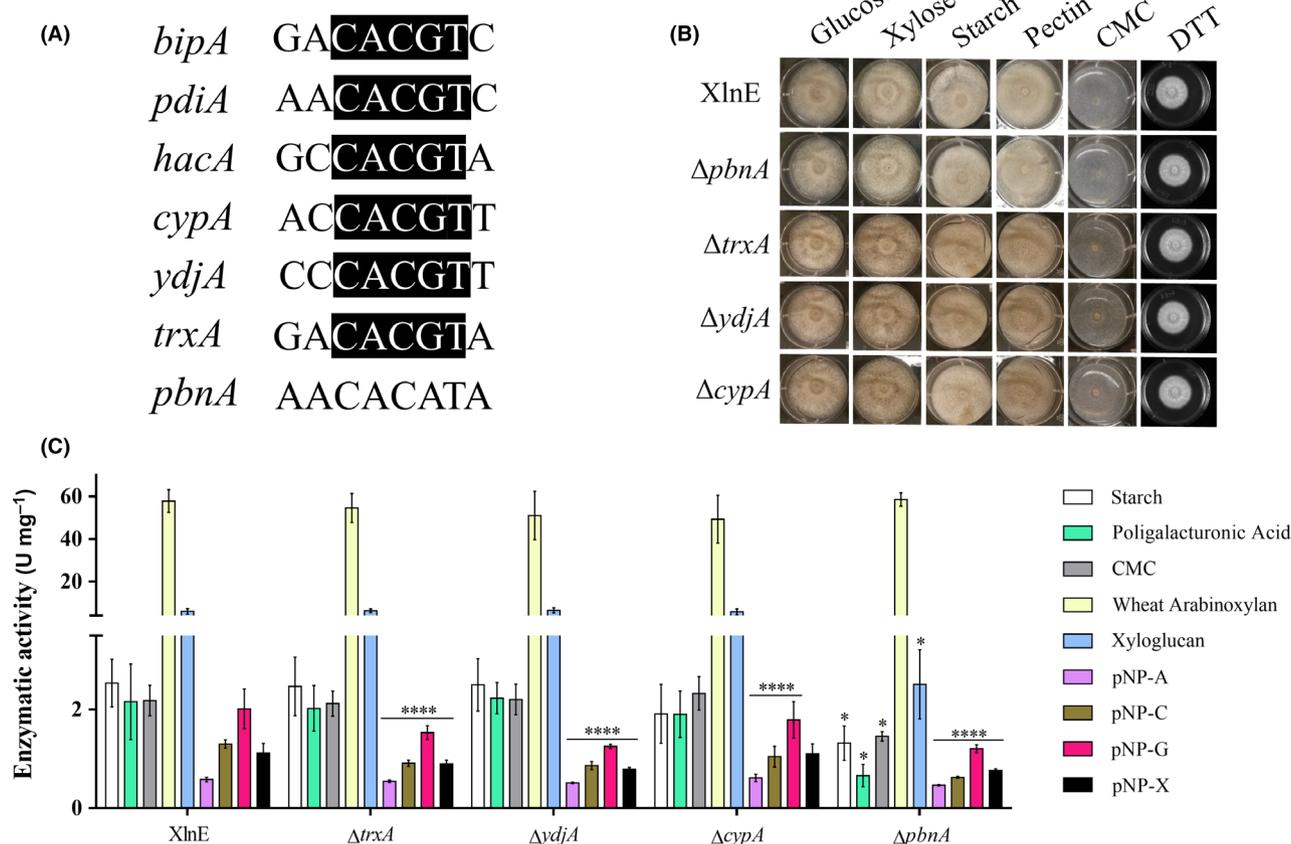


Fig. 3. Carbohydrate-active enzymes production in *A. nidulans* XlnE mutant strains.

A. Alignment of the putative promoter consensus sequences found in *bipA*, *pdiA*, *hacA*, *cypA*, *ydjA*, *trxA* and *pbnA* genes. Black box: identical sequence.

B. Growth of mutant strains on agar plates with different substrates and DTT for 3 days at 37°C.

C. Enzymatic activities in the supernatants of *A. nidulans* strains grown on 1% hydrothermal-pretreated sugarcane bagasse, pH 6.5 for 3 days at 37°C. Enzymatic activity assays were performed with different substrates, pH 5.5 and at 50°C. (*) *P*-values between 0.05 and 0.01 and (****) *P*-values <0.0001.

mutant strains seem to equally grow in the different conditions (Fig. 3B). In addition, mutant strains were grown on hydrothermal-pretreated sugarcane bagasse in order to quantify the secretion of different carbohydrate-active enzymes (CAZymes) in the crude supernatants. Arabinoxylanase, cellobiohydrolase, β -glucosidase and β -xylosidase activities were slightly lower in the XlnE mutant strains (except for β -xylosidase activity in the $\Delta cypA$ strain), whereas amylase, pectinase, endoglucanase, xylanase and xyloglucanase activities were lower only in the $\Delta pbnA$ mutant (Fig. 3C) (Appendix S1). Furthermore, no morphological differences were detected in the mutant strains hyphae (Fig. S10).

For further understanding of *pbnA*, *ydjA*, *trxA* and *cypA* functions, a weighted gene co-expression network analysis (WGCNA) was performed, resulting in the identification of 21 modules (Fig. S11). The *pbnA*, *ydjA*, *trxA* and *cypA* genes were, respectively, found in the following modules: lightyellow, brown, darkmagenta and lightcyan (Table S5). GO enrichment analysis of the modules demonstrated that these genes have potential functional links with several GO terms involved in a wide range of processes related to protein secretion (Fig. S12).

To date, *pbnA* was not characterized in *A. nidulans*, whereas the orthologous in *S. cerevisiae* (*pbn1*) encodes a chaperone-like protein, an essential component of the glycosylphosphatidylinositol-mannosyltransferase I complex. The lightyellow module was especially enriched for proteasome complex activity (Fig. S12A and Table S6). *pbn1* physically interacts with *prb1* (vacuolar proteinase B) and *ecm27* ($\text{Na}^+/\text{Ca}^{2+}$ exchanger) in *S. cerevisiae*. The expression of *pdiA* and *bipA* was measured to evaluate whether *pbnA* deletion induced UPR activation or not. The genes *pdiA* and *bipA* were not overexpressed in the knockout strain, suggesting that UPR activation is not involved in the higher production of XlnE at least in 36 h (Fig. S13). This result, in addition to the reduction in total protein secretion (Fig. 2B) and the higher XlnE secretion (Fig. 2A), suggests that the cell converged for the XlnE production by reducing the traffic of other proteins in the ER. Such a compensation mechanism could then avoid UPR activation.

A group of chaperones that includes *ydj1* is induced in strains overexpressing *hac1* (Graf *et al.*, 2008). *ydjA* was detected in the brown module (Fig. S12B and Table S6). This result indicates that this gene might have functions on intracellular protein transmembrane transport, protein refolding and ERAD pathway. In *S. cerevisiae*, *ydj1* physically interacts with *prd1*, which encodes an intracellular proteinase involved in protein degradation (Buchler *et al.*, 1994). In addition, *ydj1* genetically interacts with *pbn1* (*pbnA* in *A. nidulans*), *tsa1* and *cne1* (calnexin). Calnexin is a key ER chaperone that assists in folding and subunit assembly of most Asn-linked glycoproteins passing

through ER (Leach and Williams, 2013). Our results suggest *ydjA* is a chaperone that contributes to the folding of different *A. nidulans* proteins since *ydjA* knockout strain showed lower total protein secretion (Fig. 2B).

trxA encodes thioredoxin (*trx1* orthologous in *S. cerevisiae*) that contributes to protection against reactive oxygen species (ROS; Thön *et al.*, 2007). Detoxification mechanisms include the production of superoxide dismutases, catalases, peroxiredoxins, glutathione and the thioredoxin system, a dual system composed of thioredoxin and thioredoxin reductase (TrxR; Thön *et al.*, 2007). *trxA* was co-expressed with genes predicted to have function in cell redox homeostasis, fatty acid transport and ubiquitin-dependent protein catabolism in the darkmagenta module (Fig. S12C and Table S6). *trx1* has predicted physical interactions with *trx2* (thioredoxin), *ero1* (protein disulphide isomerase), *met16* (3'-phosphoadenylsulphate reductase), *ahp1* (thiol-specific peroxiredoxin), *tsa1* (thioredoxin peroxidase) and *mrx1* (methionine-S-sulfoxide reductase) in *S. cerevisiae*. Analysis of the genetic interactions showed crosstalk between *trx1* and *pbn1* networks through *tsa1* and *prb1* in *S. cerevisiae*. *tsa1* genetically interacts with *prb1*, which, in turn, is physically linked to *pbn1*. It is likely to suggest that, although TrxA is very important for the folding of several proteins in *A. nidulans*, it did not affect the XlnE production.

Finally, *cypA* is a homologue to *cpr1* in *S. cerevisiae*, which encodes a peptidyl-prolyl *cis-trans* isomerase (PPIases) catalysing the *cis-trans* isomerization of peptide bonds in N-terminal proline residues. *cpr1* showed physical and genetic interactions with *set3*, *hos4*, *hos2*, *sif2* and *snt1* in *S. cerevisiae*. These genes, including *cpr1*, form the Set3p complex (SET3C), in which the subunits Hos2, Set3, Sif2 and Snt1 form the core complex, whereas Hst1p, Sum1p and Cpr1p are peripherally associated (Pijnappel *et al.*, 2001). Many of these proteins are histone deacetylases (HDACs), and deacetylation has been identified as a major regulator of eukaryotic gene transcription due to a repressive chromatin structure (Kurdistani and Grunstein, 2003). Then, the SET3C complex is responsible for transcriptional repression of some genes related to the early/middle class of sporulation-specific genes, including the key meiotic regulator in yeasts (Pijnappel *et al.*, 2001). *cypA* was co-expressed with genes with predicted functions in the hyphal tip, vacuole organization and response to heat (Fig. S12D and Table S6). How the deletion of *pbnA*, *ydjA*, *trxA* and *cypA* exactly impact XlnE production, but not Tpet_0854, remains to be determined.

Cellular stress was chemically induced in *A. nidulans* using DTT and Tm. Based on the RNA-seq database, twelve genes with predicted function in the secretory pathway were deleted in *A. nidulans* recombinant strains producing homologous and heterologous GH10 endo-

xylanase. Higher enzyme production occurred only in the mutant strains ($\Delta pbnA$, $\Delta ydjA$, $\Delta trxA$ and $\Delta cypA$) producing the homologous xylanase. These four deleted genes have predicted functions in processes such as proteasome activity, protein refolding, ERAD pathway, hyphal tip, vacuole organization, autophagy, cell redox homeostasis, fatty acid transport and ubiquitin-dependent degradation. The continuing characterization of *pbnA*, *ydjA*, *trxA*, *cypA*, as well as other mutants, will enhance our understanding of protein secretion in filamentous fungi, including industrially relevant species.

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Conflict of interest

The authors declare no conflicts of interest.

References

- Brandl, J., and Andersen, M.R. (2017) Aspergilli: models for systems biology in filamentous fungi. *Curr Opin Syst Biol* **6**: 67–73.
- Buchler, M., Tislar, U., and Wolf, D.H. (1994) Proteinase yscD (oligopeptidase yscD). Structure, function and relationship of the yeast enzyme with mammalian thimet oligopeptidase (metalloendopeptidase, EP 24.15). *Eur J Biochem* **219**: 627–639.
- Carvalho, N.D., Jørgensen, T.R., Arentshorst, M., Nitsche, B.M., van den Hondel, C.A., Archer, D.B., and Ram, A.F. (2012) Genome-wide expression analysis upon constitutive activation of the HacA bZIP transcription factor in *Aspergillus niger* reveals a coordinated cellular response to counteract ER stress. *BMC Genomics* **13**: 1–17.
- Dai, Z., Aryal, U.K., Shukla, A., Qian, W.J., Smith, R.D., Magnuson, J.K., et al. (2013) Impact of *alg3* gene deletion on growth, development, pigment production, protein secretion, and functions of recombinant *Trichoderma reesei* cellobiohydrolases in *Aspergillus niger*. *Fungal Genet Biol* **61**: 120–132.
- Demain, A.L., and Vaishnav, P. (2009) Production of recombinant proteins by microbes and higher organisms. *Biotechnol Adv* **27**: 297–306.
- Fan, F., Zhang, Y., Wang, S., Han, Y., Wang, L., and Lu, D. (2018) Characterization of the oxidative protein folding activity of a unique plant oxidoreductase, Arabidopsis protein disulfide isomerase-11. *Biochem Biophys Res Commun* **495**: 1041–1047.
- Frisvad, J.C., Møller, L.L.H., Larsen, T.O., Kumar, R., and Arnaou, J. (2018) Safety of the fungal workhorses of industrial biotechnology: update on the mycotoxin and secondary metabolite potential of *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*. *Appl Microbiol Biotechnol* **102**: 9481–9515.
- Gabrielli, E., Fothergill, A.W., Brescini, L., Sutton, D.A., Marchionni, E., Orsetti, E., et al. (2014) Osteomyelitis caused by *Aspergillus* species: a review of 310 reported cases. *Clin Microbiol Infect* **20**: 559–565.
- Graf, A., Gasser, B., Dragosits, M., Sauer, M., Leparc, G.G., Tüchler, T., et al. (2008) Novel insights into the unfolded protein response using *Pichia pastoris* specific DNA microarrays. *BMC Genomics* **9**: 1–13.
- Guillemette, T., Van Peij, N.N.M.E., Goosen, T., Lanthaler, K., Robson, G.D., Van Hondel, C.A., et al. (2007) Genomic analysis of the secretion stress response in the enzyme-producing cell factory *Aspergillus niger*. *BMC Genomics* **8**: 1–17.
- Harding, H.P., Zhang, Y., Zeng, H., Novoa, I., Lu, P.D., Calton, M., et al. (2003) An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell* **11**: 619–633.
- Haynes, C.M., Titus, E.A., and Cooper, A.A. (2004) Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death. *Mol Cell* **15**: 767–776.
- Heimel, K. (2015) Unfolded protein response in filamentous fungi – implications in biotechnology. *Appl Microbiol Biotechnol* **99**: 121–132.
- Herzog, B., Popova, B., Jakobshagen, A., Shahpasandzadeh, H., and Braus, G.H. (2013) Mutual cross talk between the regulators Hac1 of the unfolded protein response and Gcn4 of the general amino acid control of *Saccharomyces cerevisiae*. *Eukaryot Cell* **12**: 1142–1154.
- Huang, M., Bao, J., Hallström, B.M., Petranovic, D., and Nielsen, J. (2017) Efficient protein production by yeast requires global tuning of metabolism. *Nat Commun* **8**: 1131.
- Khan, A., Fornes, O., Stigliani, A., Gheorghe, M., Castro-Mondragon, J.A., van der Lee, R., et al. (2018) JASPAR 2018: update of the open-access database of transcription factor binding profiles and its web framework. *Nucleic Acids Res* **46**: 260–266.
- Klein-Marcuschamer, D., Oleskowicz-Popiel, P., Simmons, B.A., and Blanch, H.W. (2012) The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnol Bioeng* **109**: 1083–1087.
- Krishnan, K., and Askew, D.S. (2014) The fungal UPR: A regulatory hub for virulence traits in the mold pathogen *Aspergillus fumigatus*. *Virulence* **5**: 334–340.
- Kurdistani, S.K., and Grunstein, M. (2003) Histone acetylation and deacetylation in yeast. *Nat Rev Mol Cell Biol* **4**: 276–284.
- Leach, M.R., and Williams, D.B. (2013) Calnexin and Calreticulin. Molecular Chaperones of the Endoplasmic Reticulum. In *Madame Curie Bioscience Database*. Austin, TX, USA: Landes Bioscience, pp. 2000–2013.

- Liu, L., Feizi, A., Österlund, T., Hjort, C., and Nielsen, J. (2014) Genome-scale analysis of the high-efficient protein secretion system of *Aspergillus oryzae*. *BMC Syst Biol* **8**: 1–13.
- Maffei, B., Francetic, O., and Subtil, A. (2017) Tracking proteins secreted by bacteria: what's in the toolbox? *Front Cell Infect Microbiol* **7**: 1–17.
- Mulder, H.J., Nikolaev, I., and Madrid, S.M. (2006) HACA, the transcriptional activator of the unfolded protein response (UPR) in *Aspergillus niger*, binds to partly palindromic UPR elements of the consensus sequence 5'-CAN (G/A)NTGT/GCCT-3'. *Fungal Genet Biol* **43**: 560–572.
- Nevalainen, H., and Peterson, R. (2014) Making recombinant proteins in filamentous fungi- are we expecting too much? *Front Microbiol* **5**: 75.
- Pakula, T.M., Laxell, M., Huuskonen, A., Uusitalo, J., Saloheimo, M., and Penttilä, M. (2003) The effects of drugs inhibiting protein secretion in the filamentous fungus *Trichoderma reesei*. Evidence for down-regulation of genes that encode secreted proteins in the stressed cells. *J Biol Chem* **278**: 45011–45020.
- Pijnappel, W.W., Schaft, D., Roguev, A., Shevchenko, A., Tekotte, H., Wilm, M., et al. (2001) The *S. cerevisiae* SET3 complex includes two histone deacetylases, Hos2 and Hst1, and is a meiotic-specific repressor of the sporulation gene program. *Genes Dev* **15**: 2991–3004.
- Pineau, L., and Ferreira, T. (2010) Lipid-induced ER stress in yeast and β cells: parallel trails to a common fate. *FEMS Yeast Res* **10**: 1035–1045.
- Qin, L., Wu, V.W., and Glass, N.L. (2017) Deciphering the regulatory network between the SREBP pathway and protein secretion in *Neurospora crassa*. *MBio* **8**: 1–16.
- Reilly, M.C., Qin, L., Craig, J.P., Starr, T.L., and Glass, N.L. (2015) Deletion of homologs of the SREBP pathway results in hyper-production of cellulases in *Neurospora crassa* and *Trichoderma reesei*. *Biotechnol Biofuels* **8**: 121.
- Schalén, M., Anyaogu, D.C., Hoof, J.B., and Workman, M. (2016) Effect of secretory pathway gene overexpression on secretion of a fluorescent reporter protein in *Aspergillus nidulans*. *Fungal Biol Biotechnol* **3**: 1–14.
- Segato, F., Damásio, A.R.L., Gonçalves, T.A., de Lucas, R.C., Squina, F.M., Decker, S.R., and Prade, R.A. (2012) High-yield secretion of multiple client proteins in *Aspergillus*. *Enzyme Microb Technol* **51**: 100–106.
- Sims, A.H., Gent, M.E., Lanthaler, K., Nigal, S., Oliver, S.G., Robson, G.D., and Dunn-coleman, N.S. (2005) Transcriptome analysis of recombinant protein secretion by *Aspergillus nidulans* and the unfolded-protein response in vivo. *Appl Environ Microbiol* **71**: 2737–2747.
- Singh, R.S., Singh, T., and Pandey, A. (2019) Microbial enzymes—an overview. In *Advances in Enzyme Technology Biomass, Biofuels, Biochemicals*. Singh, R.S., Singhania, R.R., Pandey, A., and Larroche, C. (eds). Amsterdam, the Netherlands: Elsevier, pp. 1–40.
- Sun, X., and Su, X. (2019) Harnessing the knowledge of protein secretion for enhanced protein production in filamentous fungi. *World J Microbiol Biotechnol* **35**.
- Taniwaki, M.H., Pitt, J.I., and Magan, N. (2018) *Aspergillus* species and mycotoxins: occurrence and importance in major food commodities. *Curr Opin Food Sci* **23**: 38–43.
- Thön, M., Al-Abdallah, Q., Hortschansky, P., and Brakhage, A. (2007) The thioredoxin system of the filamentous fungus *Aspergillus nidulans*: impact on development and oxidative stress response. *J Biol Chem* **282**: 27259–27269.
- Travers, K.J., Patil, C.K., Wodicka, L., Lockhart, D.J., Weissman, J.S., Walter, P., and Francisco, S. (2000) Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* **101**: 249–258.
- de Vries, R.P., Riley, R., Wiebenga, A., Aguilar-Osorio, G., Amillis, S., Uchima, C.A., et al. (2017) Comparative genomics reveals high biological diversity and specific adaptations in the industrially and medically important fungal genus *Aspergillus*. *Genome Biol* **18**: 28.
- Yoo, J., Mashalidis, E.H., Kuk, A.C.Y., Yamamoto, K., Kaeser, B., Ichikawa, S., and Lee, S.Y. (2018) GlcNAc-1-P-transferase–tunicamycin complex structure reveals basis for inhibition of N-glycosylation. *Nat Struct Mol Biol* **25**: 217–224.
- Yoon, J., Kikuma, T., Maruyama, J., and Kitamoto, K. (2013) Enhanced production of bovine chymosin by autophagy deficiency in the filamentous fungus *Aspergillus oryzae*. *PLoS One* **8**: e62512.
- Yu, X.W., Sun, W.H., Wang, Y.Z., and Xu, Y. (2017) Identification of novel factors enhancing recombinant protein production in multi-copy *Komagataella phaffii* based on transcriptomic analysis of overexpression effects. *Sci Rep* **7**: 16249.
- Zhang, G., Zhu, Y., Wei, D., and Wang, W. (2014) Enhanced production of heterologous proteins by the filamentous fungus *Trichoderma reesei* via disruption of the alkaline serine protease SPW combined with a pH control strategy. *Plasmid* **71**: 16–22.
- Zhang, X., Li, Y., Zhao, X., and Bai, F. (2017) Constitutive cellulase production from glucose using the recombinant *Trichoderma reesei* strain overexpressing an artificial transcription activator. *Bioresour Technol* **223**: 317–322.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Structure and N-glycosylation analysis of XlnE and Tpet_0854. The 3D models were constructed using Swiss Model based on *Aspergillus aculeatus* endo-xylanase PDB 6Q8M (100% identity) for AN7401 XlnE, and *T. petrophila* xylanase 10B PDB 3NIY (100% identity) for Tpet_0854. In addition, the accessible surface area (ASA) and N-glycosylation prediction were calculated for each site.

Fig. S2. Production of recombinant GH10 xylanases in *A. nidulans*. Secreted proteins were resolved by SDS-PAGE. The *A. nidulans* strains XlnE and Tpet_0854 were grown in minimum media containing 2% maltose for 72 h at 37°C. The arrow indicates the position of the homologous and heterologous xylanase.

Fig. S3. Southern blot analysis. The probe was obtained by PCR amplification of the *xlnE* gene from *A. nidulans*. A773:

reference strain; pEXPYR: A773 transformed with empty pEXPYR plasmid; AN7401 *pyrG*⁻: A773 transformed with the AN7401 gene (*xlnE*) cloned into the pEXPYR plasmid followed by *pyrG* recycling; AN7401 *pyrG*⁺: A773 transformed with the AN7401 gene (*xlnE*) cloned into the pEXPYR plasmid.

Fig. S4. Expression of UPR genes in *A. nidulans* A773 during exposure to DTT (20 mM) by qPCR analysis. (A) *hacA* total; (B) *hacA*_i; (C) *bipA*.

Fig. S5. Analysis of differentially expressed genes in *A. nidulans* exposed to cellular stress-inducing chemicals. The *A. nidulans* A773 strain was grown in MM for 24 h, exposed to DTT and Tm for 2 and 8 h, and then analyzed by RNA-seq. (A) Number of up- and down-regulated genes. (B) Venn diagram showing the DE genes. (C) Enrichment analysis of GO terms over-represented in the DE genes using the Blast2Go software. For the differential expression analysis, an adjusted *P*-value ≤ 0.01 was used as threshold, and a cutoff of log₂ fold change ≥ 1 or ≤ -1 for Tm treatment and log₂ fold change ≥ 2 or ≤ -2 for DTT treatment.

Fig. S6. Expression profile of genes with predicted function in the *A. nidulans* secretory pathway under stress conditions. Genes identified in *A. nidulans* after DTT and Tm treatment for 2 and 8 h at 37°C. (A) Number of genes up- and down-regulated. (B) Expression profile of the twelve genes chose to be deleted in *A. nidulans* recombinant strains.

Fig. S7. Strategy used for construction and confirmation of *A. nidulans* knocked out strains. (A) Δ *trxA*, (B) Δ *cyp1*, (C) Δ *ydjA* and (D) Δ *pbnA*. The *pyrG* gene from *Aspergillus fumigatus* (*pyrGAf*) was used as a selectable marker. The transformants were purified by five rounds of monospore purification and colonies containing genetic identical nuclei were submitted to two independent PCRs, one using primers anchored outside the flanking targeting sequence (F2/R1) and a second PCR using primers anchored within the cassette (F1/R1). After PCR, Southern Blot analysis was performed to confirm the deletion. Mutants gDNA were digested with *NcoI* and *BamHI* and 5' flanking regions of each gene was used as a probe. Differences among the size of the detected fragment allowed differentiation of mutant and wild-type strain. This strategy was used for all deleted genes.

Fig. S8. Extracellular xylanase activity. The *A. nidulans* XlnE strain and mutants were grown in liquid MM supplemented with 2% maltose for 48 h at 37°C. Xylanase activity was assayed with Azo-Xylan as substrate, pH 5.5 at 50°C, and the data are shown relative to the XlnE strain. (****) adjusted *P*-values < 0.0001 .

Fig. S9. Xylanase activity and protein secretion by the *A. nidulans* Tpet_0854 mutant strains. (A) Enzymatic activity measured with Azo-xylan. The assay was performed using 1 μ g of protein at pH 5.5 and 50°C and expressed in relation to the activity of the control strain. (B) Evaluation of protein secretion yield.

Fig. S10. Mycelia morphology of *A. nidulans* strains at 400 \times (right column) and 1000 \times (left column) magnifications under an optical microscope. Strains were grown at 37°C in MM for 96 h. Wild-type hyphae observed on control strain (XlnE). Mutant strains hyphae showed no morphological differences.

Fig. S11. Hierarchical cluster tree showing 21 modules of co-expressed genes. Each of the 10 586 genes is represented by a leaf in the tree, and each of the 21 modules by a major tree branch. The lower panel shows modules in designated colors after merged, such as *Black*, *Darkred*, *Pink* and others.

Fig. S12. GO Biological Process enrichment analysis of the modules *lightyellow/pbnA* (A), *brown/ydjA* (B), *darkmagenta/trxA* (C) and *lightcyan1/cypA* (D).

Fig. S13. Expression of UPR targets. The *A. nidulans* XlnE and mutant strains were grown in liquid MM supplemented with 2% maltose for 36 h at 37°C. (B) Gene expression calculated by qPCR using the Relative Standard Curve Method. The gene *tubC* (tubulin) and was used as reference.

Methodology S1. R script of the WGCNA analysis.

Table S1. Expression data of *A. nidulans* genes after DTT and TM treatment.

Table S2. Differentially expressed (DE) genes in *Aspergillus nidulans* mycelia after DTT and TM treatment. For the differential expression analysis, an adjusted *P*-value ≤ 0.01 was used as threshold, and a cutoff of log₂ fold change ≥ 1 or ≤ -1 for TM treatment and log₂ fold change ≥ 2 or ≤ -2 for DTT treatment.

Table S3. Genes encoding predicted secretory proteins based on a previously reported secretory model from *Aspergillus oryzae*. A total of 375 homologous genes were found in *A. nidulans* by performing a search in the Aspergillus Genome Database (AspGD).

Table S4. List of selected genes deleted in *A. nidulans*.

Table S5. Modules of co-expressed genes in the *A. nidulans* network.

Table S7. Strains used in this work.

Table S8. Primers used in this study.

Appendix S1. Experimental procedure.