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Transcriptome Analysis of Recombinant Protein Secretion by *Aspergillus nidulans* and the Unfolded-Protein Response In Vivo

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Filamentous fungi have a high capacity for producing large amounts of secreted proteins, a property that has been exploited for commercial production of recombinant proteins. However, the secretory pathway, which is key to the production of extracellular proteins, is rather poorly characterized in filamentous fungi compared to yeast. We report the effects of recombinant protein secretion on gene expression levels in *Aspergillus nidulans* by directly comparing a bovine chymosin-producing strain with its parental wild-type strain in continuous culture by using expressed sequence tag microarrays. This approach demonstrated more subtle and specific changes in gene expression than those observed when mimicking the effects of protein overproduction by using a secretion blocker. The impact of overexpressing a secreted recombinant protein more closely resembles the unfolded-protein response in vivo.

Filamentous fungi have a high capacity for producing large amounts of extracellular proteins. A direct result of their saprophytic lifestyle is their ability to secrete a broad range of enzymes that enable them to metabolize a wide variety of extracellular polymeric substrates. Developments in molecular techniques have given rise to the potential use of filamentous fungi for production of a wide range of heterologous products (35). Production of specific nascent proteins in some industrial strains can exceed 30 g/liter (51). However, the secretion of recombinant protein to date has been far lower. Analysis of possible limiting factors point to bottlenecks at the posttranslational level (1, 17, 35), most likely along the secretory pathway. Most of our current knowledge of protein secretion comes from the study of temperature-sensitive mutants of the yeast *Saccharomyces cerevisiae* (5). Identification of a number of homologous proteins involved in secretion in other eukaryotes, including *Aspergillus niger*, indicates that the processes are highly conserved (1, 14). Despite the reasonable assumption that protein secretion in filamentous fungi will closely resemble that in yeast, the morphological and genetic differences between these organisms suggest that additional proteins or slightly different mechanisms may be involved (34).

Overexpression of recombinant proteins causes their intracellular accumulation (57), suggesting a breakdown or overloading of the secretory pathway. Some improvements in recombinant protein production have been seen from overexpressing chaperones and foldases in yeast (18, 19). However, the overexpression of equivalent proteins in aspergilli has had variable effects: some constructs showed increased intracellular recombinant protein production but no increase in extracellular levels, while others showed no improvement at all (27, 29, 36). The conflicting evidence surrounding overexpression of secretion-related genes emphasizes the complexities of protein-folding interactions, the specific effects of different products, and the possible need for a coordinated increase in expression to optimal levels (27). Accumulation of unfolded or inactive recombinant proteins puts pressure on the secretory pathway and activates the unfolded-protein response (UPR) (28, 39). The UPR triggers enhanced expression of genes involved with protein folding, secretion, and degradation (25, 48).

Previous attempts to characterize the genes associated with recombinant protein secretion in filamentous fungi have often relied on simulating the effects by inducing the UPR using secretion blockers such as dithiothreitol (DTT), brefeldin A, and tunicamycin. These methods have identified genes involved with protein folding, such as *tigA* (52) and *cypB* (7). Secretion blockers have also been used in combination with green fluorescent protein fusions to visualize unfolded protein and locate potential bottlenecks (15).

Bovine chymosin is an aspartyl protease present in the abomasum (fourth stomach) of unweaned calves and is used as a clotting agent in cheese manufacture (55). Early attempts to use the model organisms *Escherichia coli* (10, 31) and *S. cerevisiae* (13, 26) as hosts for chymosin production suffered from the problem of intracellular accumulation. Most work has since focused on various strains of *Aspergillus* (6, 9, 49, 55). Using translational fusions (57), strain improvement by mutagenesis and multicopy transformants (9, 56) has resulted in chymosin secretion up to commercial levels of over 1 g/liter.

Functional genomics reverses the normal course of genetics research by starting with the genes and attempting to define their function (32). Genomic approaches have been used previously to look at the effect of DTT on yeast growth (11) and its capacity to induce the UPR (48), providing valuable infor-

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mation on the yeast genes involved with secretion and the coordination between the UPR and endoplasmic reticulum (ER)-associated protein degradation. Most transcriptome analysis studies are performed in batch culture, where the growth rate and environment are continually changing. However, the experimental condition being studied may have an impact on the growth rate of cells. Changes in growth rate affect the pattern of gene expression; therefore, interpretation of experimental results is inherently problematic, and the use of chemostat cultures to reduce the number of conflicting variables that affect the analysis can greatly aid in data interpretation (60). Transcript profiling can be particularly beneficial to provide an overview of a pathway not previously characterized in a species with limited genome annotation and provide evidence towards functional assignments (42). Expressed sequence tag (EST) microarrays for *Aspergillus nidulans* have been constructed and thoroughly validated in our laboratory (43). The whole-genome assembly for *A. nidulans* was released in 2003 (http://www.broad.mit.edu), and the automated genome annotation predicted 9,541 putative open reading frames (ORFs). However, the majority of these ORFs are currently described as hypothetical genes of unknown function.

We describe the first attempt to analyze the influence of heterologous protein production and secretion by comparing the transcriptome of *A. nidulans* recombinant chymosin-producing and parental strains directly in continuous culture. In addition, we compare the effects on gene expression of the production of chymosin in chemostat culture with the effects of chymosin expression in batch culture in shake flasks. Finally, we evaluated how well the commonly used secretion blocker DTT was able to mimic the response to chymosin expression. By combining transcriptome data and comparative sequence analysis along with prior knowledge of the protein secretion pathway from yeast, we attempt to characterize the holistic response to recombinant protein secretion in aspergilli.

MATERIALS AND METHODS

Transcriptome analysis in shake flask (batch) and chemostat culture. A *pyrG* mutant, single-copy recombinant chymosin-producing *A. nidulans* strain (6) was compared to its parental strain transformed with an empty vector. Shake flask cultures were grown in 500 ml of SCM medium (57) in 2l flasks, and all chemostat cultures were grown in carbon-limiting 5% SCM (2.5 g/liter maltose) medium. Fermentations were performed in a temperature-, pH-, and agitation-controlled Braun Biostat M fermenter at 30°C, pH 5.5, and 1,000 rpm with a working volume of 2.1 liters and on-line carbon dioxide monitoring as previously described (43). Dry weight samples from the vessel and overflow were taken to confirm steady-state growth (with a dilution rate of 0.1 h^{-1}). Chymosin activity was measured using a simple microtiter plate method based on the method described by Emtage et al. (10).

Inducing the UPR with dithiothreitol. The secretion blocker DTT was added to the parental strain to a final concentration of 20 mM in continuous culture at steady state, and samples were removed at various time intervals over 8 h. RNA extraction, labeling, and hybridization were performed using methods described previously (21, 43). Differential gene expression was calculated after global geometric normalization in MaxDView (http://www.bioinf.man.ac.uk/microarray/). All microarray hybridizations were performed with technical (dye flips) and biological (repeated cultures or fermentations) replicates. A protocol for determining significant changes in gene expression was followed as validated previously (43). The hybridizations for the shake flask experiment were performed on a first-generation (1G) microarray containing 4,092 ESTs from a conidial library and additional *A. nidulans* sequences deposited in GenBank (43). The chemostat and DTT-induced UPR hybridizations were performed on a larger microarray with an additional 1,722 ESTs derived from negative-subtraction hybridization (37). This second-generation (2G) microarray also included additional PCR products representing 20 known secretion-related genes. These were amplified using primers designed from the *A. nidulans* genome sequence and ClustalW (46) sequence alignments to the appropriate *A. niger* genes (42).

Annotation of the microarray. BLAST was used to identify ESTs representing predicted cDNA (ORF) sequences from the *A. nidulans* genome sequence (http://www.broad.mit.edu). The SignalP program (30) was used to look for predicted prepro signal sequences. All raw data are available at the Consortium for the Functional Genomics of Microbial Eukaryotes website (http://www.cogeme.man.ac.uk). All EST and cDNA sequences are available from Pipeonline (http://bioinfo.okstate.edu/pipeonline/) and the Oklahoma EST and cDNA database (http://www.genome.ou.edu/fungal.html). Sequences were also compared for sequence similarity to GenBank and the comprehensive yeast genome database (http://mips.gsf.de/genre/proj/yeast/index.jsp).

RESULTS

Transcriptome analysis in shake flask batch culture. The effects of recombinant protein secretion were first analyzed by comparing the gene expression profiles of chymosin-producing and parental strains in shake flasks. Chymosin activity from the recombinant strain in the growth medium was first detected 20 h after inoculation and reached a level of approximately 50 mg $liter^{-1}$ after 50 h (Fig. 1). RNA was prepared from samples taken from both strains between 20 and 66 h after inoculation. A total of 292 sequences on the 1G microarray were found to have increased expression after 30 h compared to samples taken at other time points. Of these, 179 sequences had a possible identity to genes in GenBank (using BLASTx), but only 24 ESTs or PCR products corresponded to 20 previously cloned *Aspergillus* genes (Fig. 2), demonstrating the limitations of our current view of the *Aspergillus* genome sequence. Expression profiles of non-*Aspergillus* species suggested by BLAST matches may be unreliable (43). Two of these genes, the ER chaperone *bipA* and protein disulfide isomerase *pdiA*, have been previously shown to be up-regulated during recombinant protein secretion (29, 36, 52). The unknown genes that were up-regulated included some with BLAST hits to secretion-related genes including chaperones, heat shock proteins, and GTP-binding proteins from other species.

The genes that show increased expression in the chymosin-producing strain compared to its parent mostly fall into two distinct groups: those involved with the secretory pathway and those involved in metabolism. The high proportion of genes involved with metabolism is thought to be related to the fact that the increase in chymosin production coincides with the exponential growth phase. However, continual changes in cell density and medium composition during the exponential growth phase result in changes in gene expression, and it is therefore difficult to show which changes are due to recombinant protein expression and which are due to changes in other variables.

Transcriptome analysis in chemostat culture. Both parental and chymosin-producing strains were grown in carbon-limited chemostat culture at a dilution rate (equal to the specific growth rate) of 0.1 h^{-1}. The growth rate in a continuous culture is constant; therefore, samples taken at different intervals during steady state were physiologically equivalent. By comparing any samples from the two strains and calculating the mean changes, the number of possible variables can be minimized by having many biological replicates. Transcriptome analysis of both batch and con-

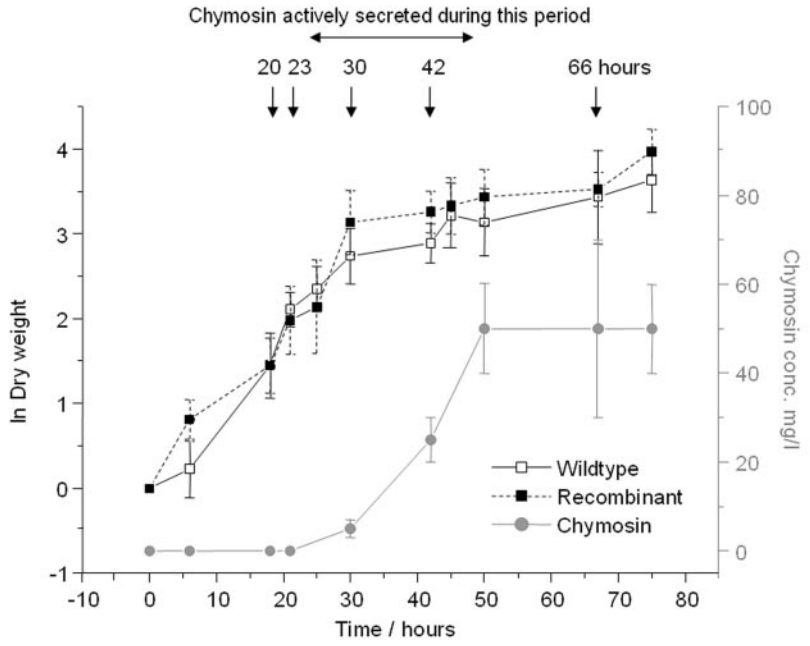

FIG. 1. Growth and chymosin production by recombinant and parental strains in batch shake flask culture. Samples were taken from both strains at times shown for comparative transcriptome analysis. ln (dry weight) and chymosin concentration are calculated from the average of three cultures, and error bars represent standard deviations.

tinuous fermentations in *A. nidulans* has shown a large reduction in noise between samples taken during steady state compared with those take during replicate batch experiments (M. E. Gent et al., unpublished observation).

Only 139 sequences present on the 1G array showed significantly increased gene expression levels in the chymosin-producing strain relative to the parental strain grown in continuous culture. This is less than half the number of transcripts

Name	GenBank Ac.	20	23	30	42	66
Alcohol dehydrogenase I *alcA*	M16196					
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Anthranilate synthase component II *trpC*	AF013602					
Branching enzyme	AB026630					
Chitin Synthase A *chsA*	D21268					
Elongation factor 3	AF035434					
ER chaperone *bipA*	Y08868					
ER chaperone *bipA*	Y08868					
ER chaperone *bipA*	Y08868					
Glyceraldehyde 3-phosphate dehydrogenase *gpdA*	M19694					
Neutral trehalase *treB*	AF043229					
Nitrate reductase *niiA*	M58289					
O-acetyl-l-homoserine sulfhydrolase *cysD*	U19394					
Phytase	U59803					
Protein disulphide isomerase *pdiA*	X98797					
Protein disulphide isomerase *pdiA*	X98797					
Pyrroline 5-carboxylate reductase *p5cr*	AJ313094					
Quinate pathway regulator *qutA*	X06252					
Ras-like protein ras?	U03025					
Septin *aspC*	AF299321					
Sterigmatocystin biosynthesis lipase/esterase *stcI*	U34740					
Terminal inverted repeat *ama1*	X78051					
Versicolorin B synthase	U51327					

FIG. 2. *Aspergillus* genes in GenBank thought to be involved in recombinant protein secretion in batch shake flask culture. Red and green represent up- and down-regulation relative to white, which is equal to no change.

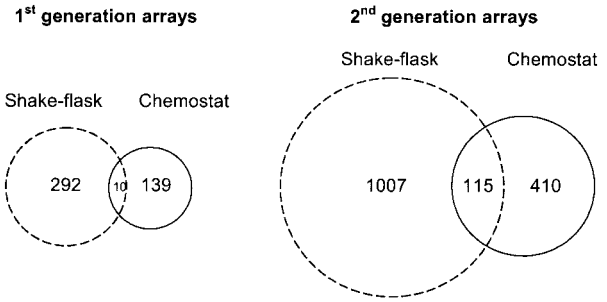

FIG. 3. Venn diagrams showing the number of sequences with increased expression when the parental and recombinant protein-producing strains in shake flask and chemostat cultures are compared. The circle area is proportional to the number of sequences. ESTs and PCR products from the first-generation arrays are also included on the second-generation arrays. Gene lists are available at http://www.cogeme.man.ac.uk.

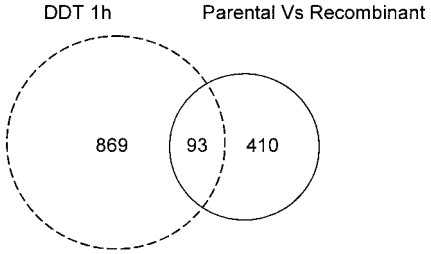

FIG. 4. Venn diagrams showing the number of up-regulated spots when different approaches for induction of UPR are compared using the G2 array. The circle area is proportional to the number of spots. Gene lists are available at http://www.cogeme.man.ac.uk.

compared to those with higher levels in the batch culture analysis (292) after 30 h (Fig. 3). However, only 10 sequences showed significantly increased levels with both batch and continuous culture methods (Fig. 3); two of these ESTs and PCR products represent the ER chaperone, *bipA*. An additional 271 (total, 410) sequences in continuous culture and over 700 additional (total, 1,007) sequences in batch culture (after 30 h) had increased expression levels in the recombinant strain compared to those in the parental strain, using the 2G array. There was a much greater overlap (115 spots [>25% of the continuous culture sequences]) of transcripts with elevated levels in both culture methods using the 2G array (Fig. 3). Analysis of the putative identities (BLAST [GenBank]) of ESTs generated by negative-subtraction hybridization appears to suggest that they represent more genes involved with secretion and the unfolded-protein response than those from the conidial library. The negative-subtraction hybridization technique actively selects for low-abundance transcripts, so many genes involved with the UPR could be expected to exhibit low levels of expression during a glucose-grown conidiating culture.

Inducing the UPR with dithiothreitol. The addition of DTT caused a significant and immediate reduction in the growth rate of the chemostat culture. However, a transcriptional response consistent with blocking secretion was not seen until 1 h after the addition of DTT, and this response was sustained at 2 and 4 h. This is slower than the response seen in similar transcriptome studies with *S. cerevisiae*. In one such study, the induction of target gene expression was essentially complete after 15 min (48), while in another study, it occurred within 30 min of DTT exposure (11). However, in both of these studies, DTT was added to the yeast cells during the mid-exponential phase, with a higher specific growth rate than that used in this study with *A. nidulans*; this would result in a quicker response. Transcription of genes encoding ribosomal proteins is directly correlated with growth rate (20, 24), and this was demonstrated 1 h after the addition of DTT to *A. nidulans*, when 34 out of 42 sequences (81%) thought to represent ribosomal genes were down-regulated.

Our transcriptome data suggest that only ~11% of the genes up-regulated in response to the addition of DTT also have increased expression levels in a chymosin-producing strain compared with its parental strain (Fig. 4). However, a greater proportion (23%) of genes with higher transcript levels in the recombinant strain also had increased expression levels in the DTT-treated culture.

Annotation of the microarray. Determining the identities of the genes that the ESTs represent is an ongoing, continually evolving process in tandem with work on the genome sequence. A major breakthrough was the release of the automated gene calls, which enabled the ESTs to be linked directly to the ORFs they represent. The estimated number of EST sequences on the array that represent ORFs (Table 1) is rather low; theoretically, every EST should represent an ORF. In reality, however, some of the ESTs may represent genes whose ORFs have not been predicted due to incorrect gene calling, small ORF size (<100 amino acids), or gaps in the genome sequence. Alternatively, poor sequencing of the ESTs themselves may mean that they fail to correspond to any part of the genome sequence. Even if all the ESTs could be matched to putative ORFs, perhaps only half of the estimated total genes in the genome would be represented on the microarray. However, not all of the predicted ORFs may result in transcribed and translated proteins.

Comparing induction of the UPR using different approaches. Transcripts of the UPR transcription factor (HacA) were significantly higher in both the chymosin-producing and DTT-treated strains, (3.2-fold and 1.7-fold, respectively). This is in accordance with the observation that, during the UPR, HacA up-regulates its own transcription (28) and demonstrates that the addition of DTT and expression of bovine chymosin both activated the UPR. Sequences representing

TABLE 1. Comparison of the annotation of the microarrays

Characteristic	No. (%) of sequences detected by microarray	
	1G	2G
Total no. of spots	4,352	6,272
No. ESTs	3,752	5,579
No. PCR products	340	363
Empty/DMSO[a]	260	330
Duplicated sequences	520 (13)	622 (10)
Unique sequences	3,572 (87)	5,320 (90)
Sequences representing ORFs	3,304 (81)	4,597 (77)
Unique *A. nidulans* ORFs represented (of predicted 9,541)	2,080 (22)	2,790 (29)

[a] DMSO, dimethyl sulfoxide.

many known *A. nidulans* GTP-binding proteins, cyclophilins, and heat shock proteins (SrgA, SrgB, SrgE, VpsB, CypA, PinA, and HscA) had increased transcript levels with both approaches (Table 2 and Fig. 5). The protein disulfide isomerase genes PdiA (AN0248.2), PrpA (AN0075.2), and TigA (AN3592.2) all had significantly elevated transcript levels in response to chymosin expression, whereas only PdiA was up-regulated in response to DTT addition. Calnexin was not seen to be up-regulated with either approach despite having a putative UPR element in its promoter sequence and a previous report of elevated mRNA levels in a strain expressing bovine prochymosin fused to the catalytic domain of glucoamylase (53). Only a few putative orthologs of genes that encode proteins in the ER-associated degradation pathway (ERAD) were represented on the array. However, transcript levels of a putative ortholog of *HRD3* were significantly increased in both the DTT-induced and chymosin-producing strains, confirming previous observations that the UPR and ERAD are intimately linked (2, 48).

Comparative genomic analysis of several *Aspergillus* species has demonstrated that the genes involved with targeting to the ER in aspergilli are highly conserved with those previously identified in yeast (40). Two routes for transporting of proteins across the ER membrane in *S. cerevisiae* have been described (44). Identification of *A. niger* homologs to *S. cerevisiae* Srp54p (47) and Kar2p/BipA (52) suggested that both signal recognition particle-dependent and -independent routes are also present in filamentous fungi. Conesa et al. (5) stated that "whether these two ER targeting mechanisms also operate in filamentous fungi remains to be determined." However, identification of putative orthologs to all the major proteins involved with ER targeting following genome sequencing (Table 2) confirms that both mechanisms are conserved in aspergilli. Multiple sequence alignments of ORFs from several fungal species demonstrated that *A. nidulans* AN7623.1 represents two independent hypothetical proteins (40). The second ORF (beginning after 308 amino acids) represents a putative ortholog to the yeast translocation protein *SEC67/72*, while the first represents a hypothetical protein with a corresponding transcript sequence (contig 597) in the University of Oklahoma cosmid and cDNA sequencing database. Prediction of multiple genes as a single ORF in the *A. nidulans* genome has been reported (12). ORF AN6720.2 clearly represents three distinct genes, specifying a homolog of fructose-2,6-bisphosphatase, AvaB (EMBL accession number AB090888), and HbrB, a probable vacuolar sorting protein involved with hyperbranching mutants (12). It is thought that AN0643.2, a predicted 258-codon ORF, represents the ortholog of Sec65p. The automated annotation also predicted an overlapping 1,388-codon ORF (AN0642.2), with the first 236 residues identical to the secretion protein, which is most likely an error in the gene prediction algorithm. Unfortunately, only one of the putative genes involved with ER targeting, the ortholog to *SEC63* (AN0834.2), appears to be present on the array. This gene was significantly up-regulated (5.47-fold) following the addition of DTT but was not differentially expressed in the comparison between the chymosin-producing and parental strains.

Additional known vesicle-trafficking genes (*sagA* and *srgD*) and sequences thought to represent orthologs of other secretion and ERAD-related genes (*SHR3*, *SLC1*, *EPT1*, *FMP50*, *UBA1*, and *RPT3*) had greater changes (severalfold) in the DTT-induced rather than recombinant protein secretion-induced UPR (Table 2 and Fig. 5). This suggests that the addition of DTT causes much greater and less-specific disruption of the secretory pathway than secretion of a recombinant protein. Secretion-related ORFs that showed particularly high (severalfold) changes in response to recombinant protein secretion were AN7683.2, a putative ortholog of *NCE102*, involved in a nonclassical protein export pathway, and AN5972.2, a putative ortholog of *SEC27*, the coatomer complex of β-chain vesicles. A putative *A. nidulans* ortholog to *INO1* (AN7625.2), an inositol-1-phosphate synthase, was significantly down-regulated by the addition of DTT but had increased transcript levels in the recombinant protein-secreting strain, confirming previous observations that the UPR signal transduction pathway is not involved directly in transcriptional regulation of *INO1* (3).

Repression of secretion under stress. To fully understand the effects of recombinant protein production on cells, it is also highly valuable to look at the transcripts that are down-regulated, particularly those encoding secreted proteins, which have previously been shown to be significantly down-regulated following inhibition of secretion (25). This observation has given rise to a novel feedback mechanism, repression of secretion under stress (33). All sequences that had reduced transcript levels and were assigned to ORFs were searched for predicted signal sequences using SignalP (30). Of the arrayed sequences significantly down-regulated after the addition of DTT and during recombinant protein secretion, 22 out of 102 (22%) (Fig. 6) were predicted to encode secreted proteins. Most of these sequences represent unknown genes, although ORFs of known function included xylanase 2 and several putative secreted proteases. Predicted secreted proteins that had significantly reduced transcript levels in the recombinant strain in the chemostat comparison included xylanase 1, α-amylase, and cellobiose hydrolase, which were all previously reported to show reduced expression when protein secretion is inhibited in *Trichoderma reesei* (33).

DISCUSSION

A large degree of variation was seen in the gene expression profiling using different approaches. However, by combining multiple approaches and excluding growth rate effects, it was possible to obtain a clearer view of the transcriptional effects of secreting recombinant proteins in vivo. Less than half the number of genes showed increased gene expression in a recombinant protein-producing strain in continuous, rather than batch, culture conditions. A chemostat approach allows experimental growth conditions to be carefully controlled, thereby greatly reducing the complexity of the system and allowing a much more focused analysis of the factors affecting gene expression (20). An additional advantage of chemostat cultures for hybridization array experiments is that the turnover rate ensures that the average transcript profile will more closely represent the current conditions.

While many of the severalfold changes appear relatively low, these values have a high degree of statistical assurance, as discussed previously (43). In addition to producing data congruent with the activation of the UPR, we have also highlighted the subtle differences between the two strains that are

TABLE 2. Changes (*n* fold) in transcript levels of *A. nidulans* secretion-related genes and putative yeast orthologs due to recombinant protein secretion and/or induction of the UPR with DTT[h]

Function	Yeast gene	*A. nidulans* ORF	ID or source	Change (fold)[e]	
				Chemostat	DTT (1h)
Protein folding/UPR					
ER chaperone ***bipA***	*KAR2*	AN2062.2	2000Sep131300_3520[a]	1.31[e]	3.90[e]
			2000Sep131300_240[a]	1.21[e]	2.64[e]
Protein disulfide isomerase ***pdiA***[h]	*PDI1*	AN7436.2	PCR	3.24[e]	1.33[e]
			2000Sep131300_1629[a]	−1.01	1.30[e]
Protein disulfide isomerase ***prpA***	—[j]	AN0248.2	PCR	2.57[e]	1.06
Protein disulfide isomerase ***tigA***	—[j]	AN0075.2	PCR	1.49[e]	−1.24[e]
Calnexin—chaperone, folding ***clxA***	*CNE1*	AN3592.2	2000Sep131300_3254[a]	1.00	−1.02
Chaperone of the ER lumen	*CER1*	AN0847.2	—[j]	—[j]	—[j]
Cyclophilin	*CPR1*	AN8605.2	2000Sep131300_1548[a]	1.02	1.79[e]
Cyclophilin ***cypA***	*CPR3*	AN3814.2	pncs916aG10.SP6[a]	1.21[e]	1.27[e]
Cyclophilin (*cis-trans*)	*CPR8*	AN4467.2	***cypB*** **(AF107254)**[d]	—[j]	—[j]
Peptidyl-prolyl *cis-trans* isomerase D	*CPR5*				
Peptidyl-prolyl *cis-trans* isomerase	*CPR2*				
Member of the cyclophilin family	*CPR6*	AN4583.2	pncs905aE03.SP6[a]	1.37[e]	4.71[e]
Member of the cyclophilin family	*CPR7*		N12F7 SP6[a]	1.23[e]	1.58[e]
Peptidyl-prolyl *cis-trans* isomerase precursor	*CPR4*		Contig 111[b]	—[j]	—[j]
PPlase, FK506-binding protein	*FPR1*	AN3598.2	2000Sep131300_1483[a]	−1.02	−1.38[e]
FK506/rapamycin-binding protein of the ER	*FPR2*	AN8343.2	—[j]	—[j]	—[j]
Proline *cis-trans* isomerase	*FPR3*	AN3908.2	k0f12a1.f1[b]	—[j]	—[j]
Nucleolar peptidyl-prolyl *cis-trans* isomerase	*FPR4*				
Mitotic peptidyl-prolyl *cis-trans* isomerase ***pinA***	*PIN1*	AN6145.2	PCR	1.68[e]	1.99[e]
Stress response transcription factor ***srrA***	*SKN7*	AN3688.2	2000Sep131300_325[a]	1.46[e]	—[j]
Heat shock protein, 70 kDa	*SSA1*	AN5129.2	N8G4.SP6[a]	1.36[e]	1.52[e]
	SSA2		SETIIpL.3-B10[a]	1.21[e]	2.03[e]
	SSA3		10_2_C5.SP6[a]	1.27[e]	1.30[e]
	SSA4		N5G5.SP6[a]	1.17	1.23[e]
Heat shock protein, 70 kDa	*SSB1*	AN4616.2	Pncs916aH11[a]	—[j]	—[j]
Heat shock protein, 70 kDa, ***hscA***	*SSB2*	X98931[c]	PCR	5.96[e]	2.07[e]
Heat shock protein, 70 kDa	*ECM10*	AN6010.2	Pncs920aB09[a]	1.37[e]	1.14[e]
Heat shock protein, 60 kDa	*HSP60*	AN6089.2	N15GE11.SP6[a]	1.34[e]	2.09[e]
			N13C11.SP6[a]	1.37[e]	2.00[e]
			N5G7.SP6[a]	1.21[e]	1.86[e]
			SETIPL2G2.SP6[a]	1.69[e]	1.72[e]
Heat shock protein, 30 kDa	—[j]	AN5781.2	N14GA2.SP6[a]	1.32[e]	2.42[e]
			10_1_C8[a]	1.30[e]	1.21[e]
UPR transcription factor ***hacA***	*HAC1*	AN9397.2	PCR	3.24[e]	1.73[e]
Serine/threonine protein kinase UPR	*IRE1*	AN0235.2	2000Sep131300_4522[a]	−1.04	−1.16
Translocation					
Translocon (alpha subunit)	*SEC61*	AN7721.2	Contig 212[b]	—[j]	—[j]
Translocon (beta subunit)	*SSS1*	AN4589.2	—[j]	—[j]	—[j]
Translocon (gamma subunit)	*SBH1*	AN0417.2	—[j]	—[j]	—[j]
ER protein-translocation complex subunit	*SEC62*	AN6269.2	—[j]	—[j]	—[j]
ER protein-translocation complex subunit	*SEC63*	AN0834.2	2000Sep131300_564[a]	1.04	5.47[e]
ER protein-translocation complex subunit	*SEC71*	AN1442.2	c8a09a1.f1[b]	—[j]	—[j]
ER protein-translocation complex subunit	*SEC72*	AN7623.2	—[j]	—[j]	—[j]
Signal sequence processing protein	*SEC11*	AN3126.2	—[j]	—[j]	—[j]
19-kDa signal recognition particle	*SEC65*	AN0643.2	—[j]	—[j]	—[j]
Signal sequence receptor alpha subunit	*SRP101*	AN6627.2	—[j]	—[j]	—[j]
Signal recognition particle protein ***srpA***	*SRP54*	AN8246.2	Contig 1155[b]	—[j]	—[j]
Vacuolar protein sorting					
Sec1-like protein (Golgi to endosome) ***vpsA***	*VPS45*	AN6531.2	f5f09a1.r1[b]	—[j]	—[j]
VPS plasma membrane to endosome ***sagA***	*END3*	AN1023.2	PCR	−1.01	1.20[e]
VPS (Golgi to plasma membrane)	*SEC1*	AN4724.2	—[j]	—[j]	—[j]
VPS (endosome to vacuole)	*VPS4*	AN3061.2	—[j]	—[j]	—[j]
VPS (Golgi to vacuole)	*VPS41*	AN4876.2	2000Sep131300_2938[a]	1.06	1.32[e]
VPS (vacuolar sorting protein)	*VPS10*	AN6118.2	—[j]	—[j]	—[j]
Probable VPS,[g] *S. pombe* (T50328)[i]	—[f]	AN0854.2	SETIPL3G1.SP6[a]	1.23[e]	1.27[e]
Sorting nexin, retrieval from endosomes	*SNX41*	AN6351.2	pncs902aB07.SP6[a]	3.23[e]	1.24[e]
Probable vacuolar sorting protein ***hbrA***	*VPS33*	AN2418.2	PCR	1.20[e]	—[j]
Protein degradation					
ER degradation	*DER1*	AN8852.2	y4h09a1.f1[b]	—[j]	—[j]
Ubiquitin-conjugating enzyme (*UBC7* ortholog)	*UBC7*	AN5351.2	—[j]	—[j]	—[j]

Continued on facing page

TABLE 2—*Continued*

Function	Yeast gene[f]	*A. hidulans* ORF	ID or source	Change (fold)[e]	
				Chemostat	DTT (1h)
Ubiquitin-conjugating enzyme (*UBC7* paralog)	—[j]	AN8258.2	Contig 1112[b]	—[j]	—[j]
Zinc finger for ER degradation E3	*HRD1*	AN1488.2	Contig 794[b]	—[j]	—[j]
HMG-CoA[g] reductase degradation	*HRD3*	AN0810.2	pncs911aB01.SP6[a]	1.49[e]	1.53[e]
Ubiquitin-specific protease	*DOA4*	AN2072.2	—[j]	—[j]	—[j]
E2 ubiquitin-conjugating enzyme—peroxin	*PEX4*	AN8258.2	—[j]	—[j]	—[j]
Ubiquitin-activating enzyme	*UBA1*	AN2174.2	2000Sep131300_1506[a]	1.02	1.87[e]
26S proteasome regulatory subunit	*RPT3*	AN2904.2	2000Sep131300_1182[a]	1.05	1.31[e]
E2 ubiquitin-conjugating enzyme	*UBC12*	AN5344.2	2000Sep131300_790[a]	1.07	1.01
Cellular export and secretion					
Reticulocyte binding protein	*SEC7*	AN6709.2	—[j]	—[j]	—[j]
Secretory component protein, putative	*SHR3*	AN1845.2	2000Sep131300_3678[a]	1.00	1.27[e]
Secretory pathway GDP-dissociation inhibitor	*GDI1*	AN5895.2	—[j]	—[j]	—[j]
Putative myo-inositol-1-phosphate synthase	*INO1*	AN7625.2	2000Sep131300_3013[a]	1.22[e]	−1.29[e]
			N1C4.SP6[a]	1.22[e]	−1.29[e]
Cytosolic factor phosphatidylinositol transfer	*SEC14*	AN0243.2	—[j]	—[j]	—[j]
Putative secretory component ***nsfA***	*SEC18*	AN3098.2	pncs902aE06.SP6[a]	1.20[e]	1.24[e]
Involved in nonclassical protein export pathway	*NCE102*	AN7683.2	2000Sep131300_1208[a]	8.90[e]	—[j]
Secretory pathway Ca^{2+}-ATPase ***pmrA***	*PMR1*	AN7464.2	—[j]	—[j]	—[j]
Glycosylation/modification					
Oligosaccharyltransferase beta subunit	*WBP1*	AN4683.2	—[j]	—[j]	—[j]
Oligosaccharyltransferase delta subunit	*SWP1*	AN1683.2	—[j]	—[j]	—[j]
Oligosaccharyltransferase epsilon subunit	*OST2*	AN4031.2	2000Sep131300_883[a]	−1.02	−1.25
Required for asparagine-linked glycosylation	*ALG11*	AN5725.2	aD02.b1.SP6[a]	1.06	−1.26
Mannosyltransferase	*ALG2*	AN6874.2	2000Sep131300_4144[a]	1.01	−5.64[e]
Regulates mannosylphosphorylation	*MNN4*	AN4030.2	2000Sep131300_2411[a]	1.01	1.02
Mannose-1-phosphate guanyltransferase	*PSA1*	AN1911.2	contig 627[b]	1.22[e]	—[j]
Vesicle trafficking/transport					
GTPase (ER to Golgi) ***sarA***	*SAR1*	AN0411.2	2000Sep131300_2639[a]	1.02	1.00
GTPase (ER to golgi Rab2 homolog) ***srgD***	*rab2*[f]	AN5106.2	2000Sep131300_2624[a]	1.06	1.35[e]
GTPase (*cis* to medial Golgi) ***srgB***	*YPT1*	AN4281.2	PCR	1.22[e]	1.43[e]
GTPase (Golgi to plasma membrane) ***srgA***	*SEC4*	AN6974.2	PCR	1.70[e]	1.38[e]
GTPase (intra-Golgi) ***srgE***	*YPT31*	AN0347.2	PCR	1.49[e]	2.27[e]
	YPT32				
GTPase (Golgi to vacuole) ***srgC***	*YPT6*	AN7602.2	—[j]	—[j]	—[j]
GTPase (late endosome to vacuole) ***avaA*/*srgF***	*YPT7*	AN0089.2	—[j]	—[j]	—[j]
GTPase (plasma membrane to endosome) *srgG*	*YPT51*	AN4915.2	2000Sep131300_3122[a]	−1.05	−1.22[e]
	YPT53				
GTPase (early to late endosome) *srgH*	*YPT52*	AN3842.2	N8G5.SP6[a]	1.24[e]	−1.03
GTPase (late endosome to plasma membrane) *srgL*	*rab11*[f]	AN0069.2	—[j]	—[j]	—[j]
Protein transport protein	*SEC13*	AN4317.2	pncs912aF05.SP6[a]	1.04	1.07
Targeting and fusion of ER-Golgi vesicles	*TRS120*	AN6533.2	2000Sep131300_717[a]	1.24[e]	−1.29[e]
ER lumen protein-retaining receptor	*ERD2*	AN9415.2	pncs920aA07.SP6[a]	−1.02	−1.29
Coatomer complex beta chain of vesicles	*SEC26*	AN1177.2	2000Sep131300_2823[a]	−1.03	1.05
Coatomer complex beta chain of vesicles	*SEC27*	AN5972.2	pncs917aB12.SP6[a]	12.82[e]	1.46[e]
ADP-ribosylation factor-like protein, *ras*	*ARL3*	AN1126.2	2000Sep131300_1470[a]	−1.06	−1.26[e]
Putative syntaxin (T-SNARE) protein (ssoA?)	*SSO1*	AN3416.2	2000Sep131300_1894[a]	−1.08	−1.22[e]
	SSO2				
Lipid/inositol metabolism					
sn-1,2-Diacylglycerol ethanolamine	*EPT1*	AN4778.2	pncs906aE03.SP6[a]	1.28[e]	−1.25[e]
Fatty acyltransferase	*SLC1*	AN6139.2	2000Sep131300_2940[a]	−1.05	1.69[e]
3-Hydroxy-3-methylglutaryl-coenzyme A reductase 2	*HMG2*	AN3817.2	2000Sep131300_2868[a]	1.27[e]	−1.84[e]
Cell wall biogenesis					
Involved in cell wall biogenesis, architecture	*ECM3*	AN0930.2	N1H1.SP6[a]	1.52[e]	1.20[e]
P-type ATPase—unknown function	*SPF1*	AN3146.2	pncs919aG12.SP6[a]	1.34[e]	1.07
Strong similarity to Chs6p FMP50	*FMP50*	AN3122.2	pncs913aA03.SP6[a]	−1.01	3.39[e]

[a] Pipeonline (http://bioinfo.okstate.edu/pipeonline).
[b] Oklahoma cDNA and EST sequencing database (http://www.genome.ou.edu/fungal.html).
[c] Not predicted by automated annotation.
[d] GenBank accession number.
[e] Significant fold change (43).
[f] No homolog in *S. cerevisiae*.
[g] VPS, vacuolar protein sorting.
[h] Boldface type indicates previously cloned *Aspergillus* genes.
[i] *Schizosaccharomyces pombe*.
[j] —, no data available.

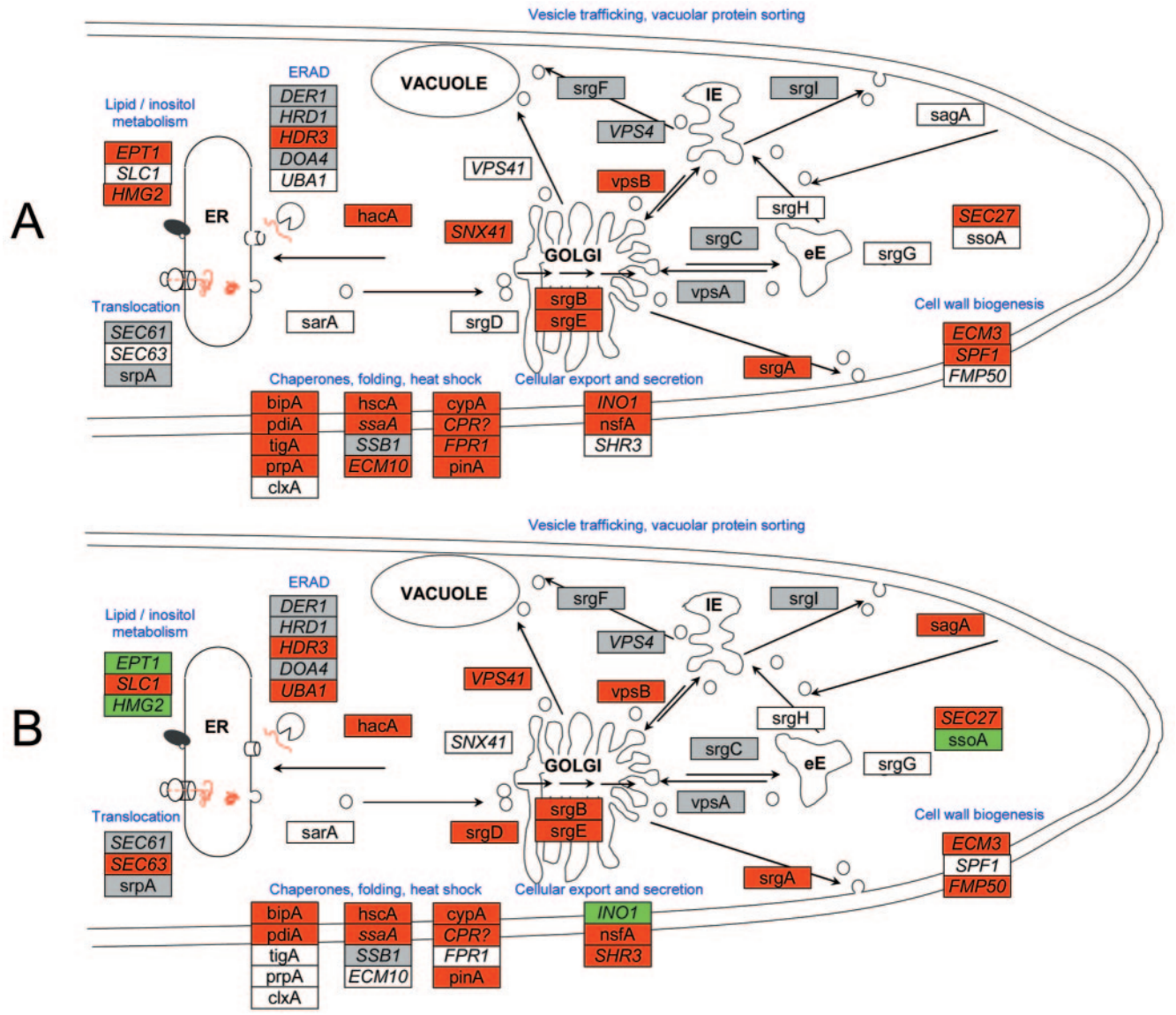

FIG. 5. Model of the secretory pathway and UPR under different conditions. (A) Recombinant protein secretion induction of UPR. (B) Secretion blocker induction of UPR. Boxes represent known and putative secretion genes. Orthologs to yeast genes are in italics. Red, genes up-regulated; green, genes down-regulated; white, no significant change; grey, not present on the microarray. lE, late endosome, eE, early endosome.

discernible under more highly controlled chemostat conditions compared to the physiologically more variable conditions in shake flasks or with nonselective secretion blockers such as DTT.

A large number of secretion-related genes previously characterized in *S. cerevisiae* appear to be highly conserved in *A. nidulans*. If predicted ORFs have significant sequence similarity and correlated expression profiles when subjected to the same conditions as those for previously characterized genes from other species, then it can be assumed that these genes represent conserved orthologs allowing putative functional assignments. Analysis of EST sequences can be highly valuable for identifying errors in the predicted coding sequences of the automated genome annotation (40). Transcriptome data can be used effectively to provide supporting evidence for func-

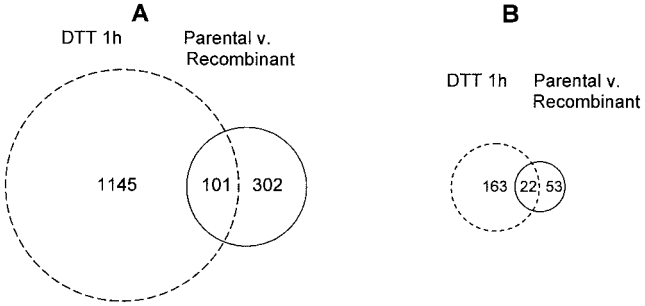

FIG. 6. Venn diagrams showing the number of transcripts with reduced levels when induction of the UPR is compared using the 2G array. (A) All spots. (B) Spots representing secreted proteins. The circle area is proportional to the number of spots. Gene lists are available at http://www.cogeme.man.ac.uk.

tional assignments and can help to identify genes which are limited to the filamentous fungi (41, 42). For example, vesicle transport in aspergilli is thought to be more complex than that in yeast due to the greater abundance of secreted proteins, the filamentous morphology, and polarized apical tip growth. For example, a homolog to mammalian *rab2* GTPase (SrgD) and to the mammalian annexin family (34) have been reported in aspergilli but are absent from the genome of *S. cerevisiae*. Many of the secretion-related genes identified for *A. nidulans* (Table 2) have also been characterized in *T. reesei* by linking the BLAST hits to ESTs with gene ontology terms (8), thus demonstrating a high level of conservation.

Prior to whole-genome sequencing, a number of *Aspergillus* orthologs to secretion-related GTPases had been identified. There are 10 or 11 Ypt/Rab genes in yeast (23) and ~50 secretion-related GTPases in mammalian cells (45). These genes are responsible for different steps in protein trafficking and were isolated from secretion-defective mutants which accumulated proteins at different stages of the secretory pathway (5). It was very straightforward to assign *A. nidulans* ORFs as orthologs to the Ypt/Rab genes previously identified in *A. niger*. Some of these GTPases, which are thought to have the same or similar function in *S. cerevisiae*, appear to have a single homolog in aspergilli. The GTPase *srgE* is an ortholog of Ypt31p and Ypt32p in yeast; these genes share an identical effector region, and single-gene disruptions are phenotypically neutral, while disruption of both is fatal (16). Two *A. nidulans* ORFs were identified as putative orthologs of two of the remaining yeast GTPases; one ORF, provisionally named SrgG (AN4915.1), appears to represent *YPT51* and *YPT53*, which are thought to be remnants of the whole-genome duplication event in yeast (59), and the other, SrgH (AN3842.1), is thought to represent *YPT52*. Fragments of cDNA that represent these ORFs were predicted to encode GTPases as described previously by Punt et al. (34) and have now been assembled to full-length cDNAs. (contigs 1227 and 1665, respectively, in the Oklahoma cDNA sequencing database). Both sequences are also represented by EST sequences on the 2G microarray but were not significantly differentially expressed either during chymosin expression or following DTT treatment. SrgI is a putative homologue of Rab11A, which is found only in mammals and targets to the membrane from the recycling complex (38). The only GTPase showing differential expression in response to UPR induction in *S. cerevisiae* was Ypt10p (48), but this does not appear to be conserved in filamentous fungi. Both *YPT10* and *YTP11* have no mutant phenotypes and appear to be nonessential in yeast (23). Many of the previously identified GTPases had increased transcript levels upon UPR induction in this study, suggesting a greater role for vesicle trafficking in the *Aspergillus* UPR.

Peptidylpropyl isomerases catalyze the isomerization of *cis* and *trans* peptide bonds on the N-terminal side of proline residues (5) and are thought to be involved in secretion and the unfolded-protein response. In yeast, eight cyclophilins and four FK506-binding proteins have been identified. However, in the Aspergilli, putative orthologs of only four cyclophilins and three FK506-binding proteins could be found in the whole-genome sequences (Table 2). It is not clear whether these discrepancies are due to duplication of the yeast genes or errors in gene calling for aspergilli. The cyclophillin CypB has been previously isolated from both *A. niger* and *A. nidulans* (7, 22), but it appears to be homologous to three distinct genes in yeast. The CypB protein has a 23-amino-acid ER retention sequence, and the 5′-untranslated region includes three sequences resembling UPR elements. Expression of *cypB* is upregulated in the presence of tunicamycin and DTT (7) and during the production of tissue plasminogen activator (58). However, no representative sequences were present on the microarray, and when *cypB* was overexpressed in a tissue plasminogen activator-producing strain, the level of the product was not increased. The heat shock protein HscA, a 70-kDa homolog to yeast Ssb2p (Table 2), is conserved in *A. nidulans* (GenBank accession no. X98931). However, it appears that the gene overlaps the far end of supercontig 1.22 and so has not been assigned an ORF by the Broad annotation. Only one ORF was identified to represent the yeast heat shock proteins Ssa1p to Ssa4p. This ORF (AN5129.2), putatively named SsaA, was represented by four different ESTs that all had increased transcript levels under both DTT and recombinant protein UPR induction.

Chymosin contains three disulfide bonds, one of which is indispensable for correct protein folding (4), which would explain why mRNAs for the protein disulfide isomerases PdiA, TigA, and PrpA were all significantly higher in the recombinant protein-producing strain than in the parental strain (Table 2). Disruption of *prpA* has been shown to reduce bovine chymosin production in *A. niger*, but overexpression had little effect (54). Perhaps coregulation is important, since constitutive expression of the entire UPR in a recombinant *Aspergillus niger* var. *awamori* strain resulted in a 2.8-fold increase in chymosin yield (50).

Our global view of transcript levels implicate many secretion-related genes being involved with the UPR response as a result of recombinant protein secretion. Previous studies have shown that secretion of a variety of recombinant proteins can induce the UPR (28, 50) and may indicate that there are both general and recombinant product-specific responses, which cannot be mimicked by using a secretion blocker.

From our study and previous work involving yeast (11, 48), it seems clear that a large number of genes are involved in the UPR. Travers et al. (48) reported transcriptome analysis of the UPR induced by DTT and tunicamycin in *S. cerevisiae*. Their study identified 381 genes apparently involved in the UPR, with just over a quarter of these genes (103) known to be associated with the secretory pathway, UPR, or ERAD. They conceded that the high stringency of their data analysis criteria resulted in an underestimation of the full scope of the UPR (48). Indeed, transcript levels for *KAR2*, *INO1*, and *HAC1* were not significantly elevated, despite the observations of other studies (11, 28). Our data lead us to question whether all of the genes identified in previous experiments are really associated with the UPR and suggest that many may be artifacts of the particular experimental approach employed.

From the data presented here, expression profiling of different strains, either those lacking secretion-related genes or those secreting alternative recombinant products, may represent the most reliable approaches for the future. Our data are consistent with the idea that the UPR has at least three effects: to improve protein folding and transport, to degrade unfolded proteins, and to allow fewer secretory proteins to enter the ER

(25). We have demonstrated both similarities and differences in the genes induced by the UPR using different approaches and have highlighted the conservation of the secretory pathway in filamentous fungi. Our data stress the importance of minimizing the number of confounding variables in microarray experiments. While the addition of DTT does induce the UPR, it also retards growth of the mycelia, causing wide-ranging effects on the gene expression profile. A much smaller number of genes were significantly differentially expressed by inducing the UPR with the secretion of a recombinant protein than by the addition of a secretion blocker. A greater proportion of the genes identified represent known UPR or secretion-related genes, demonstrating the more subtle and specific effects of the UPR in vivo.

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