Applied and Environmental Microbiology, Dec. 2003, p. 6979–6986 Vol. 69, No. 12
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.12.6979–6986.2003
Copyright © 2003, American Society for Microbiology. All Rights Reserved.

Improvement of Foreign-Protein Production in *Aspergillus niger* var. *awamori* by Constitutive Induction of the Unfolded-Protein Response

Mari Valkonen,[1]* Michael Ward,[2] Huaming Wang,[2] Merja Penttilä,[1] and Markku Saloheimo[1]

VTT Biotechnology, Espoo, Finland,[1] and Genencor International, Inc., Palo Alto, California[2]

Received 9 June 2003/Accepted 2 September 2003

Unfolded-protein response (UPR) denotes the upregulation of endoplasmic reticulum (ER)-resident chaperone and foldase genes and numerous other genes involved in secretory functions during the accumulation of unfolded proteins into the ER. Overexpression of individual foldases and chaperones has been used in attempts to improve protein production in different production systems. We describe here a novel strategy to improve foreign-protein production. We show that the constitutive induction of the UPR pathway in *Aspergillus niger* var. *awamori* can be achieved by expressing the activated form of the transcription factor *hacA*. This induction enhances the production of *Trametes versicolor* laccase by up to sevenfold and of bovine preprochymosin by up to 2.8-fold in this biotechnically important fungus. The regulatory range of UPR was studied by analyzing the mRNA levels of novel *A. niger* var. *awamori* genes involved in different secretory functions. This revealed both similarities and differences to corresponding studies in *Saccharomyces cerevisiae*.

Folding, disulfide bond formation, and subunit assembly, as well as core glycosylation of secreted proteins, takes place in the endoplasmic reticulum (ER). The protein-folding capacity of the ER is able to react to external signals; when unfolded proteins overload the ER, a signal transduction pathway called the unfolded-protein response (UPR) is activated. The UPR controls the expression of genes of several ER-resident chaperones and foldases (reviewed in references 19 and 28) and numerous other genes involved in other secretory functions (38).

The *Saccharomyces cerevisiae HAC1* gene encodes the transcription factor of the UPR pathway that binds to a specific region of the promoters of the target genes. An unconventional splicing mechanism involving Ire1p and a tRNA ligase removes a 250-nucleotide (nt) intron from the *HAC1* mRNA (37). This splicing removes translational attenuation caused by the intron and causes a replacement of the C-terminal portion of the Hac1p, creating a transcriptionally active Hac1p (9). In higher eukaryotes it has been shown that the XBP1 transcription factor binds to the mammalian ER stress-response element (ERSE) (48), and an unconventional intron is cleaved from the XBP-1 mRNA by Ire1 (6, 22). Another ERSE-binding protein in mammals is ATF-6 (47), an ER transmembrane protein that has been shown to be proteolytically processed upon activation of the UPR (17, 48). Thus, it seems that the UPR activation is more complex in higher eukaryotes than in yeast. We have cloned the functional homologues of the yeast *HAC1* from *Trichoderma reesei*, *Aspergillus nidulans* (33), and *Aspergillus niger* var. *awamori* (unpublished data). Our studies have indicated that the activation of the *hac1*/*hacA* genes in filamentous fungi includes two events: splicing of a 20-nt intron analogously to yeast *HAC1* and truncation of the mRNA at the 5′ flanking region, removing an upstream open reading frame (33).

* Corresponding author. Mailing address: VTT Biotechnology, P.O. Box 1500, FIN-02044 VTT, Finland. Phone: 358-9-456-5825. Fax: 358-9-455-2103. E-mail: mari.valkonen@vtt.fi.

Filamentous fungi have been used as protein production hosts because of their high secretion capacities. *Aspergillus* spp. and the fungus *T. reesei* are able to secrete several tens of grams per liter of native hydrolytic enzymes, but the production of heterologous proteins, except for those from closely related fungal species, often results in yields of only tens of milligrams per liter (14, 29). There is some indication that problems can occur at different steps of protein synthesis and secretion, translation initiation or elongation, translocation into the ER, folding, transport, processing, or secretion (14).

Many approaches have been used to improve foreign-protein production in various expression systems. These include strain improvement by mutagenesis and screening and genetic modifications such as the deletion of proteases from the production strain (43). Yield improvement has also been obtained by the introduction of multiple copies of expressed genes, the use of strong promoters, gene fusions to well-secreted proteins, the use of native signal sequences, and overexpression of individual ER foldase or chaperone genes (1, 8, 11, 31, 36). Although these strategies have been successful for some heterologous proteins, the production of most foreign proteins still remains problematic. In large-scale protein production, the ER may encounter stress that results from the high level of protein expression. It has been shown that the expression of heterologous proteins can activate the UPR pathway (32).

We describe here a general approach for the improvement of heterologous proteins by overexpressing the UPR pathway regulator. Rather than by overexpressing just one limiting factor in heterologous protein production, we induced some functions throughout the secretory pathway by overexpressing the UPR-induced form of the *A. niger* var. *awamori hacA* gene in *A. niger* var. *awamori* strains producing a laccase from a basidiomycete fungus *Trametes versicolor* or bovine chymosin. Basidiomycete laccases are generally proteins whose expression in heterologous hosts can be problematic (18). Calf chymosin has been difficult to express in *A. niger* but, with the use of efficient strain improvement program, commercial levels of production have been reached (11).

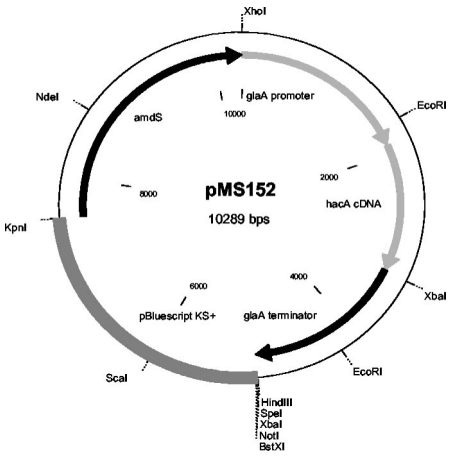

FIG. 1. Schematic presentation of the pMS152 expression vector containing the *A. niger* var. *awamori hacA* gene in constitutively active form. The construction of the vector is explained in the text.

MATERIALS AND METHODS

Construction of strains and plasmids. The *A. niger* var. *awamori* ΔAP3 and ΔAP4 (5) strains are equivalent strains with the *pepA* gene (encoding the major extracellular aspartic proteinase) deleted and having a *pyrG*-null mutation. ΔAP3 was transformed with the vector pUC*pyr*GRG3 to create strain ΔAP3pUC*pyr*GRG3#11. pUC*pyr*GRG3 consists of the GRG3 expression cassette (bovine preprochymosin gene between the *A. niger glaA* promoter and terminator) obtained from pGRG3 (10) and the *Neurospora crassa pyr4* gene in pUC19. ΔAP4 was transformed with pGPT-LCC1 to create strain ΔAP4:pGPT-laccase producing *Trametes versicolor* laccase 1. pGPT-LCC1 is a derivative of pGPT*pyrG1* (4) containing the *N. crassa pyr4* gene and the *A. niger glaA* promoter and terminator. To create pGPT-LCC1, the *Trametes versicolor lcc1* open reading frame (27) was inserted between the *glaA* promoter and terminator.

To make a construct for constitutive UPR induction, the induced form of the *A. niger* var. *awamori hacA* cDNA was first created by deleting the 20-bp intron and truncating the 5′ flanking region by ca. 150 bp. A truncated *hacA* PCR fragment was created with the oligonucleotides TCGATTGAATTCGCTGTGT CGACCTACATCACC (forward primer with an *Eco*RI site) and CGGGGTCG AAATCAACCATA (reverse primer). This fragment was digested with *Eco*RI and *Pst*I and ligated into *Eco*RI-*Not*I-digested pZERO (Invitrogen), together with a 3′-end fragment of *hacA* digested by *Pst*I and *Not*I. The *hacA* gene has two *Pst*I sites precisely at the borders of the 20-bp intron. Thus, the ligation described above created a fragment in which the 5′ end was truncated and the 20-bp intron was removed. This fragment was cloned into the *Bgl*II site of the expression vector pGPT-pyrG1 between the *glaA* promoter and terminator. The *hacA* expression cassette was cloned as a *Hin*dIII-*Xho*I fragment into the *Hin*dIII-*Xho*I-digested pBluescript II KS(+) (Stratagene) fragment harboring the *A. nidulans amdS* marker. The *hacA* overexpression construct (pMS152; Fig. 1) was transformed into the *A. niger* var. *awamori* strains ΔAP3pUCpyrGRG3#11 and ΔAP4:pGPTlaccase as described previously (30).

The preprochymosin transformants were cultivated in Clofine special medium (described in international patent application WO 98/31821). The laccase-producing transformants were cultivated in a medium containing (per liter) 8 g of Bacto Soytone (Difco), 12 g of tryptone peptone (Difco), 15 g of $(NH_4)_2SO_4$, 12.1 g of $NaH_2PO_4 \cdot H_2O$, and 3.3 g of $Na_2HPO_4 \cdot 7H_2O$ to which were added (per liter) 5 ml of 20% $MgSO_4$ solution, 2 ml of copper citrate solution (110 g of citrate $\cdot H_2O$/liter, 125 g of $CuSO_4 \cdot 5H_2O$/liter), 1 ml of Tween 80, 300 ml of 50% maltose solution, and 200 ml of a 100-mg/liter arginine solution after autoclaving. The cultivations were done in 250-ml shake flasks with 70 ml of medium (28°C, 200 rpm).

Nucleic acid methods. DNA was isolated with the DNAeasy kit (Invitrogen) according to the manufacturer's instructions. Total RNA's were isolated by using the Trizol reagent (Gibco-BRL) as instructed by the manufacturer. Southern and Northern hybridizations were done as described previously (34). The *A. niger* var. *awamori* genes encoding secretory functions that were used as probes in Northern hybridizatios, were digested from the plasmid pCMV.SPORT 6 (Life Technologies) with *Sal*I/*Not*I digestion. The probe fragments were *ino1* (GenBank accession no. AY365137), *snc1* (AY365139), *ktr1* (AY365138), *sec61* (AY365136), and *nsfA* (AF263922). A 950-bp fragment of *bipA* gene (GenBank accession no. Y08868) was created by PCR with GTCTCCGCCATGGTTC TTGG (forward primer) and GGTGGGCTGGTTATCAGCGG (reverse primer) oligonucleotides with *A. niger* var. *awamori* genomic DNA as a template. The *lcc1* cDNA (GenBank accession no. U44430) fragment used as a probe was excised from pBK117 plasmid by *Eco*RI/*Xho*I digestion. The *glaA* PCR fragment was created with oligonucleotides CCTGAGCGGCCTCGTCTGCAC (forward primer) and GTCGTATTGCTCGGACATGG (reverse primer) by using pGASKHi vector as a template. The *amyB* gene fragment encoding α-amylase B from pAMY3 (21) vector with *Hin*dIII digestion. Transcript levels were quantified with the PhosphorImager S1 (Molecular Dynamics).

Enzyme activity and protein measurements. Chymosin activity was measured from samples diluted into 1% sodium acetate buffer. A total of 200 μl of the diluted sample was incubated in 5 ml of buffer containing 11% skim milk (Difco) at 30°C. The milk clotting time was correlated to known standards. Laccase activity was measured as described previously (26). α-Amylase activity was measured with the Phadebas amylase test (Pharmacia) according to the manufacturer's instructions. β-Glucosidase activities were measured as described previously (3). The total protein measurements were done from trichloroacetic acid-precipitated samples by using the Bio-Rad protein assay (Bio-Rad).

To study the quantity of the chymosin protein produced, equal amounts of samples from the culture supernatants of the *hacA*-overexpressing transformants and the controls were analyzed by Western blotting with a chymosin antibody K336 (Novo). Sodium dodecyl sulfate gels were run from culture medium samples containing 10 μg of total protein, and Western detection was carried out with horseradish peroxidase-conjugated secondary antibody (Bio-Rad). The films from the Western filters were scanned by using a GS-710 imaging densitometer (Bio-Rad).

RESULTS

To obtain constitutive UPR induction in *A. niger* var. *awamori*, the UPR-induced form of *hacA* cDNA that lacked the 20-nt intron and had a truncation of ca. 150 bp at the 5′ end was constructed. This gene was cloned into an expression vector under the control of the *A. niger* glucoamylase *glaA* promoter. The resulting plasmid (Fig. 1) was transformed into *A. niger* var. *awamori* strains producing *Trametes versicolor* laccase 1 or bovine preprochymosin. The transformants were identified by Southern hybridization (data not shown).

In order to study the effect of constitutive UPR induction on chymosin production, seven transformants shown by Southern hybridization to contain the *hacA* overexpression construct and the parental strain (ΔAP3pUC*pyr*GRG3#11) were cultivated in shake flasks, and the chymosin activities were measured from the culture supernatants. The results are shown for the fifth day of cultivation (Fig. 2A). All of the seven transformants studied produced equal or higher chymosin levels than the parental strain. Four transformants produced 1.3- to 2.8-fold more chymosin than the control. Samples from the culture supernatants were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto membrane filters, and probed with chymosin antibody. A band of the size of the mature chymosin was observed in all of the samples. In the parental strain, there were two bands, the smaller one representing the mature chymosin and the bigger one prochymosin. A faint prochymosin signal was also detected in two of the *hacA* transformants. The results showed that there seems to be more mature chymosin produced by most of the transformants (Fig. 2B).

Transformants from the laccase-producing strain were studied in more detail. The strains were cultivated for Northern analysis and measurement of the produced enzyme activities. Northern hybridization from the second culture day of the laccase-producing strains revealed an mRNA of the expected

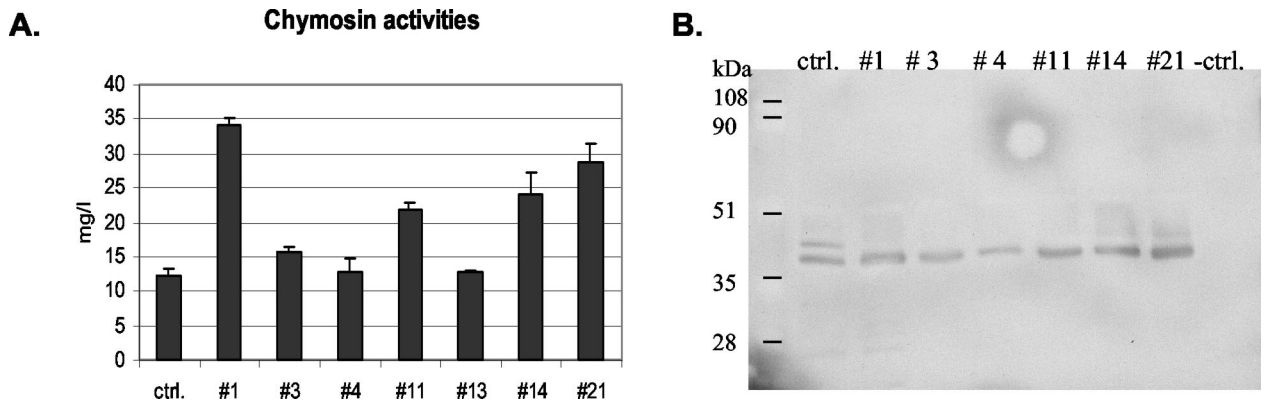

FIG. 2. Chymosin activities measured from the culture supernatants of the *hacA*-overexpressing transformants and the parental strain. (A) Chymosin activities. The results shown are averages (± the standard deviation [±SD]) from two parallel cultures. (B) Western blot analysis of the culture supernatants. The "−ctrl." lane shows a sample (10 μg of total protein) from the culture supernatant of a laccase-producing control strain that was used as a negative control for the chymosin antibody.

size from the *hacA* overexpression cassette in all of the transformants studied, in addition to the 1.7-kb band derived from the native *hacA* gene (Fig. 3). In the 2-day samples derived from the parental strain, a faint *hacA* band somewhat smaller than the full-length mRNA signal was detected. This suggests that the native UPR pathway might be partially induced in the parental strain, possibly caused by production of laccase. Similarly, the *hacA* mRNA derived from the overexpression construct was detected in transformants of the preprochymosin strain but not in the parental strain (data not shown).

In order to show that the inducing form of *hacA* had a regulatory effect on the downstream genes of the UPR, Northerns were probed with the *bipA* gene encoding the major ER chaperone. *HacA* overexpression led to a significant increase in the *bipA* transcript levels (two- to fourfold) on the second culture day in all of the *hacA*-transformants compared to the parental strains (Fig. 3), thus demonstrating that the UPR pathway is induced in the transformants. The results also show that the extent of *bipA* induction correlates with the expression level of the induced *hacA*. In samples from later time points (days 5 and 7), the induction of the *bipA* gene in the *hacA* transformants was reduced. The induction was 1.25- to 2-fold compared to the parental strain (data not shown). A fragment of the *Trametes versicolor lcc1* gene was also used as a probe, and the results show that laccase expression levels were higher in the parental strain than in any of the transformants (Fig. 3). The lower amount of *lcc1* mRNA in the transformants may be due to a titration effect of transcription factors since both the laccase gene and *hacA* was produced under the *A. niger glaA* promoter (42). The Northerns were also probed with a *glaA* and α-amylase gene fragments. The results show that the *glaA* is expressed at ~2-fold-higher levels in the parental strain than in any of the transformants (Fig. 4D). Probing with the *amyB* a gene fragment showed two bands. This is because the α-amylase a and b genes are almost identical (21). Quantification and normalization of the signals showed that their expression was not affected by *hacA* overexpression. This further confirms the titration of the transcription factors for *glaA* promoter.

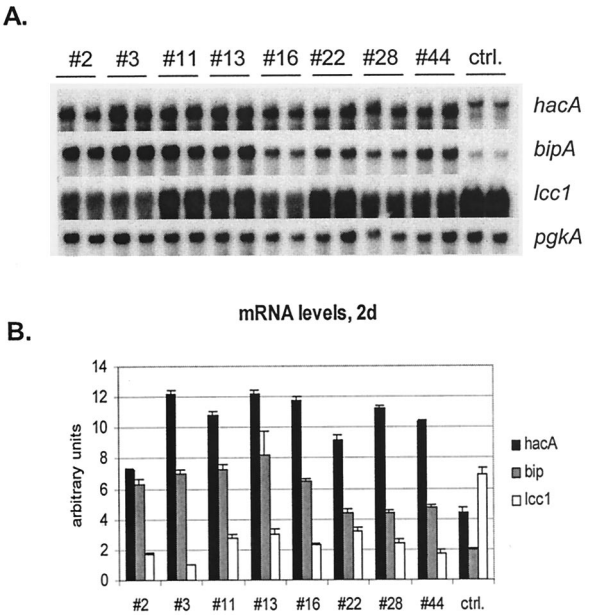

FIG. 3. (A) Expression levels of *hacA*, *bipA*, and the laccase gene *lcc1* in the transformants and parental strain (ctrl.). The results of two parallel cultures are shown for each strain. (B) The signal intensities of the Northern blots were quantified and normalized to the *pgkA* signal intensities. The panels show the averages (±SD) from the two parallel cultures of each strain.

It has been shown that the UPR pathway regulates many functions throughout the whole secretory pathway in *S. cerevisiae* and *Arabidopsis* spp. (23, 38). Northern blots made from the UPR-induced *A. niger* var. *awamori* strains were probed with novel genes encoding different functions in the secretory pathway. The orthologs of these genes in yeast have been shown to be induced by UPR (38). The *ino1* encoding inositol-3-phosphate synthase involved in lipid biosynthesis and the *snc1* encoding a v-SNARE functioning in exocytosis were not induced in the *hacA*-overexpressing transformants (Fig. 4A). The *ktr1* gene encoding mannosyltransferase involved in protein glycosylation in the Golgi complex and *sec61* encoding a major component of the translocon complex were clearly induced in all of the transformants compared to the parental

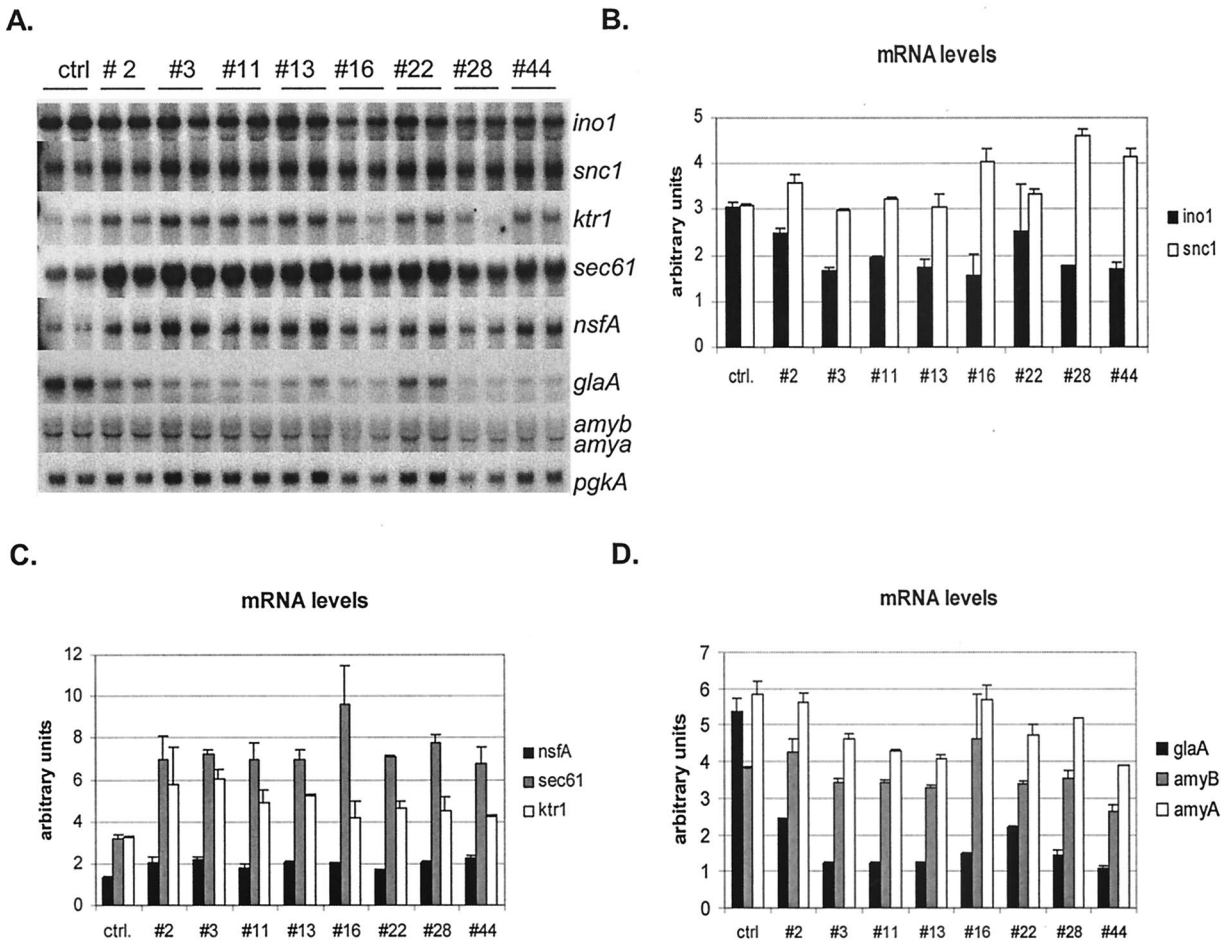

FIG. 4. (A) Expression of *ino1*, *snc1*, *ktr1*, *sec61*, *nsfA*, *glaA*, and amylase genes in the transformants and the parental strain (ctrl). Two parallel cultures are shown for each strain. (B to D) Signal intensities of *ino1* and *snc1* (B); *nsfA*, *sec61*, and *ktr1* (C); and *glaA*, *amyA*, and *amyB* (D) genes. The signal intensities of the Northern blots were quantified and normalized to the *pgkA* signal intensities. The panels show the averages (±SD) from the two parallel cultures of each strain.

strain (Fig. 4B). The induction of these genes was 1.5- to 3-fold in the transformants. The *nsfA* gene that is the homologue of yeast *SEC18* encoding general fusion factor functioning at different steps of the vesicle transport was also slightly induced in the transformants compared to the parental strain (Fig. 4B).

Laccase activity measurements from shake flask cultures in a complex medium showed that all eight *hacA* transformants studied produced more laccase than the parental strain (Fig. 5A). The laccase levels of the transformants in the fifth day samples were 3- to 7.6-fold higher than those of the parental strain. The transformants produced laccase much more rapidly than the parental strain, reaching the peak already on the fifth day, whereas the parental strain continued to produce more laccase until the end of the culture.

The growth and native protein production of the laccase-producing strains were analyzed in detail. The pH was measured from the shake flask cultures described above and showed that the parental strain acidified the culture medium more rapidly than any of the transformants (Fig. 5B), indicating that it was growing faster. To verify this, two transformants and the parental strain were grown in larger shake flask cultures in which the growth could be studied by dry-weight measurements. The *hacA* transformants grew more slowly than the parental strain but reached a slightly higher final biomass (Fig. 5C). This is in accordance with the previously reported result that the constitutive UPR induction retards growth in yeast (9, 20).

The level of total secreted protein was measured from the shake flask cultures, and the parental strain produced more secreted protein than any of the transformants (Fig. 6A). The transformants that produced the most laccase secreted the least total protein. At the end of the cultures the difference between the parental strain and the transformants was 1.5- to 1.9-fold. The production of specific enzyme activities, i.e., α-amylase and β-glucosidase, was also measured. All *hacA* transformants produced three- to sevenfold less α-amylase than the control in all time points tested (Fig. 6B) and also less β-glucosidase, although this was only obvious at a later stage of culture (Fig. 6C).

DISCUSSION

The synthesis and secretion processes of proteins in filamentous fungi have been studied widely to understand the prob-

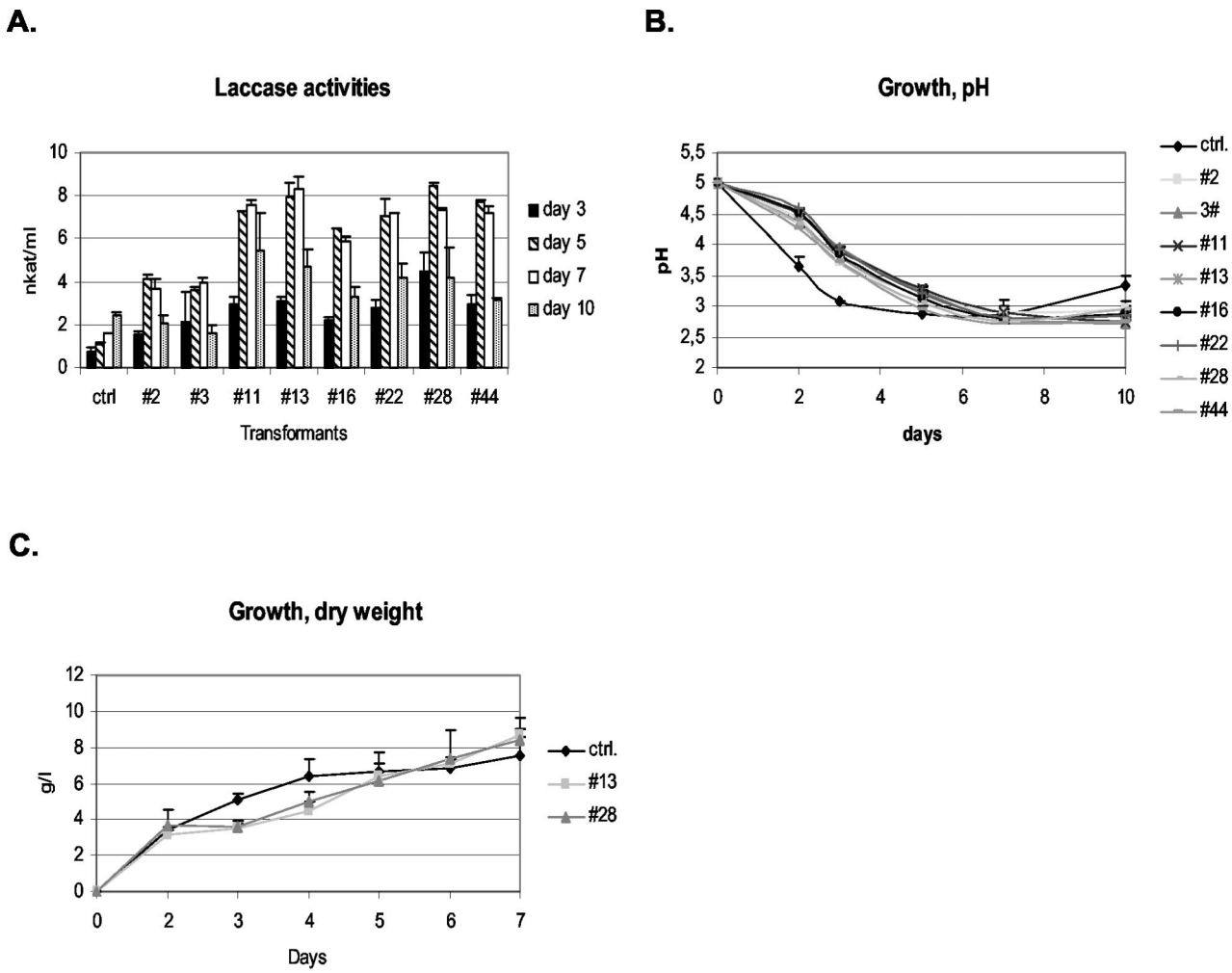

FIG. 5. (A) Laccase activities measured from the culture supernatants of the *hacA*-overexpressing transformants and the parental strain. The results shown are averages (±SD) from two parallel cultures. (B and C) pH values (±SD) from two parallel cultures (B) and dry weights (±SD) from three parallel cultures (C) of laccase-producing strains. For the pH measurements, the strains were grown in 50-ml shake flask cultures. For the dry-weight measurements, two *hacA*-overexpressing transformants and the parental strain were grown in 350-ml shake flask cultures.

lems connected to heterologous protein production. Although the information about the fungal secretion pathway has increased remarkably in recent years (reviewed in reference 8), there still is only limited knowledge of the factors affecting protein production.

One rate-limiting step in the secretion of foreign proteins is in the ER. Limiting amounts of chaperones and foldases involved in proper folding and assembly of proteins may lead to accumulation of misfolded proteins into the ER. It has been shown that this may cause routing of the proteins into the degradation pathway (2, 13). For this reason, the effects of foldase and chaperone expression have been studied in relation to heterologous protein production. Overexpression protein disulfide isomerase (PDI) or KAR2/Bip can increase the production of some heterologous proteins in *S. cerevisiae* (15, 16, 31, 35, 36). Work along the same lines has been carried out also in filamentous fungi. Overproduction of Bip in strains expressing cutinase variants from *Fusarium solani pisi* did not affect the protein production capacity in *A. awamori* (40, 41). On the other hand, overproduction of another ER lumenal chaperone, calnexin, has been shown to increase the production of *Phanerochaete chrysosporium* manganese peroxidase in *A. niger* up to fivefold (7). Thaumatin production in *A. awamori* was improved by PDIA overexpression up to fivefold. The highest improvement was observed in a strain where the relative PDIA level was between two and four compared to a strain with one copy of *pdiA* gene (24). On the other hand, overexpression of *pdiA* (25) or the PDI-related gene *prpA* (44) in *A. niger* had no effect on heterologous protein production. Also, overexpression of another foldase, cyclophilin, in *A. niger* did not increase tissue plasminogen activator production (45). This would suggest that the particular secreted protein under investigation greatly affects the outcome.

We have studied the effect of constitutive activation of the UPR pathway on the production of heterologous protein in *A. niger* var. *awamori*. It was first demonstrated that the expression of a UPR target gene, *bipA*, was elevated in the transformants expressing the UPR-activated form of *hacA* (Fig. 3). The extent of *bipA* induction would appear to correlate with the expression level of the induced *hacA*. In the 2-day samples

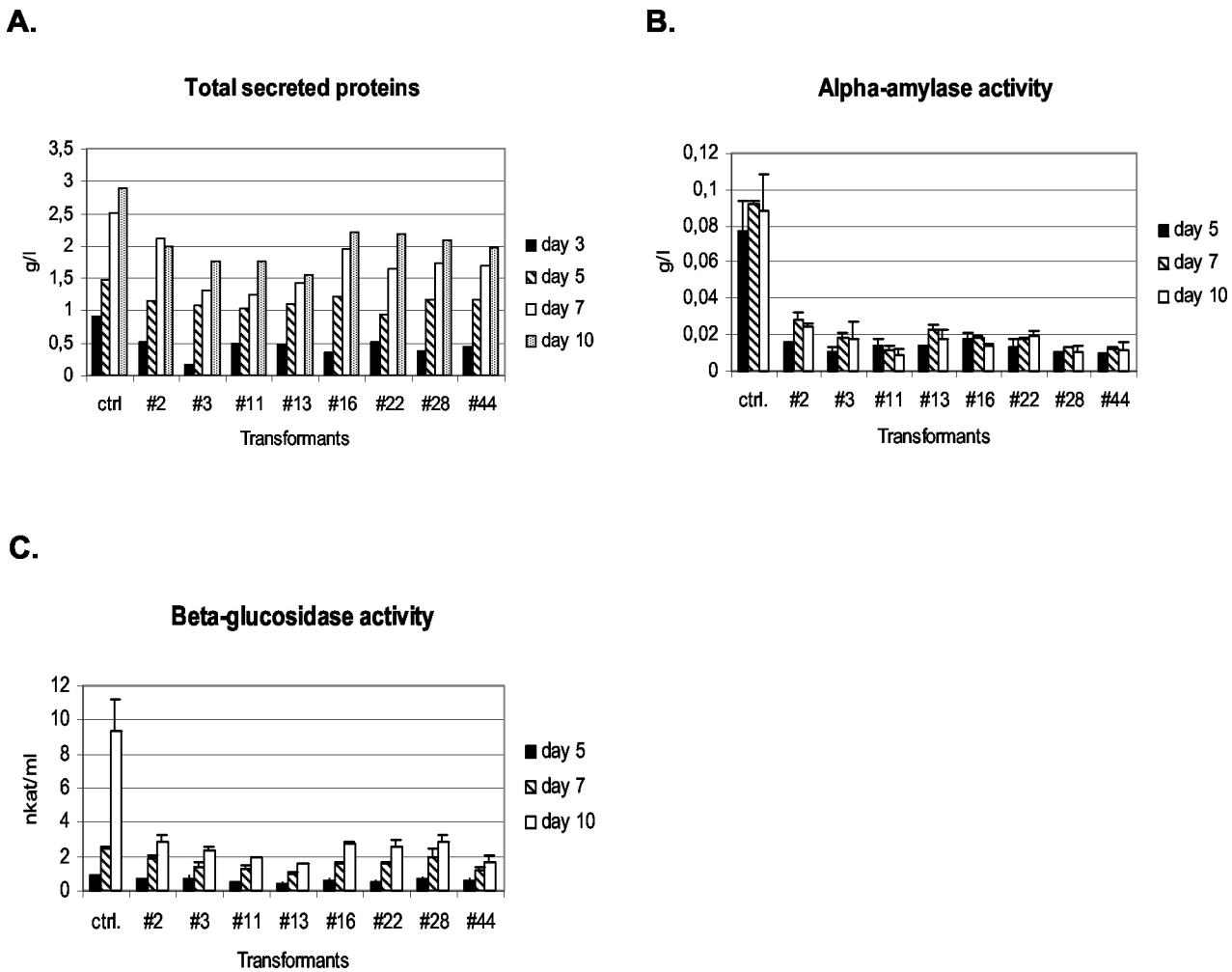

FIG. 6. (A) Amounts of total secreted protein measured from the culture supernatants of the *hacA*-overexpressing transformants of the laccase-producing strain and the parental strain grown in 50-ml shake flask cultures. (B and C) α-Amylase (B) and β-glucosidase (C) activities measured form the supernatants of the same cultures. The results (±SD) are from two parallel cultures.

derived from the parental strain, a faint *hacA* band somewhat smaller than the full-length mRNA signal can be detected. This indicates that the UPR pathway may be induced in the parental strain, possibly by expression of the laccase gene. However, a remarkable increase in the *bipA* expression levels was achieved by the *hacA* overexpression.

By expressing the induced form of *hacA* in a strain producing *Trametes versicolor* laccase 1 (27), we were able to increase not only the levels of mRNAs encoding an ER-resident chaperone but also the production of both laccase and chymosin compared to the parental strain. Interestingly, even though the laccase mRNA levels were lower in all of the transformants studied compared to the parental strain (Fig. 3), the transformants still secreted more active laccase into the culture medium (Fig. 5A). Thus, the posttranscriptional events of laccase production in the *hacA*-overexpressing strains were actually enhanced more than what can be concluded just from the secreted activity data.

It would appear that there is an optimal level of *hacA* expression with respect to laccase production, since the highest levels of laccase were secreted by transformants with intermediate levels of UPR induction. In the present study, we saw more pronounced improvement of laccase production compared to chymosin. This can be due to the intrinsic nature of the expressed proteins that cause problems in their secretion at different stages of secretion. The Western analysis from the culture supernatants of the chymosin-producing strains showed that there seems to be more of mature chymosin produced by the *hacA* transformants (Fig. 2B). Thus, it is possible that the *hacA* overexpression enhances the production of the correctly folded chymosin, which is more prone to autocatalytic processing to give mature chymosin. It should be noted that the *hacA* expression cassette was not directed to a specific locus in the genome. Therefore, it may be that the variations seen in the effects on the production of the two model proteins originate from the site at which the expression cassette was integrated. In order to further optimize this method, screening of the transformants for best producers, perhaps by an automated system, would help in finding the optimal level of the UPR induction versus foreign-protein production.

It has been observed that at least some secreted proteins in filamentous fungi are attached to the cell wall and not secreted

efficiently to the culture medium (46). Also a glucoamylase-green fluorescent protein fusion protein has been shown to be partially retained within the cell wall (12). Therefore, we made a simple plate assay with the chromogenic substrate ABTS [2,2′azinobis(3-ethylbenzthiazolinesulfonic acid)] for laccase production and were able to detect similar differences in the laccase activities between the transformants and the parental strain that were observed in the culture supernatants of shake flask cultures. This indicates that the differences seen in the laccase production between the transformants and the parental strain are due to the enhanced secretion of laccase and not, for example, due to the altered structure of the cell walls.

According to pH (Fig. 5B) and dry-weight measurements (Fig. 5C), the growth rate of the transformants with constitutive UPR induction was slower than that of the parental strain. Even so, the rate of laccase production of the transformants was by far faster than the production rate of the parental strain (Fig. 5A). Therefore, the constitutive UPR induction could bring about a remarkable improvement in the specific production rate of laccase (per biomass), a very important measure describing a biotechnical process.

At the same time with enhanced laccase production, the production of native proteins was lessened in the transformants with constitutive UPR induction (Fig. 6). This may be in part due to the slower growth observed in the HACA transformant strains compared to the parental strain. Another factor that should be taken into account is that the α-amylase activity test also measures glucoamylase activity. Thus, since both *hacA* and *Trametes versicolor* laccase were expressed from the *glaA* promoter and since *glaA* gene is expressed at a lower level in the transformants, the results can be explained by the titration of the regulatory factors as mentioned earlier. On the other hand, it has been shown that induction of UPR in the filamentous fungus *T. reesei* results in downregulation of the genes encoding secreted proteins (27a). The results presented in the present study do not show such downregulation, since the genes encoding α-amylase were not repressed in the *hacA* transformants.

It has been shown in *S. cerevisiae* that the UPR pathway regulates the transcription of approximately 380 genes. Of the functionally characterized genes, 103 are involved in secretion or biogenesis of secretory organelles. As could be expected, these included genes for ER-resident chaperones and foldases. More surprising was that a set of genes encoding for protein functions throughout the whole secretory pathway were induced (38). In our strategy to improve the heterologous protein production in filamentous fungi, the aim was to upregulate the ER folding machinery, as well as other genes from the secretory pathway, by constitutive UPR induction. The results from Northern analysis support the idea that, by constitutive UPR induction, functions throughout the secretory pathway can be induced in *A. niger* var. *awamori*. This is seen in the induction of *nsfA*, *sec61*, and *ktr1* genes that encode functions at different levels of the secretory pathway. In contrast, the expression of *ino1* and *snc1* genes in *A. niger* var. *awamori* was not induced in the *hacA* transformants. Yeast *INO1* is among the genes most strongly induced by UPR; the induction at its highest was ~10-fold. The gene *SNC1* is also induced by UPR in yeast, although the level of induction was lower (ca. two- to threefold higher than in the control). These results indicate that there are both similarities and differences in the range of UPR induction between yeast and *A. niger* var. *awamori*.

In another study we obtained improved foreign-protein production by constitutive UPR induction in *S. cerevisiae*. The overexpression of the UPR-induced form of yeast *HAC1* or *T. reesei hac1* caused a clear increase in the production of *Bacillus amyloliquefaciens* α-amylase (39). The experiments reported here indicate that similar induction mechanism of the UPR pathway exists in *A. niger* var. *awamori* and in yeast and that, by manipulating the UPR pathway, the protein production can be improved in both organisms. Thus, it would seem that our strategy can be used more generally for the improvement of protein production. The difference between the two organisms is that in yeast the production of a native protein, invertase, was also increased, whereas we did not detect a beneficial effect in *A. niger* var. *awamori* on the production of native proteins. This may be due to the low secretion capacity of yeast, in which fine-tuning of the secretory machinery can cause a clear effect on the production of native proteins, as well as differences in the regulation of the secretory machinery as described above.

ACKNOWLEDGMENTS

Riitta Nurmi is acknowledged for excellent technical assistance.

The study was partially supported by the Finnish Technology Agency (Tekes).

REFERENCES

1. **Archer, D. B., D. J. Jeenes, and D. A. Mackenzie.** 1994. Strategies for improving heterologous protein production from filamentous fungi. Antonie Leeuwenhoek **65:**245–250.
2. **Archer, D. B., D. A. MacKenzie, D. J. Jeenes, and I. N. Roberts.** 1992. Proteolytic degradation of heterologous proteins expressed in *Aspergillus niger.* Biotechnol. Lett. **14:**357–362.
3. **Bailey, M. J., and K. M. H. Nevalainen.** 1981. Induction, isolation, and testing of stable *Trichoderma reesei* mutants with improved production of solubilizing cellulase. Enzyme Microb. Technol. **3:**153–157.
4. **Berka, R. M., and C. C. Barnett.** 1989. The development of gene expression systems for filamentous fungi. Biotechnol. Adv. **7:**127–154.
5. **Berka, R. M., M. Ward, L. J. Wilson, K. J. Hayenga, K. H. Kodama, L. P. Carlomagno, and S. A. Thompson.** 1990. Molecular cloning and deletion of the gene encoding aspergillopepsin A from *Aspergillus awamori.* Gene **86:** 153–162.
6. **Calfon, M., H. Zeng, F. Urano, J. H. Till, S. R. Hubbard, H. P. Harding, S. G. Clark, and D. Ron.** 2002. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature **415:**92–96.
7. **Conesa, A., D. Jeenes, D. B. Archer, C. A. van den Hondel, and P. J. Punt.** 2002. Calnexin overexpression increases manganese peroxidase production in *Aspergillus niger*. Appl. Environ. Microbiol. **68:**846–851.
8. **Conesa, A., P. J. Punt, N. van Luijk, and C. A. van den Hondel.** 2001. The secretion pathway in filamentous fungi: a biotechnological view. Fungal Genet. Biol. **33:**155–171.
9. **Cox, J. S., and P. Walter.** 1996. A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. Cell **87:** 391–404.
10. **Cullen, D., G. L. Gray, L. J. Wilson, K. J. Hayenga, M. H. Lamsa, M. W. Rey, S. Norton, and R. B. Berka.** 1987. Controlled expression and secretion of bovine chymosin in *Asperillus nidulans.* Bio/Technology **5:**369–376.
11. **Dunn-Coleman, N. S., P. Bloebaum, R. M. Berka, E. Bodie, N. Robinson, G. Armstrong, M. Ward, M. Przetak, G. L. Carter, and R. LaCost.** 1991. Commercial levels of chymosin production by *Aspergillus*. Bio/Technology **9:**976–981.
12. **Gordon, C. L., D. B. Archer, D. J. Jeenes, J. H. Doonan, B. Wells, A. P. Trinci, and G. D. Robson.** 2000. A glucoamylase::GFP gene fusion to study protein secretion by individual hyphae of *Aspergillus niger*. J. Microbiol. Methods **42:**39–48.
13. **Gouka, R. J., P. J. Punt, J. G. Hessing, and C. A. van den Hondel.** 1996. Analysis of heterologous protein production in defined recombinant *Aspergillus awamori* strains. Appl. Environ. Microbiol. **62:**1951–1957.
14. **Gouka, R. J., P. J. Punt, and C. A. van den Hondel.** 1997. Efficient production of secreted proteins by *Aspergillus*: progress, limitations, and prospects. Appl. Microbiol. Biotechnol. **47:**1–11.

15. **Harmsen, M. M., M. I. Bruyne, H. A. Raue, and J. Maat.** 1996. Overexpression of binding protein and disruption of the PMR1 gene synergistically stimulate secretion of bovine prochymosin but not plant thaumatin in yeast. Appl. Microbiol. Biotechnol. **46:**365–370.
16. **Hayano, T., M. Hirose, and M. Kikuchi.** 1995. Protein disulfide isomerase mutant lacking its isomerase activity accelerates protein folding in the cell. FEBS Lett. **377:**505–511.
17. **Haze, K., H. Yoshida, H. Yanagi, T. Yura, and K. Mori.** 1999. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol. Biol. Cell **10:**3787–3799.
18. **Jönsson, L. J., M. Saloheimo, and M. Penttilä.** 1997. Laccase from the white-rot fungus *Trametes versicolor*: cDNA cloning of *lcc1* and expression in *Pichia pastoris*. Curr. Genet. **32:**425–430.
19. **Kaufman, R. J.** 1999. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev. **13:**1211–1233.
20. **Kawahara, T., H. Yanagi, T. Yura, and K. Mori.** 1997. Endoplasmic reticulum stress-induced mRNA splicing permits synthesis of transcription factor Hac1p/Ern4p that activates the unfolded protein response. Mol. Biol. Cell **8:**1845–1862.
21. **Korman, D. R., F. T. Bayliss, C. C. Barnett, C. L. Carmona, K. H. Kodama, T. J. Royer, S. A. Thompson, M. Ward, L. J. Wilson, and R. M. Berka.** 1990. Cloning, characterization, and expression of two alpha-amylase genes from *Aspergillus niger* var. *awamori.* Curr. Genet. **17:**203–212.
22. **Lee, K., W. Tirasophon, X. Shen, M. Michalak, R. Prywes, T. Okada, H. Yoshida, K. Mori, and R. J. Kaufman.** 2002. IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. Genes Dev. **16:**452–466.
23. **Martinez, I. M., and M. J. Chrispeels.** 2003. Genomic analysis of the unfolded protein response in *Arabidopsis* shows its connection to important cellular processes. Plant Cell **15:**561–576.
24. **Moralejo, F. J., A. J. Watson, D. J. Jeenes, D. B. Archer, and J. F. Martin.** 2001. A defined level of protein disulfide isomerase expression is required for optimal secretion of thaumatin by *Aspergillus awamori*. Mol. Genet. Genomics **266:**246–253.
25. **Ngiam, C., D. J. Jeenes, P. J. Punt, C. A. Van Den Hondel, and D. B. Archer.** 2000. Characterization of a foldase, protein disulfide isomerase A, in the protein secretory pathway of *Aspergillus niger*. Appl. Environ. Microbiol. **66:**775–782.
26. **Niku-Paavola, M. L., E. Karhunen, P. Salola, and V. Raunio.** 1988. Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. Biochem. J. **254:** 877–884.
27. **Ong, E., W. B. Pollock, and M. Smith.** 1997. Cloning and sequence analysis of two laccase complementary DNAs from the ligninolytic basidiomycete *Trametes versicolor*. Gene **196:**113–119.

27a. **Pakula, T., M. Laxell, A. Huuskonen, J. Uusitalo, M. Saloheimo, and M. Pentillä.** 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., in press.

28. **Patil, C., and P. Walter.** 2001. Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Curr. Opin. Cell Biol. **13:**349–355.
29. **Penttilä, M.** 1998. Heterologous protein production in *Trichoderma*, p. 365–382. *In* G. E. Harman and C. P. Kubicek (ed.), *Trichoderma* and *Gliocladium*, vol. 2. Taylor & Francis, Ltd., London, United Kingdom.
30. **Penttilä, M., H. Nevalainen, M. Rattö, E. Salminen, and J. Knowles.** 1987. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene **61:**155–164.
31. **Robinson, A. S., V. Hines, and K. D. Wittrup.** 1994. Protein disulfide isomerase overexpression increases secretion of foreign proteins in *Saccharomyces cerevisiae*. Bio/Technology **12:**381–384.
32. **Saloheimo, M., M. Lund, and M. E. Penttilä.** 1999. The protein disulphide isomerase gene of the fungus *Trichoderma reesei* is induced by endoplasmic reticulum stress and regulated by the carbon source. Mol. Gen. Genet. **262:**35–45.
33. **Saloheimo, M., M. Valkonen, and M. Penttilä.** 2003. Activation mechanisms of the HACI-mediated unfolded protein response in filamentous fungi. Mol. Microbiol. **47:**1149–1161.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Schultz, L. D., H. Z. Markus, K. J. Hofmann, D. L. Montgomery, C. T. Dunwiddie, P. J. Kniskern, R. B. Freedman, R. W. Ellis, and M. F. Tuite.** 1994. Using molecular genetics to improve the production of recombinant proteins by the yeast *Saccharomyces cerevisiae*. Ann. N. Y. Acad. Sci. **721:** 148–157.
36. **Shusta, E. V., R. T. Raines, A. Pluckthun, and K. D. Wittrup.** 1998. Increasing the secretory capacity of *Saccharomyces cerevisiae* for production of single-chain antibody fragments. Nat. Biotechnol. **16:**773–777.
37. **Sidrauski, C., P. Walter.** 1997. The transmembrane kinase Ire1p is a site-specific endonuclease that initiates mRNA splicing in the unfolded protein response. Cell **90:**1031–1039.
38. **Travers, K. J., C. K. Patil, L. Wodicka, D. J. Lockhart, J. S. Weissman, and P. Walter.** 2000. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell **101:**249–258.
39. **Valkonen, M., M. Penttilä, and M. Saloheimo.** 2003. Effects of inactivation and constitutive expression of the unfolded-protein response pathway on protein production in the yeast *Saccharomyces cerevisiae.* Appl. Environ. Microbiol. **69:**2065–2072.
40. **van Gemeren, I. A., A. Beijersbergen, C. A. van den Hondel, and C. T. Verrips.** 1998. Expression and secretion of defined cutinase variants by *Aspergillus awamori*. Appl. Environ. Microbiol. **64:**2794–2799.
41. **van Gemeren, I. A., P. J. Punt, A. Drint-Kuyvenhoven, M. P. Broekhuijsen, A. van't Hoog, A. Beijersbergen, C. T. Verrips, and C. A. van den Hondel.** 1997. The ER chaperone encoding *bipA* gene of black aspergilli is induced by heat shock and unfolded proteins. Gene **198:**43–52.
42. **Verdoes, J. C., A. D. van Diepeningen, P. J. Punt, A. J. Debets, A. H. Stouthamer, and C. A. van den Hondel.** 1994. Evaluation of molecular and genetic approaches to generate glucoamylase overproducing strains of *Aspergillus niger.* J. Biotechnol. **36:**165–175.
43. **Ward, M.** 1989. Heterologous gene expression in *Aspergillus*. EMBO-ALKO Workshop on Molecular Biology of Filamentous Fungi. ALKO, Espoo, Finland.
44. **Ward, M., L. J. Wilson, K. H. Kodama, M. W. Rey, and R. M. Berka.** 1990. Improved production of chymosin in *Aspergillus* by expression as a glucoamylase-chymosin fusion. Bio/Technology **8:**435–440.
45. **Wiebe, M. G., A. Karandikar, G. D. Robson, A. P. Trinci, J. L. Candia, S. Trappe, G. Wallis, U. Rinas, P. M. Derkx, S. M. Madrid, H. Sisniega, I. Faus, R. Montijn, C. A. van den Hondel, and P. J. Punt.** 2001. Production of tissue plasminogen activator (t-PA) in *Aspergillus niger*. Biotechnol. Bioeng. **76:**164–174.
46. **Witteveen, C. F. B., M. Veenhuis, and J. Visser.** 1992. Localization of glucose oxidase and catalase activities in *Aspergillus niger*. Appl. Environ. Microbiol. **58:**1190–1194.
47. **Yoshida, H., K. Haze, H. Yanagi, T. Yura, and K. Mori.** 1998. Identification of the *cis*-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins: involvement of basic leucine zipper transcription factors. J. Biol. Chem. **273:** 33741–33749.
48. **Yoshida, H., T. Okada, K. Haze, H. Yanagi, T. Yura, M. Negishi, and K. Mori.** 2000. ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the *cis*-acting element responsible for the mammalian unfolded protein response. Mol. Cell. Biol. **20:**6755–6767.