THE IMPACT OF N-GLYCOSYLATION ON THE FUNCTION AND SECRETION OF FUNGAL RECOMBINANT ENZYMES

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ABSTRACT

Filamentous fungi are cell factories traditionally used for enzyme production in various industrial sectors, including food and beverages, biopolymers, biofuels, and animal feed. Genetic engineering of genes involved in the fungal secretory pathway has been applied to overcome production barriers, including post-translational modifications like N-glycosylation of proteins. N-glycosylation can significantly influence proteins' stability, yield, and function. Previously, we observed that mutations in N-glycosylation sites affected the secretion and catalytic efficiency of a GH3 β-xylosidase (BxlB) produced by *A. nidulans*. Then, we hypothesized that deleting genes involved in N-glycans assembly or the mechanism of protein quality control into the ER (ERQC) could impact the secretion of client proteins. Genes related to N-glycan assembly and ERQC in *A. nidulans* were deleted, resulting in eight viable mutants. Next, the *bxlb* gene (AN8401) was transformed into these mutant strains. Single deletion of most target genes did not affect protein secretion and fungal growth. Interestingly, the specific activity of BxlB measured in the secretome of single mutant strains was significantly affected in a time-dependent manner, while BxlB secretion remained unaffected. In contrast, the combined deletion of *alg3* and *alg9* increased the BxlB secretion significantly while the kinetic parameters remained unaffected. Conversely, the multiple deletion of *alg3*, *alg6*, and *alg9* did not affect BxlB secretion, but reduced enzymatic kinetics by 20%. Manipulating genes involved in the N-glycan assembly pathway can have varying effects on enzyme secretion and activity, opening new avenues for enhancing enzyme production in industrially relevant fungi.

Keywords: N-glycosylation, CRISPR/Cas9, carbohydrate-active enzymes, *A. nidulans*, protein secretion

1 INTRODUCTION

Filamentous fungi have emerged as exceptionally efficient organisms in producing recombinant proteins and enzymes, establishing themselves as remarkable microbial cell factories. Because of their superior protein secretion capabilities, high protein levels are achieved and the versatile genetic manipulation tools further enhance their potential. During the process of producing proteins, nascent proteins experience different post-translational modifications (PTMs). The most common type of PTM that occurs on extracellular proteins is protein glycosylation, which is highly prevalent in filamentous fungi[1,2]. N-glycosylation refers to the attachment of a glycan to an asparagine (N) residue catalyzed by oligosaccharyltransferases in the endoplasmic reticulum (ER).

N-glycosylation pathway contributes to the appropriate modification and maturation of nascent proteins, ultimately contributing to their functionality and biological activity. Indeed, glycosylation influences various parameters such as protein secretion, stability, activity, signaling, and protease protection[3]. The deletion of a gene involved in N-glycan synthesis (alg3) in Aspergillus niger had a significant impact on biological aspects, including spore germination, hyphal growth, sporulation, as well as pigment and protein production. At the same time, noticeable effects were observed on the hydrolytic activity of a heterologous recombinant cellobiohydrolase (rCel7A). Moreover, the position of N-linked glycans influences either positively or negatively the enzymatic activity of a homologous GH3 β-xylosidase produced in *Aspergillus nidulans*[4].

Despite significant research efforts, a systematic study correlating the loss or gain-of-function of N-glycosylation and ER protein quality control (ERQC) on the secretion and activity of CAZymes is still needed. Therefore, this study aimed to investigate the involvement of genes with predicted function in N-glycan assembly as well as genes coding for ERQC components on the secretion and activity of β-xylosidase and CAZymes in *A. nidulans*. To achieve this goal, we analyzed phenotypic characteristics, such as fungal growth and protein secretion. Furthermore, we evaluated the functional properties and the secretion yield of a recombinant β-xylosidase produced by the mutant strains. This enzyme is glycosylated and secreted by *A. nidulans* in the presence of plant biomass, making it a convenient model of glycoprotein to study.

2 MATERIAL & METHODS

Deletion of N-glycosylation and quality control pathway genes by CRISPR/Cas9 technology: CRISPR/Cas9 vectors were constructed as previously described[5], using primers designed based on the A. nidulans genome obtained from https://fungidb.org. All vectors were assembled using USER cloning[6] and USER fusion techniques. To generate each fragment for vector construction, the DNA polymerase Pfu X7, was utilized with the vector pFC902 serving as a template. The resulting amplified fragments were purified using a 2% agarose gel and cloned into the vector pFC330 previously digested with the PacI and Nt.BbvcI enzymes. For transformation using A. nidulans Δku protoplasts, constructed vectors and synthesized repairing oligonucleotides for homologous recombination, composed of upstream and downstream sequences of the target genes were utilized. Transformants growing withoutin the absence of uracil and uridine were isolated and subjected to five rounds of monosporic purification. Sequential rounds of deletion were carried out to obtain double and triple knockout strains after confirmation of each mutant. Gene deletions were confirmed by diagnostic PCR and Southern blot was conducted using the genomic DNA of knockout strains.

Cloning and expression of BxlB: The *bxlb* (AN8401) full-length ORF was PCR amplified from *A. nidulans* Δku gDNA using Phusion DNA polymerase and standard molecular biology protocols. The resulting amplicons were ligated into pUC2115-1 expression vector; this vector is composed of a glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter, which enabled constitutive expression of the target gene, and the terminator of tryptophan synthase gene (*antrpCt*). The native signal peptide of BxlB was maintained at the N-terminus and a His tag was added to the C-terminus. As a selected marker, the vector contains the orotidine 5-phosphate carboxylase (*pyrG*) gene from the fungus *A. niger*, used to complement the truncated *pyrG89* gene from *A. nidulans*. In addition, pUC2115-1 has a 2kb flanking region of the target sequence – TSI (upstream) and TSII (downstream) – which ensures the integration of the gene in a specific location in the genome. After confirmation by sequencing, the vector was transformed into *A. nidulans* Δku protoplasts, as described above.

3 RESULTS & DISCUSSION

Genes at the early stage of N-glycan synthesis are essential for fungal growth and sporulation: For a comprehensive understanding of the importance of different genes involved in the N-glycosylation pathway in *A. nidulans*, fourteen genes with distinct cellular localizations were selected for their predicted function in N-glycan assembly, processing, and ERQC. The chosen targets were genes encoding proteins predicted in the cytoplasmic (alg7, alg13, alg1, alg2, alg11, and rft1) as well as luminal sections of the ER (alg3, alg9, alg12, and alg6). A predicted mannosidase in the Golgi (AN5748) and genes associated with ERQC (clxA, gtbA, and uggt) were also included. CRISPR/Cas9 vectors were designed to individually delete target genes into the reference strain (REF), resulting in eight mutants: Δrft1, Δalg2, Δalg3, Δalg9, Δalg6, ΔgtbA, ΔclxA and ΔAN5748. The first analysis of the glycomutants on glucose minimal medium (GMM) revealed differences in fungal growth. The strains ΔgtbA, ΔclxA and ΔAN5748 exhibited a notable decline in radial growth compared to the REF strain. Additionally, the strains Δrft1 and Δalg2 were severely affected, showing drastic impairments in sporulation and radial growth. Due to the severity of growth impairments, these latter strains were unsuitable for further investigations (**Figure 1A-B**).

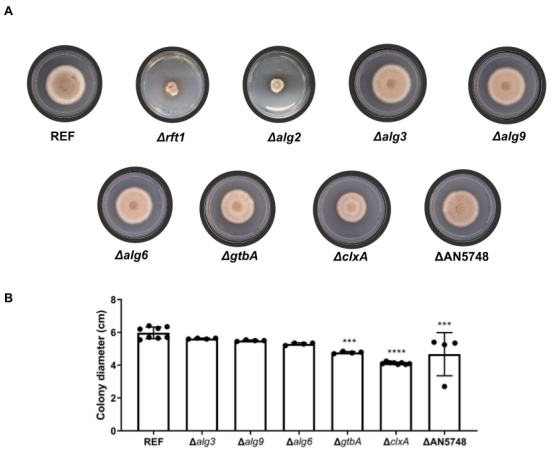

Figure 1. Growth of *A. nidulans* glycomutants on agar plates. Representative image (A) and diameter measurement (B) of REF, Δrft1, Δalg2, Δalg3, Δalg9, Δalg6, ΔgtbA, ΔclxA and ΔAN5748 after cultivation in GMM pH 6.5 for 120 h at 37 ºC. *ANOVA with post hoc Bonferroni test (P value 0.1234 (ns); 0.0332, *; 0.0021, **; 0.0002, ***; <0.0001, ****).

The deletion of genes linked to N-glycosylation and ERQC has no impact on the overall secretion of proteins nor on the fungal biomass: To assess further the impact of the single deletions in protein secretion by A. nidulans, the recombinant production of a homologous GH3 β-xylosidase (AN8401/bxlb) was investigated in REF and the six viable mutant strains (Δalg3, Δalg9, Δalg6, ΔgtbA, ΔclxA and ΔAN5748). The glycomutants expressing the recombinant enzyme were cultivated in liquid GMM for 48 and 72h. To evaluate the impact of gene deletions on BxlB function, extracellular activity was measured using ρNP-X as the substrate. Enzyme secretion was assessed through Western blot (WB) analysis using a polyclonal BxlB antibody. The results revealed a significant decrease in BxlB activity across most of the mutants after 48 h of cultivation, which was evident when data was normalized by both total protein secreted (mg/mL) and fungal biomass (mg dry mass). However, after 72 h, the BxlB activity was similar to REF_OE:bxlb. Notably, despite the reduced enzymatic activity at 48 h, WB analysis indicated that the secretion profile of most mutants was comparable to REF_OE:bxlb, except for ΔclxA_OE:bxlb and ΔAN5748_OE:bxlb at 48 h cultivation, which showed decreased BxlB secretion.

The combined deletion of alg3 and alg9 increased the BxlB secretion: After analyzing the disruption of N-glycosylation and ERQC pathways through single deletions, we investigated the potential impact of multiple gene deletions on BxlB kinetics and secretion, as well as total protein secretion by the glycomutants. To achieve this, we constructed a double mutant (Δalg3, Δalg9) and a triple mutant (Δalg3, Δalg6, Δalg9). This approach was applied recently to generate glycoproteins with shorter glycans attached using A. nidulans as a model organism[7]. To gain insights into the effects of truncated N-glycans on recombinant protein secretion, the bxlb gene was transformed in the double and triple glycomutants, generating the Double_OE:bxlb and Triple_OE:bxlb strains. The glycomutants secretomes were produced in GMM, and BxlB secretion and kinetic parameters were evaluated. No changes were observed in fungal biomass or protein secretion compared to the reference strain REF_OE:bxlb as reported for the single mutants. A significant increase in the extracellular BxlB activity was observed in the Double_OE:bxlb secretome at both 48 and 72 h of cultivation. This increase was particularly significant after normalization by total protein secretion (mg/mL) and fungal biomass (mg dry mass) (**Figure 2A**). Moreover, the WB further confirmed that the combined deletion of alg3 and alg9 positively affected BxlB secretion in the Double_OE:bxlb strain, resulting in higher activity in the secretome (**Figure 2B**).

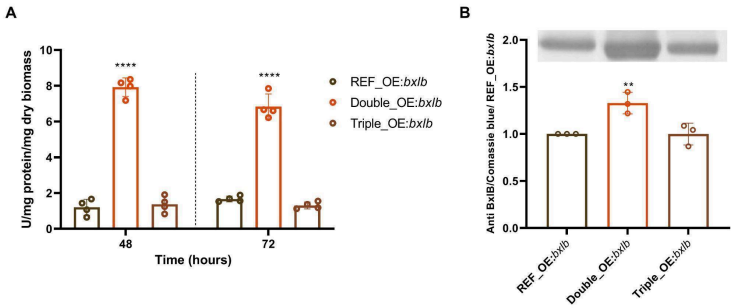

Figure 2. Evaluation of BxlB enzymatic activity and secretion in A. nidulans glycomutants. (A) The ratio enzymatic activity (U/mL), protein secretion (mg/mL), and fungal dry biomass (mg) were analyzed in the REF_OE:bxlb, Double_OE:bxlb and Triple_OE:bxlb strains by cultivation in GMM pH 6.5 for 48 and 72 h. The BxlB enzymatic was assayed using ρNP-X as the substrate at pH 5.0 for 30 minutes at 50°C. (B) The secretion of BxlB was assessed by WBs analysis using the A. nidulans secretomes (20 µg of protein, 48h) and a polyclonal BxlB antibody. The protein amount corresponding to BxlB band was normalized relative to SDS-PAGE stained with Coomassie blue. Top of graph: WB representative membrane. Relative quantification was performed using the ImageJ software. *ANOVA with post hoc Bonferroni test (P value 0.1234 (ns); 0.0332, *; 0.0021, **; 0.0002, ***; <0.0001, ****). Reference strain: REF_OE:bxlb.

CONCLUSION

In this study, we explored the impact of deleting genes involved in N-glycan assembly and protein quality control on the secretion and function of an A. nidulans homologous enzyme (BxlB) and the overall secretion of CAZymes. Our findings demonstrate that rft1 and alg2 genes are essential for A. nidulans growth and sporulation. Moreover, single deletions of alg3, alg9, alg6, gtbA, clxA and AN5748 have no significant effect on fungal biomass or protein secretion. Although most mutants exhibit a decline in recombinant BxlB activity (U/mg) in the secretomes, this profile was time-dependent and reverted at 72h. Moreover, the BxlB secretion was not affected in the majority of the single mutants compared to the reference strain REF_OE:bxlb, except for ΔclxA_OE:bxlb and ΔAN5748_OE:bxlb. Notably, the multiple deletion of alg3 and alg9 enhances BxlB secretion by the Double_OE:bxlb, maintaining the kinetic parameters comparable to REF_OE:bxlb. This effect is likely due to reduced protein retention in the quality control system and the conformational influence of the truncated N-glycan on substrate interaction. On the other hand, the additional deletion of alg6 does not affect BxlB secretion in the Triple_OE:bxlb mutant but reduces enzymatic kinetics by 20%. Finally, deleting genes involved in N-glycan assembly and clxA deletion decreases the secretion of various CAZymes. In contrast, a cluster of up-regulated GHs and AAs were found in the glycomutants, demonstrating a severe alteration in the secretomes profile.

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