

# Random and direct mutagenesis to enhance protein secretion in *Ashbya gossypii*

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To improve the general secretion ability of the biotechnologically relevant fungus *Ashbya gossypii*, random mutagenesis with ethyl methane sulfonate (EMS) was performed. The selection and screening strategy followed revealed mutants with improved secretion of heterologous *Trichoderma reesei* endoglucanase I (EGI), native  $\alpha$ -amylase and/or native  $\beta$ -glucosidase. One mutant, S436, presented 1.4- to 2-fold increases in all extracellular enzymatic activities measured, when compared with the parent strain, pointing to a global improvement in protein secretion. Three other mutants exhibited 2- to 3-fold improvements in only one (S397, B390) or two (S466) of the measured activities.

A targeted genetic approach was also followed. Two homologs of the *Saccharomyces cerevisiae* *GAS1*, *AgGAS1A* (*AGL351W*) and *AgGAS1B* (*AGL352W*), were deleted from the *A. gossypii* genome. For both copies deletion, a new antibiotic marker cassette conferring resistance to phleomycin, BLE3, was constructed. *GAS1* encodes an  $\beta$ -1,3-glucanosyltransglycosylase involved in cell wall assembly. Higher permeability of the cell wall was expected to increase the protein secretion capacity. However, total protein secreted to culture supernatants and secreted EGI activity did not increase in the *Aggas1AΔ* mutants. Deletion of the *AgGAS1B* copy affected cellular morphology and resulted in severe retardation of growth, similarly to what has been reported for *GAS1*-deficient yeast. Thus, secretion could not be tested in these mutants.

## Introduction

Because of their natural ability to secrete high amounts of extracellular proteins, filamentous fungi have been extensively exploited for the production of homologous and heterologous proteins. Although homologous protein production can reach the level of hundreds of grams per liter, the production levels of heterologous proteins can be several orders of magnitude lower.<sup>1,2</sup> In the search to further improve the properties of fungi as protein producers, many strategies have been employed and optimized, including signal sequence optimization, use of strong promoters, co-expression of chaperones and foldases, genetic modifications which improve secretion capabilities (such as the knockout of specific genes or random mutagenesis), reduction of proteolytic activity,<sup>3</sup> optimization of fermentation conditions, among others.<sup>4–10</sup> Improvement of the secretion of extracellular enzymes by the application of random mutagenesis and screening has been successfully performed in various filamentous fungi.<sup>11,12</sup> Many of the high-secreting mutants isolated are currently used for industrial production of fungal enzymes and applied as expression hosts for recombinant gene products.<sup>13</sup>

It is a routine practice to develop mutants through random mutagenesis. Strain improvement by random mutagenesis is a successful method, but it is mainly a trial-and-error process, involving laborious procedures. Moreover, in many cases improved

performance is a black box, and the underlying mechanism is not easily identified.<sup>14</sup> The main effect of mutagenic agents (X-rays, UV-rays, nitrous acid, dimethyl sulphonate, ethyl methane sulfonate (EMS) and acridine mustards) relies on creating a lesion or a modification in the base sequence of the DNA molecule. If this lesion is not repaired a mutation is caused.<sup>15</sup> The nature of the mutagenic agent is such that changes are not directed exclusively at the loci which will generate beneficial change, requiring the screening of large numbers of strains for the desired phenotypes. Different methods have been used to introduce random mutations in different microorganisms,<sup>16</sup> including mutagenesis using nitrous acid, hydroxylamine, UV radiation, transposons and EMS.<sup>17</sup> Mutagenic procedures can be optimized in terms of type of mutagen, dose and temperature. EMS is an alkylating agent that induces point mutations by A-T transition to G-C.<sup>18</sup> This mutagenic agent has been used to increase glucose oxidase activity,<sup>19</sup> phytase,<sup>20</sup> pectinase,<sup>21</sup> catalase,<sup>19</sup> lipase,<sup>22</sup> laccase<sup>23</sup> and citric acid production in *Aspergillus niger*,<sup>24,25</sup> as well as to improve recombinant strains of *Saccharomyces cerevisiae*<sup>26</sup> and *Trichoderma viride*,<sup>27</sup> among others.

Secretion of a heterologous protein often represents a major bottleneck. Once a protein has been released out of the ER–Golgi system, it has to cross the cell wall. Mutations in genes involved in the construction and in the maintenance of the cell wall, such as *PMR1*, *SEC14*, *ERD1*, *MNN9* and *MNN10*, have in some cases been demonstrated to lead to supersecreting mutants

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in *S. cerevisiae* and other yeast.<sup>28</sup> In addition, the deletion of the cell wall cross-linking enzyme glycopospholipid-anchored surface GasIp led to an almost 7-fold increase in the level of human insulin-like growth factor 1 (hIGF1) in *S. cerevisiae*.<sup>29</sup> In *Pichia pastoris*, deletion of the *GAS1* homolog resulted in a supersecreting phenotype for the heterologous *Rhizopus oryzae* lipase, but had no effect on the secretion of recombinant human trypsinogen and albumin.<sup>30</sup> More recently, deletion of the *Zygosaccharomyces bailii* *GAS1* homolog almost doubled the amount of recombinant *Candida rugosa* lipase and *Yarrowia lipolytica* protease extracellular enzymatic activity, but no relevant effect was observed on recombinant human IL-1b secretion.<sup>31</sup>

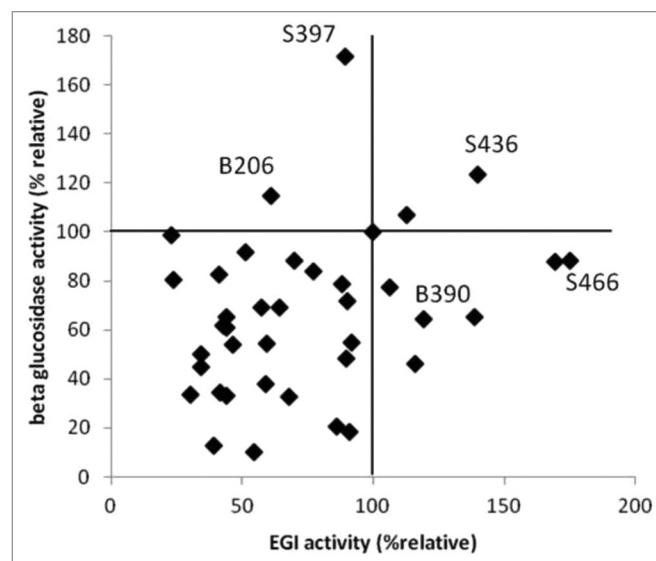
*Ashbya gossypii* is a filamentous hemiascomycete<sup>32,33</sup> with a natural capacity to produce riboflavin (vitamin B2),<sup>34,35</sup> which is used for the industrial production of this vitamin. The potential of *A. gossypii* as a host for recombinant protein production was previously evaluated by expressing cellobiohydrolase I (CBHI) and endoglucanase I (EGI) from *Trichoderma reesei*.<sup>36</sup> However, the secretion levels obtained were very low, particularly in the case of CBHI.<sup>36</sup> Here, we describe the improvement of the general secretion capability of *A. gossypii*. Two approaches were used: random mutagenesis with EMS and direct mutagenesis by individual and multiple deletion from the genome of both copies of the *GAS1* gene, *AgGAS1A* (*AGL351W*) and *AgGAS1B* (*AGL352W*).

## Results

**EMS treatment parameters for mutagenesis of recombinant *A. gossypii*.** Since *A. gossypii* spores cluster together in groups of needle shape spores difficult to separate, treatment with EMS resulted in highly variable numbers of colonies per plate after treatment. Between 46–100% lethality was obtained when using 5–20% (v/v) EMS during 1 and 2 h of incubation at room temperature. One hundred per cent mortality was recorded after 3 h of EMS treatment. Average killing rates of 50–90% were obtained after treatment with 5% (v/v) EMS for 90 min, which were considered to be suitable for mutant screening (data not shown).

**Mutant screening and characterization of putative *A. gossypii* mutants with improved secretion capacity.** Primary selection of hyperproducing mutants was done based on the diameter of the clearing zone surrounding the colony on the screening media containing 0.1% (w/v) carboxymethylcellulose (CMC) and G418 (200  $\mu\text{gml}^{-1}$ ). In addition to CMC, mutants were also screened on starch and tributyrin agar plates for  $\alpha$ -amylase and lipase activity, respectively. Screening on medium containing CMC gave a fairly reliable indication of increased cellulolytic activity, whereas screening on starch and tributyrin plates was less conclusive (data not shown).

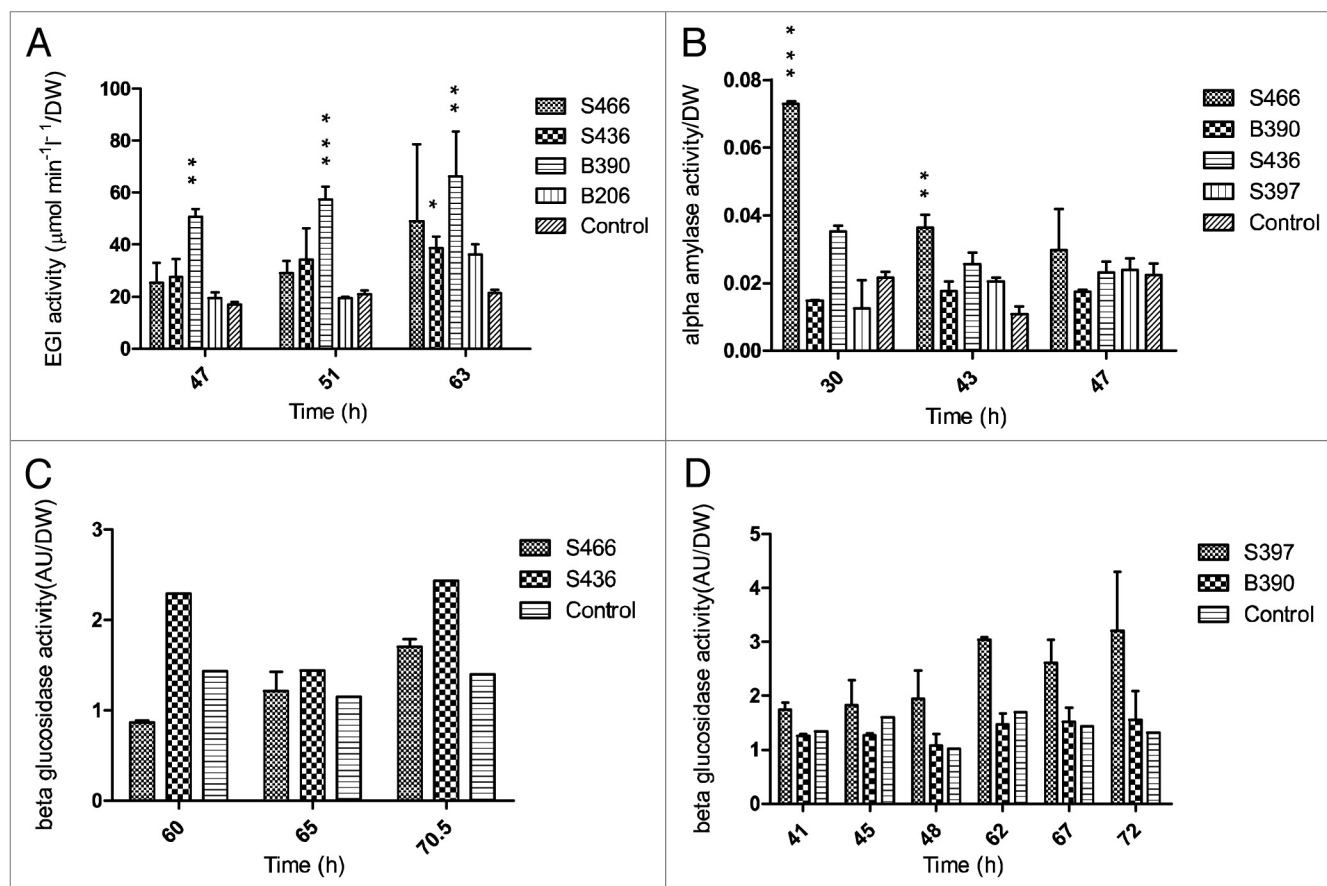
In the primary selection, approximately 1000 mutant colonies showing larger clearing zones than the control strain were isolated from the screening plates. Of these, the 39 mutants that presented the biggest haloes in the CMC plates were selected and enzyme production was determined in submerged cultures (Fig. 1). Comparison of the EGI and  $\beta$ -glucosidase extracellular activities between the control strain and the 39 selected mutants



**Figure 1.** EGI and  $\beta$ -glucosidase extracellular activity from 39 selected EMS treated *A. gossypii* mutants in small scale batch cultures. Activities are expressed in percentage relative to those of the parent strain ( $\text{U mg}^{-1}$  protein for  $\beta$ -glucosidase and  $\text{nkat mg}^{-1}$  protein for EGI activity). The mutants B206, B390, S397, S436 and S466 identified in the figure were selected for further analysis. Mutants B390, S436 and S466 showed improved EGI activity, whereas mutants B206, S397 and S436 presented increased  $\beta$ -glucosidase activity.

was made at several times after inoculation, to take into account differences in the lag phase or specific growth rate on enzyme production and/or secretion. The EGI and  $\beta$ -glucosidase specific activities (per gram of dry cell weight) quantified from the culture supernatants increased along the culture time reaching their maximum levels at the stationary phase (data not shown). From these 39 mutants, the most promising 5 were selected for further analysis, which showed increase in both EGI and  $\beta$ -glucosidase activity (mutant S436), only in EGI activity (mutants B390 and S466) or only in  $\beta$ -glucosidase activity (mutants B206 and S397) (Fig. 1).

The EGI,  $\alpha$ -amylase and  $\beta$ -glucosidase specific activities measured from the supernatants of the selected mutants at different cultivation times are depicted in Figure 2. There were no significant ( $p > 0.05$ ) differences in the specific growth rate of the mutants when compared with the parent strain. The specific growth rate varied between 0.09–0.16  $\text{h}^{-1}$  in AFM, which was in agreement with values reported for the recombinant *A. gossypii* VTT D-101398.<sup>36</sup> Three mutants presented enhanced EGI activity ( $p < 0.05$ ), with mutant B390 showing nearly a 3-fold increase and mutants S436 and S466 a 2-fold increase compared with the parent in flask culture after 63 h of growth (Fig. 2A). At this point all cultures had reached the stationary phase (data not shown). In comparison to the control strain, mutant S466 also presented increased  $\alpha$ -amylase activity ( $p < 0.01$ ) at the deceleration phase (after 30 h and 43 h of growth) and mutant S436 presented an increase in both  $\alpha$ -amylase and  $\beta$ -glucosidase activities as well (Fig. 2B and C). The mutant with the highest  $\beta$ -glucosidase activity was mutant S397, which produced 2-fold ( $p < 0.05$ )



**Figure 2.** Extracellular enzyme activity from *A. gossypii* mutants obtained by random mutagenesis. EGI (A),  $\alpha$ -amylase (B) and  $\beta$ -glucosidase (C and D) extracellular specific activities (per gram of dry weight) from *A. gossypii* EMS mutants and parent strain (control, VTT D-101398) in small scale batch cultures. Values represent the standard error of the mean of two independent experiments with triplicates (n = 6). Activities were analyzed with ANOVA using Graphpad from Prism5 and significant differences ( $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ ) are indicated by asterisks (\*, \*\* and \*\*\*, respectively).

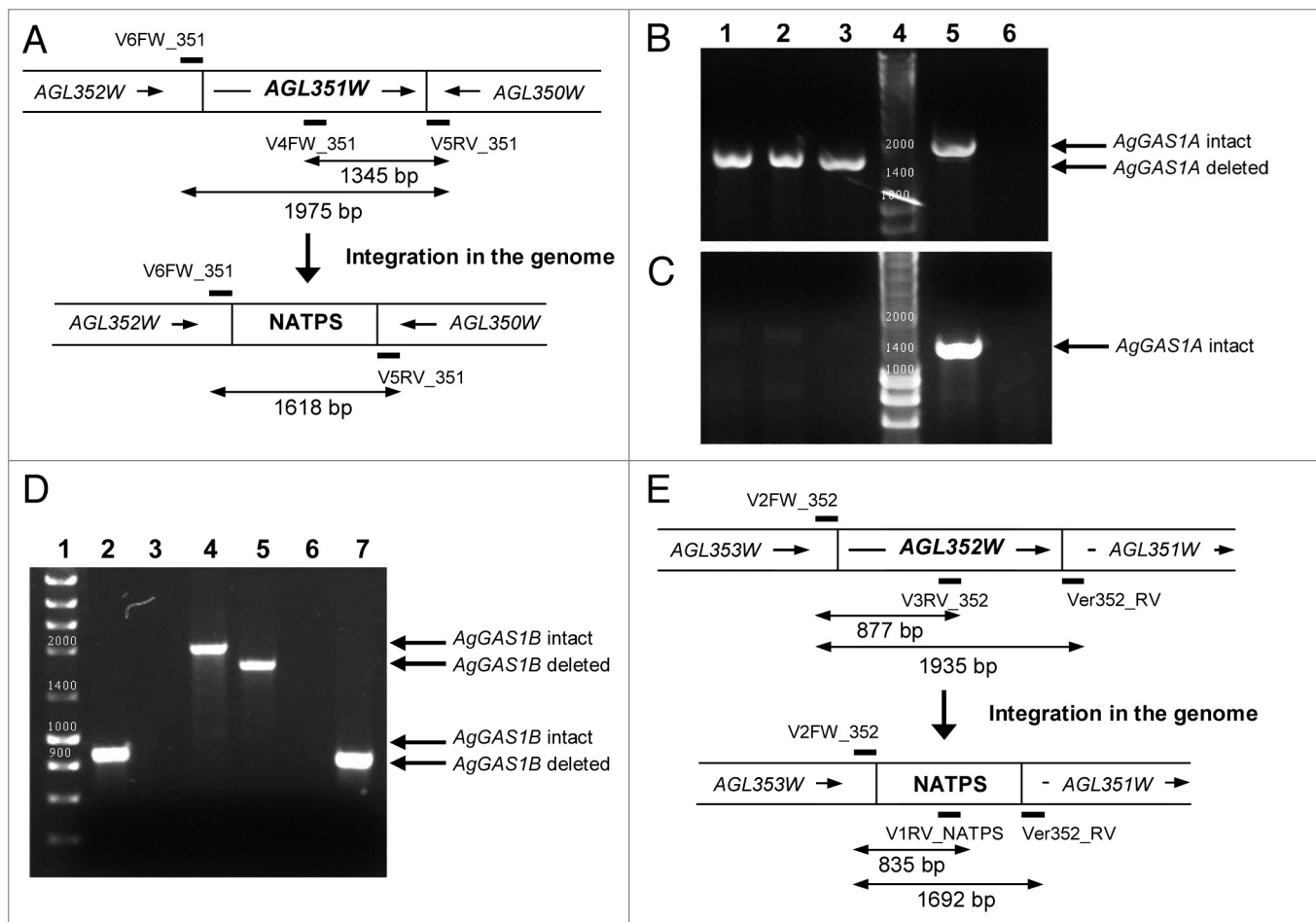
more compared with the non-mutagenized strain (Fig. 2D). Thus, four mutants were isolated with improved extracellular enzyme activity when compared with the parent strain.

**Effect of *AgGAS1* gene deletion on recombinant protein production.** For the inactivation of *A. gossypii* ATCC10895 *AgGAS1A* and *AgGAS1B*, the entire open reading frames *AGL351W* and *AGL352W* were replaced with the NATPS cassette flanked by 66 bp and 67 bp upstream and downstream of *AGL351W* and *AGL352W* sequences, to enable homologous recombination and genomic insertion using a PCR strategy.<sup>37</sup> As can be seen in Figure 3, *AgGAS1A* elimination was confirmed in three mutants. The expected size fragments (Fig. 3B) or no amplification (Fig. 3C), were obtained. The mutants did not show any visible phenotype variation in comparison to the parent strain when grown on solid or in liquid AFM with glucose (Fig. 4A and B). However, microscopically it was observed that their mycelia presented more vacuoles than the parent strain mycelia (Fig. 4E and F).

Elimination of the *AgGAS1B* was also accomplished, as can be seen by the expected sizes of the products obtained from the verification PCR (Fig. 3D and E). The resulting mutants exhibited an extremely slow growth rate on solid AFM medium (Fig. 4C and D) and altered morphology (Fig. 4G and H), even

on plates containing 1 M sorbitol as osmotic support. Moreover, the heterokaryotic *Aggas1A* $\Delta$ *gas1B* $\Delta$  double mutant also presented defects in sporulation. The mycelia of the *Aggas1B* $\Delta$  single mutant (Fig. 4G) presented granular cytoplasm and higher number of vacuoles than the parent strain (Fig. 4E). These were also bigger than the vacuoles observed in the mycelia from the parent strain. Similarly, intracellular granules and altered vacuoles were also visible in the heterokaryotic *Aggas1A* $\Delta$ *gas1B* $\Delta$  mutant (Fig. 4H), for which it was observed that a large fraction of cells underwent lysis even in the presence of an osmotic stabilizer. The extremely slow growth rates presented by the *Aggas1B* $\Delta$  null mutants imposed several experimental and processing constraints, hampering the evaluation of the effect of *AgGAS1B* deletion on protein secretion.

The *S. cerevisiae ADH1* terminator has been reported to display autonomous replicating sequence activity in *A. gossypii*.<sup>38</sup> In this study, the modification of the plasmid pMI519, which also included the removal of the *S. cerevisiae ADH1* terminator sequence present, resulted in a 2-fold increase in the extracellular EGI activity, when compared with the strains expressing EGI from the original pMI519 plasmid (VTT D-101398 strain) (Fig. 5). Therefore, the modified pMI519 plasmid was chosen to be introduced in the *A. gossypii* NQ89 strain to assess the effect of the *AgGAS1A* deletion in the production of *T. reesei* EGI. Two



**Figure 3.** PCR confirmation of *GAS1* deletion in *A. gossypii*. **(A)** Schematic representation with the location of the primers used for verification of *AgGAS1A* gene deletion and corresponding amplicon sizes. **(B)** PCR with primers V6FW\_351 and V5RV\_351 was made using as template: lanes 1, 2 and 3 genomic DNA (gDNA) from homokaryotic transformants (predicted fragment size of 1618 bp); lane 5 parental gDNA (predicted fragment size of 1975 bp). Lane 4 molecular marker (NZYTech) and lane 6 negative control. **(C)** PCR with primers V4FW\_351 and V5RV\_351 was made using as template: lanes 1, 2 and 3 gDNA from homokaryotic transformants (no amplification); and lane 5 parental gDNA (predicted fragment size of 1345 bp). Lane 4 molecular marker (NZYTech) and lane 6 negative control. **(D)** Lane 1 molecular marker (NZYTech). Lanes 2, 4 and 6 parental gDNA respectively amplified with primers: V2FW\_352 and V3RV\_352 (predicted fragment size of 877 bp for the parent and no amplification in the deleted strain), V2FW\_352 and Ver352\_RV (predicted fragment size of 1935 bp for the parent and 1692 bp when the deletion cassette has been integrated in the genome), and V2FW\_352 and V1RV\_NATPS (predicted fragment size of 835 bp for the deleted strain and no amplification in the parental strain). Lanes 3, 5 and 7 correspond to gDNA from a homokaryotic *Agga1BΔ* mutant amplified with the same sets of primers used with the parental strain. **(E)** Schematic representation with the location of the primers used for verification of *AgGAS1B* deletion and corresponding amplicon sizes.

*Aggas1AΔ* transformants expressing EGI and the negative control containing the empty vector were grown in 250 ml flasks in AFM supplemented with G418, in order to evaluate total extracellular EGI protein production. The amount of total protein secreted into the culture medium was very similar between the mutant (61–440 mg ml<sup>-1</sup>) and the parent VTT D-101398 strain (66–377 mg ml<sup>-1</sup>). EGI activity, measured with the fluorogenic substrate MULac from the two *Aggas1AΔ* transformants and from the parent EGI strain containing the modified vector did not differ significantly ( $p > 0.05$ ).

## Discussion

Several mutants of *A. gossypii* have been generated using UV radiation and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)

mutagenesis with the goal of increasing riboflavin production.<sup>39,40</sup> Although there are numerous studies on the application of EMS to induce mutagenesis in other microorganisms, this is the first time that *A. gossypii* secretion mutants have been produced by means of EMS mutagenesis.

EMS treatment was effective in generating *A. gossypii* mutants with improved extracellular enzymatic activities (Figs. 1 and 2), even though only 50–90% of killing was obtained after 90 min of incubation at room temperature with 5% (v/v) EMS. As no significant differences were observed between the growth of the parent and of the isolated mutant strains, the enhanced extracellular enzyme production by the mutants was not due to an increase in growth, but to an improvement in protein production and/or secretion. Three mutants were isolated with enhanced EGI extracellular activity (Fig. 2A). One of these mutants (S436)

also presented increased  $\alpha$ -amylase and  $\beta$ -glucosidase extracellular activities, as compared with the parent strain (Fig. 2B and C), indicating that the mutation(s) affected the secretory pathway in general. Other mutant (S466) showed a 3-fold increased extracellular  $\alpha$ -amylase activity as well, whereas mutant B390 only presented increased EGI activity. Additionally, one mutant was isolated which did not have improved EGI activity, but which produced more extracellular  $\beta$ -glucosidase (S397). In mutants B390, S397 and S466, as the mutation(s) only affected the production and/or secretion of some enzymes, the activity increase observed corresponds to a specific event which may not be associated with an improvement on the secretory pathway per se, but be protein specific.

Mutant S436, with 1.4- to 2-fold improved secreted activity (Fig. 2), would be useful for further development of *A. gossypii* protein production. 2-fold improvements in secreted hydrolytic activity are generally observed for other mutants obtained by random mutagenesis. For example, using different mutagens (ethidium bromide, NNMG and UV) to treat *Trichoderma* sp. and *Aspergillus* sp. spores, Chand et al.<sup>41</sup> reported 1.9- to 2.9-fold increase in cellulase production. Dillon et al.<sup>42</sup> reported 1.5-fold more cellulase productivity in *Penicillium echinulatum* by three repeated mutagenic treatment steps with UV radiation. *T. viride* TL-124 was subjected to successive mutagenic treatments, to obtain mutants with 2- to 2.6-fold increased cellulase production relative to the wild type strain.<sup>43</sup> *Aspergillus* sp. has been improved for cellulase production by sequential treatments with  $\gamma$  radiation, UV radiation and NNMG to obtain improved mutants which produced 2- to 3-fold improvements in cellulolytic activity.<sup>44</sup> In this regard, the range of improvement obtained here is similar to what has been obtained in other microorganisms, although secretion is low compared with e.g., *Trichoderma*.

The low secretory ability of *A. gossypii* made screening for secretory mutants difficult. The difficulty of rapidly screening for cellulase hyperproducing mutants of *Neurospora* and *Trichoderma* has previously been noted.<sup>45</sup> For *A. gossypii*, the haloes in agar plate assays were small and affected by the depth of agar and size of fungal colony. Germination time following mutagenesis was variable and thus colonies of different sizes needed to be compared. It was also not practical to restrict colony expansion by inclusion of agents such as Tween 20 or Triton X100, which have been used with *T. reesei*,<sup>9</sup> because they inhibited *A. gossypii* spores germination. Nonetheless, the screening method used did allow direct selection of hyperproducing mutants of the target protein from the colonies after mutagenesis, even though false positives were also isolated (Figs. 1 and 2).

Targeted genetic modification, by deletion of the *A. gossypii* *GAS1* homologs, was less successful than random mutagenesis. *GAS1* was chosen as a target for *A. gossypii* strain improvement because in yeast it encodes a cross-linking enzyme known to affect the structure and permeability of the cell wall. Cell wall defective strains deleted in the *GAS1* gene of *S. cerevisiae*,<sup>29</sup> *P. pastoris*<sup>30</sup> and *Z. bailii*<sup>31</sup> have been reported as super-secreting. Deletion of *GAS1* in *S. cerevisiae* resulted in an almost 7-fold increase in the level of human hIGF1.<sup>29</sup> Similarly, secreted enzymatic activity was almost doubled when *GAS1* was deleted in

*Z. bailii* producing *C. rugosa* lipase Crl1p or *Y. lipolytica* protease Xpr2p.<sup>31</sup> In *A. fumigatus*, an increase in glycoprotein secretion has also been reported for *gel2* $\Delta$  mutants.<sup>46</sup>

Since phylogenetically *A. gossypii* is closely related to *S. cerevisiae*<sup>33</sup> and both show similar secretion characteristics,<sup>36</sup> deletion of its *GAS1* homologs might have been expected to result in similar improvements in secretion. However, the deletion of *AgGAS1A* from *A. gossypii* genome did not cause any effect on the capacity of *A. gossypii* to secrete heterologous EGI (Fig. 4) or native  $\alpha$ -amylase (data not shown). Unlike in *S. cerevisiae*, there are two tandem copies of the *GAS1* gene in the *A. gossypii* genome. Here we have only tested the effect of the *AgGAS1A* deletion on protein secretion, because both *Aggas1B* $\Delta$  and *Aggas1A* $\Delta$ *gas1B* $\Delta$  mutants exhibited severe limitations in terms of growth. Therefore, there may still be sufficient protein in the wall of the *Aggas1A* $\Delta$  mutant to explain the lack of improved secretion. It is worth noting that also in *P. pastoris* the disruption of *GAS1* had no effect on the secretion of human trypsinogen or human serum albumin, although the amount of *R. oryzae* lipase released from the cells was doubled.<sup>31</sup> Similarly, *GAS1* deletion in *Z. bailii* did not increase human IL-1b secretion.<sup>32</sup> Improvements in *GAS1* deletion strains have been protein dependent and EGI is not a readily secreted protein. Altogether, this may have contributed to the lack of improvement.

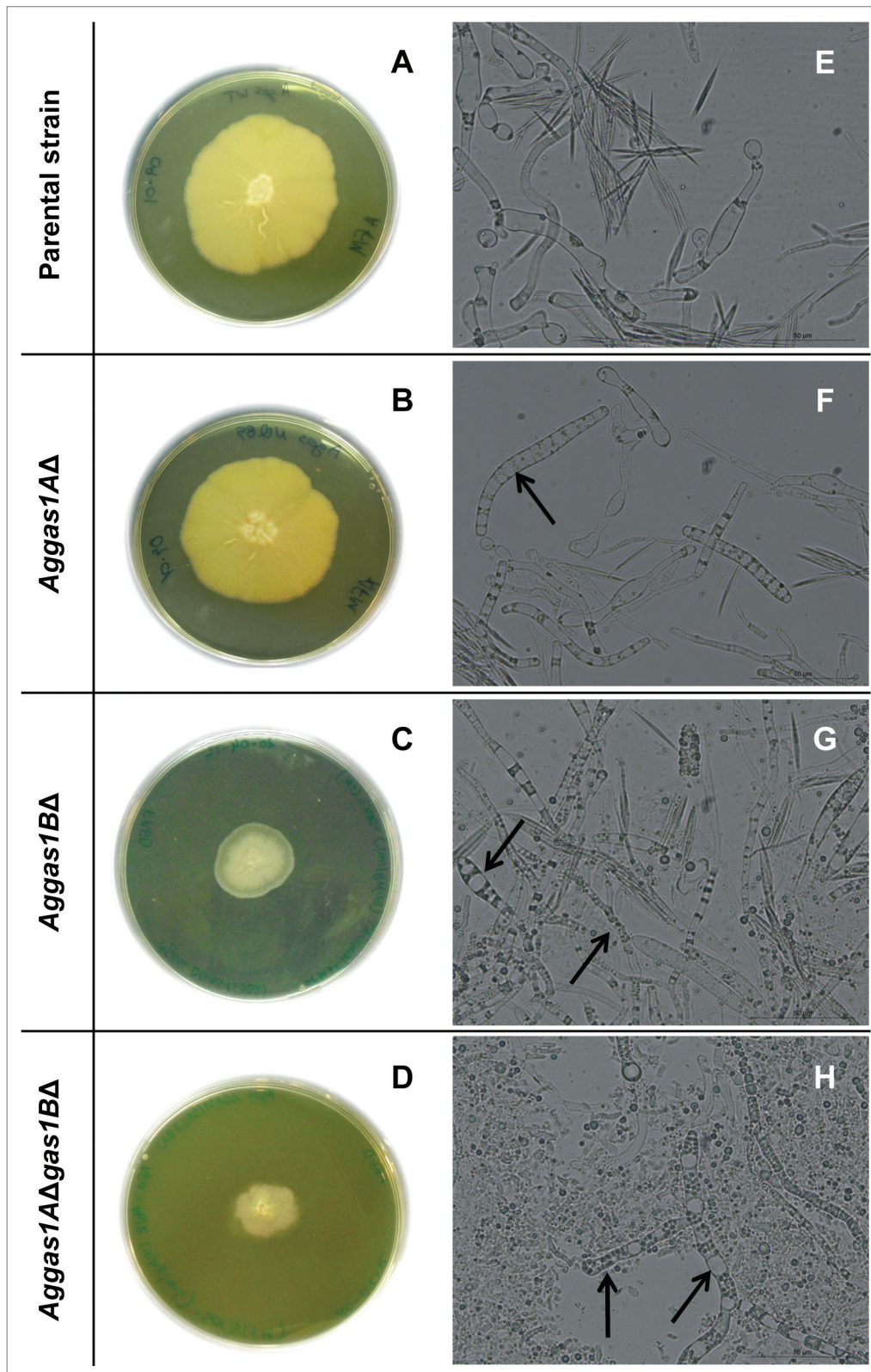
The inactivation of the *AgGAS1* homologs affected *A. gossypii* morphology and growth rate, as already reported for other *GAS1*-deficient organisms. Similarly to what has been observed in yeast,<sup>29,30,32,47</sup> *A. gossypii* *Aggas1B* $\Delta$  mutants exhibited severe growth retardation and abnormal cells with granular cytoplasm and big vacuoles. Moreover, the *Aggas1A* $\Delta$ *gas1B* $\Delta$  double mutant presented sporulation deficiency and a high percentage of cellular lysis even when osmotic support was present. In *A. fumigatus*, abnormal conidiogenesis and slower growth were observed when *GEL2*, a ortholog of the *GAS* gene family, was deleted.<sup>46</sup> These peculiar morphological alterations have been associated with altered structure and composition of the mutants cell wall.<sup>32,46,47</sup>

The major output of this study was one mutant with overall improved secretion capability obtained by random mutagenesis. Moreover, a new antibiotic marker cassette, BLE3, consisting of the *ble*<sup>r</sup> gene from the bacterial transposon Tn5 flanked by the promoter and terminator sequences of the *S. cerevisiae* *TEF2* gene, has also been constructed for use in *A. gossypii* gene deletions, allowing selection of phleomycin-resistant transformants. Targeted deletion of *AgGAS1A* had no significant effect on the overall *A. gossypii* secretion ability and the deletion of *AgGAS1B* led to defects in cellular growth, morphology and sporulation.

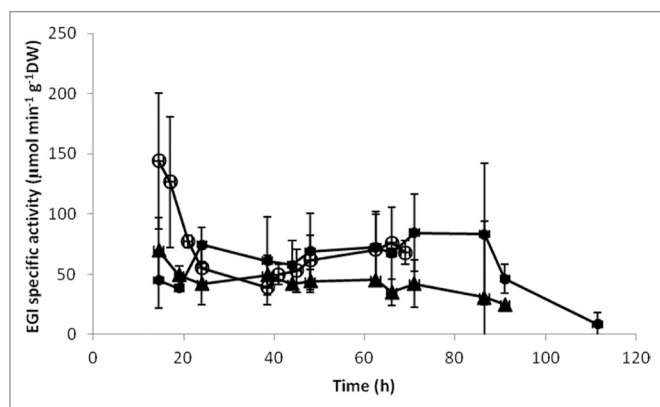
## Materials and Methods

**Strains.** Recombinant *A. gossypii* VTT D-101398 expressing EGI from *T. reesei*, constructed previously,<sup>36</sup> was used throughout this study. *A. gossypii* ATCC10895 (kindly provided by Prof P Philippsen from Basel) was used for gene deletion experiments.

**Media.** *Asbyya* Full Medium (AFM; 10 g l<sup>-1</sup> peptone, 10 g l<sup>-1</sup> yeast extract, 1 g l<sup>-1</sup> myo-inositol plus a carbon source) was used for growth of *A. gossypii*. Starch (10 g l<sup>-1</sup>), carboxymethylcellulose



**Figure 4.** Colony and cellular morphology of *A. gossypii* parental and *gas1* null mutant strains. From (A) to (D), *A. gossypii* parental and *gas1* null mutant colonies are shown, which were inoculated in the middle of AFM plus glucose plates containing 1 M sorbitol and grown for one week at 30°C. Panels (E) to (H) show microscope images of *A. gossypii* parental and *gas1* null mutant mycelia and needle shaped spores harvested from the center of the colonies on the right. Arrows indicate granules and enlarged vacuoles in mutant hyphae. In panel (H), extensive cellular lysis is observed.



**Figure 5.** Average EGI activity from supernatant of recombinant *A. gossypii* growing in flasks with AFM plus glucose at 30°C and 200 rpm. Activities were measured with the MULac assay, described in materials and methods. Data represent average  $\pm$  standard error of the mean obtained from three independent cultures. *Aggas1A* $\Delta$  mutant strain (NQ89) expressing EGI from the modified pMI519 plasmid (open circle), parent strain expressing EGI from the modified pMI519 plasmid (closed circle) and parent strain expressing EGI from the original pMI519 plasmid (closed triangle).

(CMC) (1 g l<sup>-1</sup>) or tributyrin (10 ml l<sup>-1</sup>) were provided as carbon sources for mutants screening. Media were solidified with agar (20 g l<sup>-1</sup>) and when indicated 1 M sorbitol was added to AFM plates. Glucose (20 g l<sup>-1</sup>) was the carbon source used for liquid cultures.

**Mutagenesis and selection.** Mutations were induced in *A. gossypii* VTT D-101398<sup>36</sup> with EMS. Several concentrations of EMS and different incubation periods were tested to determine a treatment suitable for 90–99% killing. Based on the results, mutagenesis was subsequently performed by diluting a spore suspension of *A. gossypii* ten times in sodium phosphate buffer and incubating with 5% (v/v) EMS for 90 min at room temperature, with shaking. Mutagenized spores were diluted in 0.9% (w/v) NaCl and plated on agar-solidified AFM containing 0.1% (w/v) CMC plus 200  $\mu$ g ml<sup>-1</sup> G418 (Sigma) in order to keep the plasmid containing the recombinant EGI. A control was treated with water instead of EMS. Colonies appeared after 3 d.

For rapid screening of EGI production, mutants and the control strain of *A. gossypii* were incubated for 48 h on agar medium containing 0.1% (w/v) CMC as the sole carbon source and then flooded with 1% (w/v) congo red solution according to the method of Teather and Wood.<sup>48</sup> From this procedure, zones of CMC hydrolysis were clearly visible indicating cellulase production and its activity on the substrate (CMC). Mutants were selected on the basis of clearing zone size after the congo red treatment. Before flooding the plate with congo red, colonies were transferred to agar-solidified AFM plus G418, with glucose as carbon source, in order to store the putative mutants. Selected mutants and *A. gossypii* VTT D-101398 were further assessed for cell growth and production of extracellular enzymes in AFM containing either CMC 0.1% (w/v), starch (10 g l<sup>-1</sup>) or tributyrin (10 ml l<sup>-1</sup>).

**Culture conditions.** Spores were prepared by collecting 8–10 d old mycelia from agar-solidified medium, digesting with

zymolyase (75 mg ml<sup>-1</sup>) for 2 h and washing with a solution of 0.8% (w/v) NaCl, 20% (v/v) glycerol and 0.025% (v/v) Tween 20.

Submerged cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml AFM with glucose and 200  $\mu$ g ml<sup>-1</sup> G418 (Sigma) and incubated at 30°C, 200 rpm. Flasks were inoculated with spores which had been stored at -80°C. Dry cell weight was determined as previously described.<sup>36</sup>

**Protein and enzymatic assays.** *Endoglucanase I (EGI).* Endoglucanase I (EGI) activity was assayed as described previously<sup>36</sup> by using 4-methylumbelliferyl- $\beta$ -D-lactoside (MULac, Sigma) as substrate.

$\beta$ -glucosidase activity was assayed according to the method described by Bailey and Nevalainen<sup>11</sup> with 1 mM 4-nitrophenyl- $\beta$ -D-glucopyranoside (Sigma) as substrate.

$\alpha$ -amylase. The production of starch degrading enzymes on agar-solidified medium was detected by staining the surface with iodine solution<sup>49</sup> to visualize zones in which starch had been broken down. Amylase activity by mutants exhibiting the largest hydrolysis haloes were then tested in liquid cultures. Alpha-amylase was measured by incubating 0.5 ml supernatant at 25°C for 3–4 min to achieve temperature equilibration, after which 0.5 ml of 1% (w/v) starch solution (at 25°C) was added. After 3 min of incubation, 1 ml dinitrosalicylic acid color reagent was added. Samples were incubated in a boiling water bath for 5 min. After cooling to room temperature, 1 ml of water was added, samples were mixed and absorbance at 540 nm read vs. a blank. Maltose release was determined from a standard curve. One unit was defined as the amount of enzyme that released from soluble starch one micromole of reducing groups (calculated as maltose) per minute at 25°C and pH 6.9 under the specified conditions.

*Lipase.* Lipase was detected by growing the fungus on agar solidified tributyrin AFM. Degradation of this compound gives rise to clear zones surrounding the lipolytic colonies in the otherwise turbid culture medium.

*Protein content.* The protein concentration present in the supernatant was determined by the Bradford assay using bovine serum albumin (BSA; Sigma) as the standard.

***GAS1*-homolog genes deletion from the *A. gossypii* genome.** In the *A. gossypii* genome, *GAS1* is present as a tandem gene duplication, namely *AgGAS1A* (*AGL351W*) and *AgGAS1B* (*AGL352W*). In this study, *A. gossypii gas1* null mutants were created using a PCR-based, one-step gene targeting approach.<sup>37,50</sup>

For individual gene deletion, the disruption cassettes containing the NATPS marker, which confers resistance to nourseothricin/clonNAT, were amplified by PCR from pUC19NATPS (Hoepfner D, personal communication), using the primers 351FW and 351RV and 352FW and 352RV (Table 1). These primers were designed to have 66 bp and 67 bp flanking regions corresponding to the 5' and 3' flanking regions of *AgGAS1A* and *AgGAS1B*, respectively. Phusion® High-Fidelity DNA Polymerase (Finnzymes) was used and the PCR conditions were 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 50°C for 30 sec, 72°C for 1 min and a final step of 72°C for 10 min. The amplified deletion modules were purified using the QIAquick PCR purification kit (Qiagen) and used to transform *A. gossypii* ATCC 10895 mycelia

**Table 1.** Oligonucleotides used in this study

| Primer name     | Sequence   |
|-----------------|--|
| 351FW           | 5' GTT CAG TGG ACA CGC ATT CAA ACA ATC GTG AGG TTT GCG GAG CTT TGA ATT TGG TTG AAA ATC GTA gtt acg gta ttt tac tat gg<br>3'                    |
| 351RV           | 5' GTA CAT TTC TTT AAG CTA AAT GTG TCA AAG CTT GAG GCG ACA ATA GAG TGC TCA AGA CAT AGC TAT Gca aca gtg ttc ctt aat<br>caa gg 3'                |
| 352FW           | 5' TAA GTT GGG GCC TTG CGC ATA TTC GGA AGG TAA CAG CAG TCG CTA TAT TTG TCA ATC GCT ATG TTA TTC AAT AAG CTA GCC<br>ctg cag aac cgt tac ggt a 3' |
| 352RV           | 5' AAA GAA CAT AGA GCT TCG CCT TAT AGA GTA ATA TAG AAG AGG CTG CAT GAA AAT CTA TTT TTA AAT GAA GAT TAG CCC GAc<br>ctg cag cca aac agt gtt 3'   |
| GEN3_FW         | 5' GCT AGG GAT AAC AGG GTA AT 3'   |
| GEN3_XhoI_RV    | 5' CCG CTC GAG GAT CTG ATG AGG CCG TCT 3'  |
| ScTEFt_EcoRI_FW | 5' CGG AAT TCT CGA TGA GTT TTT CTA AGA G 3'  |
| ScTEFp_NcoI_RV  | 5' TCT ATT CCA TGG TAA TTA TAG TTC GTT GAC CG 3'   |
| Ble_FW          | 5' GCA GGT CGA CAA CCC TTA AT 3'   |
| Ble_EcoRI_RV    | 5' CGG AAT TCT CAT GAG ATG CCT GCA AG 3'   |
| 352BleFW        | 5' TAA GTT GGG GCC TTG CGC ATA TTC GGA AGG TAA CAG CAG TCG CTA TAT TTG TCA ATC GCT ATG TTA TTC AAT AAG CTA GCg<br>cag gtc gac aac cct taa t 3' |
| 352BleRV        | 5' AAA GAA CAT AGA GCT TCG CCT TAT AGA GTA ATA TAG AAG AGG CTG CAT GAA AAT CTA TTT TTA AAT GAA GAT TAG CCC GAg<br>cat agg cca cta gtg gat c 3' |
| V6FW_351        | 5' CTA GTG GCA TGT CTC GAG 3'  |
| V4FW_351        | 5' GCA GGT GTG GTT GAC GAA ATG CAG 3'  |
| V5RV_351        | 5' GCA GAT GAA GTG CAC ATC TTA CTG C 3'  |
| V2FW_352        | 5' CAA CGA TCC CGT CTG TCA TTG 3'  |
| V3RV_352        | 5' GTA GAC GTT GAA GCC GTA GAA GTC AG 3'   |
| Ver352_RV       | 5' GCT GGA CTT TGG TCA TAG CC 3'   |
| V1RV_NATPS      | 5' GAT TCG TCG TCC GAT TCG TC 3'   |

Lower case letters indicate the sequences complementary to the template

by electroporation, as previously described.<sup>37</sup> Transformants were selected on AFM agar containing 100  $\mu\text{g ml}^{-1}$  clonNAT (WERNER BioAgent).

The *A. gossypii* *Aggas1AΔ* homokaryotic strain was named *A. gossypii* NQ89. As this strain was resistant to clonNAT, the cassette used for the deletion of the second *AgGAS1* copy (*AgGAS1B*) conferred resistance to phleomycin (*ble<sup>r</sup>*). Plasmid pUGGEN3 was created by amplification of the GEN3 module from pGEN3<sup>37</sup> with the primers GEN3\_FW and GEN3\_XhoI\_RV (Table 1), which was digested with *Bgl*III and *Xho*I (New England BioLabs) and ligated to pUG66<sup>51</sup> digested with the same enzymes. Using the primers ScTEFt\_EcoRI\_FW and ScTEFp\_NcoI\_RV (Table 1), all of the pUGGEN3 sequence, with the exception of the *kan<sup>r</sup>* gene, was amplified with the Phusion® High-Fidelity DNA Polymerase (Finnzymes) as described above. The *ble<sup>r</sup>* gene was obtained by PCR from plasmid pUG66 with the primers Ble\_FW and Ble\_EcoRI\_RV (Table 1). Both PCR fragments were digested with *Eco*RI and *Nco*I (New England BioLabs), and ligation was performed with T4 DNA Ligase (Promega) according to the manufacturer instructions. The resulting plasmid was named pUGBLE3.

The deletion cassette BLE3 (resistance module for phleomycin) used for the elimination of the second copy of *AgGAS1*,

*AgGAS1B*, was obtained by PCR from the plasmid pUGBLE3 with the primers 352BleFW and 352BleRV (Table 1). The deletion modules were used to transform *A. gossypii* NQ89 mycelia by electroporation as previously described.<sup>37</sup> Transformants were selected on AFM agar containing 10  $\mu\text{g ml}^{-1}$  phleomycin (InvivoGen).

Transformation of multinucleate mycelia leads to heterokaryotic mutants, which contain a mixture of transformed and parental nuclei. Clonal selection of homokaryotic mutants was achieved by isolating and growing single germinating spores.

**Confirmation of *GAS1*-homolog genes deletion from the *A. gossypii* genome.** Correct integration of the disruption cassette on the *AgGAS1A* region was verified by analytical PCR with the primers V6FW\_351 and V5RV\_351, which anneal outside the deleted gene, and V4FW\_351 and V5RV\_351, which anneal inside the deleted region (Table 1; Figure 3A).

The single deletion of *AgGAS1B* was confirmed by PCR using following primers (Table 1; Figure 3E): V2FW\_352 and V3RV\_352, which anneal inside the deleted region; V2FW\_352 and Ver352\_RV, annealing outside of the deleted region; and V2FW\_352 and V1RV\_NATPS, which anneal outside of the deleted region and in the middle of the NATPS module, respectively.



Microscope images were taken by a Leica DMI3000 B inverted microscope, using the Leica Application Suite (LAS) software v4.0.

**Modified EGI expression plasmid construction.** In order to evaluate the production of EGI by the *Aggas1Δ* null mutant, one isolate, NQ89, was transformed with an EGI expression plasmid, or with the empty vector, pMI516<sup>36</sup> as negative control. The expression plasmid pMI519<sup>36</sup> containing EGI from *T. reesei* was modified by replacing the kanMX module, where *kan<sup>r</sup>* gene was flanked by the *A. gossypii* *TEF1* promoter and *S. cerevisiae* *ADHI* terminator, with the GEN3 module, in which the *kan<sup>r</sup>* gene is under the *S. cerevisiae* *TEF2* promoter and terminator. The kanMX cassette together with the *URA3* selection marker were

excised from pMI519 with *Bgl*II (NZYTech) and substituted with the GEN3 module obtained from pGEN3<sup>37</sup> digestion with the same enzyme. This plasmid is referred here as modified pMI519.

#### Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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#### References

- Punt PJ, van Biezen N, Conesa A, Albers A, Mangnus J, van den Hondel CA. Filamentous fungi as cell factories for heterologous protein production. *Trends Biotechnol* 2002; 20:200-6; PMID:11943375; [http://dx.doi.org/10.1016/S0167-7799\(02\)01933-9](http://dx.doi.org/10.1016/S0167-7799(02)01933-9).
- Cherry JR, Fidantsef AL. Directed evolution of industrial enzymes: an update. *Curr Opin Biotechnol* 2003; 14:438-43; PMID:12943855; [http://dx.doi.org/10.1016/S0958-1669\(03\)00099-5](http://dx.doi.org/10.1016/S0958-1669(03)00099-5).
- Chow TY, Ash JJ, Dignard D, Thomas DY. Screening and identification of a gene, PSE-1, that affects protein secretion in *Saccharomyces cerevisiae*. *J Cell Sci* 1992; 101:709-19; PMID:1522152.
- Jeenes DJ, Mackenzie DA, Roberts IN, Archer DB. Heterologous protein production by filamentous fungi. *Biotechnol Genet Eng Rev* 1991; 9:327-67; PMID:1839127.
- Archer DB, Jeenes DJ, Mackenzie DA. Strategies for improving heterologous protein production from filamentous fungi. *Antonie Van Leeuwenhoek* 1994; 65:245-50; PMID:7847891; <http://dx.doi.org/10.1007/BF00871952>.
- Punt PJ, Veldhuis G, van den Hondel CA. Protein targeting and secretion in filamentous fungi. A progress report. *Antonie Van Leeuwenhoek* 1994; 65:211-6; PMID:7847888; <http://dx.doi.org/10.1007/BF00871949>.
- Gouka RJ, Punt PJ, van den Hondel CA. Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects. *Appl Microbiol Biotechnol* 1997; 47:1-11; PMID:9035405; <http://dx.doi.org/10.1007/s002530050880>.
- Nemoto T, Maruyama J, Kitamoto K. Improvement of heterologous protein production in *Aspergillus oryzae* by RNA interference with alpha-amylase genes. *Biosci Biotechnol Biochem* 2009; 73:2370-3; PMID:19897917; <http://dx.doi.org/10.1271/bbb.90177>.
- Nakari-Setälä T, Paloheimo M, Kallio J, Vehmaanperä J, Penttilä M, Saloheimo M. Genetic modification of carbon catabolite repression in *Trichoderma reesei* for improved protein production. *Appl Environ Microbiol* 2009; 75:4853-60; PMID:19447952; <http://dx.doi.org/10.1128/AEM.00282-09>.
- Meyer VRA, Punt PJ. Genetics, genetic manipulation and approaches to strain improvement of filamentous fungi. In: Demain AL DJe, ed. *Manual of industrial microbiology and biotechnology*. New York: Wiley, 2010:318-29.
- Bailey MJ, Nevalainen KMH. Induction, isolation and testing of stable *Trichoderma reesei* mutants with improved production of solubilizing cellulase. *Enzyme Microb Technol* 1981; 3:153-7; [http://dx.doi.org/10.1016/0141-0229\(81\)90076-4](http://dx.doi.org/10.1016/0141-0229(81)90076-4).
- Mattern IE, van Noort JM, van den Berg P, Archer DB, Roberts IN, van den Hondel CA. Isolation and characterization of mutants of *Aspergillus niger* deficient in extracellular proteases. *Mol Gen Genet* 1992; 234:332-6; PMID:1508158; <http://dx.doi.org/10.1007/BF00283855>.
- Mäntylä A, Paloheimo M, Suominen P. Industrial mutants and recombinant strains of *Trichoderma reesei*. In: Kubicek CR, Harman GR (eds.), *Trichoderma and Gliocladium* vol.2. London: Taylor and Francis Ltd, 1998:291-309.
- Baker HV. GCR1 of *Saccharomyces cerevisiae* encodes a DNA binding protein whose binding is abolished by mutations in the CTTCC sequence motif. *Proc Natl Acad Sci U S A* 1991; 88:9443-7; PMID:1946357; <http://dx.doi.org/10.1073/pnas.88.21.9443>.
- Devchand M, Gwynne DI. Expression of heterologous proteins in *Aspergillus*. *J Biotechnol* 1991; 17:3-9; PMID:1367014; [http://dx.doi.org/10.1016/0168-1656\(91\)90022-N](http://dx.doi.org/10.1016/0168-1656(91)90022-N).
- Takahashi T, Shimoi H, Ito K. Identification of genes required for growth under ethanol stress using transposon mutagenesis in *Saccharomyces cerevisiae*. *Mol Genet Genomics* 2001; 265:1112-9; PMID:11523784; <http://dx.doi.org/10.1007/s004380100510>.
- Walton C, Booth RK, Stockley PG. Random chemical mutagenesis and the non-selective isolation of mutated DNA sequences in vitro. In: McPherson MJ (ed.), *Directed mutagenesis: A practical approach*. Oxford: Oxford University Press, 1991:135-62.
- French CT, Ross CD, Keysar SB, Joshi DD, Lim CU, Fox MH. Comparison of the mutagenic potential of 17 physical and chemical agents analyzed by the flow cytometry mutation assay. *Mutat Res* 2006; 602:14-25; PMID:17045307; <http://dx.doi.org/10.1016/j.mrfmmm.2006.07.009>.
- Fiedurek J, Gromada A. Selection of biochemical mutants of *Aspergillus niger* with enhanced catalase production. *Appl Microbiol Biotechnol* 1997; 47:313-6; PMID:9114519; <http://dx.doi.org/10.1007/s002530050933>.
- Chelius RJ. Strain improvement of *Aspergillus niger* for phytase production. *Appl Microbiol Biotechnol* 1994; 41:79-83; <http://dx.doi.org/10.1007/BF00166085>.
- Minjares-Carranco A, Trejo-Aguilar BA, Auilar G, Vinięgra-Gonzalez G. Physiological comparison between pectinase-producing mutants of *Aspergillus niger* adopted either to solid-state fermentation or submerged fermentation. *Enzyme Microb Technol* 3 1997; 21:25-31; [http://dx.doi.org/10.1016/S0141-0229\(96\)00212-8](http://dx.doi.org/10.1016/S0141-0229(96)00212-8).
- Sandana Mala JG, Kamini NR, Puvanakrishnan R. Strain improvement of *Aspergillus niger* for enhanced lipase production. *J Gen Appl Microbiol* 2001; 47:181-6; PMID:12483617; <http://dx.doi.org/10.2323/jgam.47.181>.
- Weenink XO, Punt PJ, van den Hondel CA, Ram AF. A new method for screening and isolation of hypersecretion mutants in *Aspergillus niger*. *Appl Microbiol Biotechnol* 2006; 69:711-7; PMID:16021486; <http://dx.doi.org/10.1007/s00253-005-0013-y>.
- Lotfy WA, Ghanem KM, El-Helou ER. Citric acid production by a novel *Aspergillus niger* isolate: I. Mutagenesis and cost reduction studies. *Bioresour Technol* 2007; 98:3464-9; PMID:17223558; <http://dx.doi.org/10.1016/j.biortech.2006.11.007>.
- Khattab AA, Bazaraa WA. Screening, mutagenesis and protoplast fusion of *Aspergillus niger* for the enhancement of extracellular glucose oxidase production. *J Ind Microbiol Biotechnol* 2005; 32:289-94; PMID:15952011; <http://dx.doi.org/10.1007/s10295-005-0249-7>.
- Lussier M, White AM, Sheraton J, di Paolo T, Treadwell J, Southard SB, et al. Large scale identification of genes involved in cell surface biosynthesis and architecture in *Saccharomyces cerevisiae*. *Genetics* 1997; 147:435-50; PMID:9355584.
- Mandels M, Weber J, Parizek R. Enhanced cellulase production by a mutant of *Trichoderma viride*. *Appl Microbiol* 1971; 21:152-4; PMID:5099761.
- Bartkevičute D, Sasnauskas K. Disruption of the *MNN10* gene enhances protein secretion in *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2004; 4:833-40; PMID:15450190; <http://dx.doi.org/10.1016/j.femsyr.2004.03.001>.
- Vai M, Brambilla L, Orlandi I, Rota N, Ranzi BM, Alberghina L, et al. Improved secretion of native human insulin-like growth factor 1 from gas1 mutant *Saccharomyces cerevisiae* cells. *Appl Environ Microbiol* 2000; 66:5477-9; PMID:11097931; <http://dx.doi.org/10.1128/AEM.66.12.5477-5479.2000>.
- Marx H, Sauer M, Resina D, Vai M, Porro D, Valero F, et al. Cloning, disruption and protein secretory phenotype of the *GAS1* homologue of *Pichia pastoris*. *FEMS Microbiol Lett* 2006; 264:40-7; PMID:17020547; <http://dx.doi.org/10.1111/j.1574-6968.2006.00427.x>.
- Passolunghi S, Riboldi L, Dato L, Porro D, Branduardi P. Cloning of the *Zygosaccharomyces bailii* *GAS1* homologue and effect of cell wall engineering on protein secretory phenotype. *Microb Cell Fact* 2010; 9:7; PMID:20102600; <http://dx.doi.org/10.1186/1475-2859-9-7>.
- Tanner FW Jr, Vojnovich C, Van Lanen JM. Factors affecting riboflavin production by *Ashbya gossypii*. *J Bacteriol* 1949; 58:737-45; PMID:15395174.
- Kurtzman CP. Relationships among the genera *Ashbya*, *Eremothecium*, *Holleya* and *Nematospora* determined from rDNA sequence divergence. *J Ind Microbiol* 1995; 14:523-30; PMID:7662294; <http://dx.doi.org/10.1007/BF01573968>.
- Wickerham LJ, Flickinger MH, Johnston RM. The production of riboflavin by *Ashbya gossypii*. *Arch Biochem* 1946; 9:95-8; PMID:21009578.

35. Demain AL. Riboflavin oversynthesis. *Annu Rev Microbiol* 1972; 26:369-88; PMID:4567521; <http://dx.doi.org/10.1146/annurev.mi.26.100172.002101>.
36. Ribeiro O, Wiebe M, Ilmén M, Domingues L, Penttilä M. Expression of *Trichoderma reesei* cellulases CBHI and EGI in *Ashbya gossypii*. *Appl Microbiol Biotechnol* 2010; 87:1437-46; PMID:20422178; <http://dx.doi.org/10.1007/s00253-010-2610-7>.
37. Wendland J, Ayad-Durieux Y, Knechtle P, Rebischung C, Philippsen P. PCR-based gene targeting in the filamentous fungus *Ashbya gossypii*. *Gene* 2000; 242:381-91; PMID:10721732; [http://dx.doi.org/10.1016/S0378-1119\(99\)00509-0](http://dx.doi.org/10.1016/S0378-1119(99)00509-0).
38. Knechtle P, Dietrich F, Philippsen P. Maximal polar growth potential depends on the polarisome component AgSpa2 in the filamentous fungus *Ashbya gossypii*. *Mol Biol Cell* 2003; 14:4140-54; PMID:12937275; <http://dx.doi.org/10.1091/mbc.E03-03-0167>.
39. Park EY, Zhang JH, Tajima S, Dwiarti L. Isolation of *Ashbya gossypii* mutant for an improved riboflavin production targeting for biorefinery technology. *J Appl Microbiol* 2007; 103:468-76; PMID:17650208; <http://dx.doi.org/10.1111/j.1365-2672.2006.03264.x>.
40. Tajima S, Itoh Y, Sugimoto T, Kato T, Park EY. Increased riboflavin production from activated bleaching earth by a mutant strain of *Ashbya gossypii*. *J Biosci Bioeng* 2009; 108:325-9; PMID:19716523; <http://dx.doi.org/10.1016/j.jbiosc.2009.04.021>.
41. Chand P, Aruna A, Maqsood AM, Rao LV. Novel mutation method for increased cellulase production. *J Appl Microbiol* 2005; 98:318-23; PMID:15659186; <http://dx.doi.org/10.1111/j.1365-2672.2004.02453.x>.
42. Dillon AJ, Zorgi C, Camassola M, Henriques JA. Use of 2-deoxyglucose in liquid media for the selection of mutant strains of *Penicillium echinulatum* producing increased cellulase and beta-glucosidase activities. *Appl Microbiol Biotechnol* 2006; 70:740-6; PMID:16151800; <http://dx.doi.org/10.1007/s00253-005-0122-7>.
43. Xu F, Wang J, Chen S, Qin W, Yu Z, Zhao H, et al. Strain improvement for enhanced production of cellulase in *Trichoderma viride*. *Prikl Biokhim Mikrobiol* 2011; 47:61-5; PMID:21438472.
44. Vu VH, Pham TA, Kim K. Fungal strain improvement for cellulase production using repeated and sequential mutagenesis. *Mycobiology* 2009; 37:267-71; <http://dx.doi.org/10.4489/MYCO.2009.37.4.267>.
45. Adsl MG, Bastawde KB, Varma AJ, Gokhale DV. Strain improvement of *Penicillium jantbinellum* NCIM 1171 for increased cellulase production. *Bioresour Technol* 2007; 98:1467-73; PMID:17097876; <http://dx.doi.org/10.1016/j.biortech.2006.02.036>.
46. Mouyna I, Morelle W, Vai M, Monod M, Léchenne B, Fontaine T, et al. Deletion of *GEL2* encoding for a  $\beta(1-3)$ glucanotransferase affects morphogenesis and virulence in *Aspergillus fumigatus*. *Mol Microbiol* 2005; 56:1675-88; PMID:15916615; <http://dx.doi.org/10.1111/j.1365-2958.2005.04654.x>.
47. de Medina-Redondo M, Arnáiz-Pita Y, Clavaud C, Fontaine T, del Rey F, Latgé JP, et al.  $\beta(1,3)$ -glucanotransferase activity is essential for cell wall integrity and viability of *Schizosaccharomyces pombe*. *PLoS One* 2010; 5:e14046; PMID:21124977; <http://dx.doi.org/10.1371/journal.pone.0014046>.
48. Teather RM, Wood PJ. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl Environ Microbiol* 1982; 43:777-80; PMID:7081984.
49. Carlsen M, Marcher J, Nielsen J. An improved FIA method for measuring  $\alpha$ -amylase in cultivation media. *Biotechnol Tech* 1994; 8:479-82; <http://dx.doi.org/10.1007/BF00222839>.
50. Wach A. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. *Yeast* 1996; 12:259-65; PMID:8904338; [http://dx.doi.org/10.1002/\(SICI\)1097-0061\(19960315\)12:3<259::AID-YEA901>3.0.CO;2-C](http://dx.doi.org/10.1002/(SICI)1097-0061(19960315)12:3<259::AID-YEA901>3.0.CO;2-C).
51. Gueldener U, Heinisch J, Koehler GJ, Voss D, Hegemann JH. A second set of *loxP* marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res* 2002; 30:e23; PMID:11884642; <http://dx.doi.org/10.1093/nar/30.6.e23>.