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BIOTECHNOLOGY

FlbA-Regulated Gene *rpnR* Is Involved in Stress Resistance and Impacts Protein Secretion when *Aspergillus niger* Is Grown on Xylose

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ABSTRACT Proteins are secreted throughout the mycelium of *Aspergillus niger* except for the sporulating zone. A link between sporulation and repression of protein secretion was underlined by the finding that inactivation of the sporulation gene *flbA* results in mycelial colonies that secrete proteins throughout the colony. However, Δ*flbA* strain hyphae also lyse and have thinner cell walls. This pleiotropic phenotype is associated with differential expression of 36 predicted transcription factor genes, one of which, *rpnR*, was inactivated in this study. Sporulation, biomass, and secretome complexity were not affected in the Δ*rpnR* deletion strain of the fungus. In contrast, ribosomal subunit expression and protein secretion into the medium were reduced when *A. niger* was grown on xylose. Moreover, the Δ*rpnR* strain showed decreased resistance to H_2O_2 and the proteotoxic stress-inducing agent dithiothreitol. Taking the data together, RpnR is involved in proteotoxic stress resistance and impacts protein secretion when *A. niger* is grown on xylose.

IMPORTANCE *Aspergillus niger* secretes a large amount and diversity of industrially relevant enzymes into the culture medium. This makes the fungus a widely used industrial cell factory. For instance, carbohydrate-active enzymes of *A. niger* are used in biofuel production from lignocellulosic feedstock. These enzymes represent a major cost factor in this process. Higher production yields could substantially reduce these costs and therefore contribute to a more sustainable economy and less dependence on fossil fuels. Enzyme secretion is inhibited in *A. niger* by asexual reproduction. The sporulation protein FlbA is involved in this process by impacting the expression of 36 predicted transcription factor genes. Here, we show that one of these predicted transcriptional regulators, RpnR, regulates protein secretion and proteotoxic stress resistance. The gene is thus an interesting target to improve enzyme production in *A. niger*.

KEYWORDS asexual development, aspergillus, *flbA*, fungus, protein secretion, proteotoxic stress

Fungi in the genus *Aspergillus* feed on organic waste and can be opportunistic pathogens of plants, animals, and humans (1). They secrete a large amount and diversity of enzymes that are instrumental in these lifestyles and that also make *Aspergillus* species, such as *Aspergillus niger*, important cell factories (2, 3). However, the costs of these enzymes can still be high. For instance, they represent up to 28% of the costs of biofuel production from lignocellulosic biomass (4). The biofuel industry, therefore, is in need of reduced production costs.

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Asexual development in *A. niger* is initiated as a response to changing environmental conditions and as a result of an environment-independent differentiation program (1). It results in conidiophores that form conidia. These spores are heterogeneous in cellular composition and germination rate (5); are dispersed by wind, water, and insects; and give rise to new mycelia. The mycelia, also known as colonies, consist of zones that show heterogeneity in function and composition (3). For instance, the sporulation zone within a colony does not secrete proteins, while the neighboring nonsporulating zones do release proteins into the medium (6). Covering the mycelium with a porous membrane prevents sporulation but does not affect spatial secretion within the mycelium. Apparently, the capacity to sporulate, but not the sporulation process itself, represses secretion.

FlbA was shown to control asexual reproduction, to repress protein secretion in the sporulation zone, to activate mycotoxin production, to inhibit vegetative growth and autolysis, and to impact cell wall architecture (6–9). It does so by activating the Gα subunit FadA, which is at the start of a signaling pathway. A total of 36 predicted transcription factor genes are differentially expressed when the Δ*flbA* strain is grown on xylose medium, 18 of which are downregulated and 18 upregulated (10, 11). The most downregulated predicted transcription factor gene, *fum21*, is involved in production of the mycotoxins fumonisin and pyranonigrin A, but it does not affect sporulation, secretion, vegetative growth, autolysis, and cell wall architecture (9). Here, we show that the predicted transcription factor *rpnR*, which is upregulated in the Δ*flbA* strain (10), is involved in proteotoxic stress resistance and impacts protein secretion when *A. niger* is grown on xylose.

RESULTS

Inactivation of *rpnR*. The predicted transcription factor gene An08g06850 (12) is upregulated 9-, 11-, and 6-fold in the central, middle, and outer concentric zones, respectively, of Δ*flbA* strain colonies, making it the most upregulated transcription factor in the strain (10). The protein encoded by An08g06850 showed a bidirectional hit with Rpn4p of *Saccharomyces cerevisiae* (13) and Rpn4 of *Neurospora crassa* (14). The former protein was shown to be an activator of 26S proteasome subunit expression. The function of the *N. crassa* orthologue is not known, but it binds to the same DNA sequences as Rpn4p of *S. cerevisiae* (14). The proteins of *A. niger*, *S. cerevisiae*, and *N. crassa* are 701, 531, and 714 amino acids long. The zinc finger C2H2 domains share 44% and 77% identity, respectively, with that of An08g06850, while the overall identities are 16% and 45%. Based on these data, An08g06850 is now called *rpnR*. The gene was inactivated in the wild-type strain MA234.1, resulting in the Δ*rpnR* strain. Deletion of the gene was verified by PCR (see Fig. S1 in the supplemental material). Two independent complemented strains showed restoration of the wild-type phenotype compared to the Δ*rpnR* strain phenotypes described below.

Phenotypic analysis of the Δ*rpnR* strain. Growth of the Δ*rpnR* strain was assessed using minimal medium with xylose as a carbon source (MM-X). The sugar was chosen because differential expression of *rpnR* in the Δ*flbA* strain was found on this carbon source (10). On MM-X agar (MM-XA) plates, the Δ*rpnR* strain formed colonies with a smaller diameter, but the total biomass was greater than that of the wild type. The diameters of 7-day-old wild-type and Δ*rpnR* strain colonies were 6.08 $\pm$ 0.20 cm and 5.11 $\pm$ 0.34 cm ($n = 6$; $P < 0.05$), whereas the biomasses were 27.4 $\pm$ 7.5 mg and 37.4 $\pm$ 4.5 mg ($n = 8$; $P < 0.05$), respectively. When xylose was replaced with 1% beechwood xylan, no difference in biomass was found, but the Δ*rpnR* strain diameter was again smaller (3.96 $\pm$ 0.05 versus 3.66 $\pm$ 0.11 cm [$n = 5$; $P < 0.05$]). Together, these data show that the Δ*rpnR* strain grows more compactly than the wild type. Both the wild type and the Δ*rpnR* strain sporulated in the subperipheral zone and the centers of the colonies (Fig. 1A and B), with similar production of spores per square centimeter (Fig. 1C).

Since Rpn4 of *S. cerevisiae* activates expression of 26S proteasome subunits, it was hypothesized that the Δ*rpnR* strain would cope less well with unfolded proteins, i.e., its

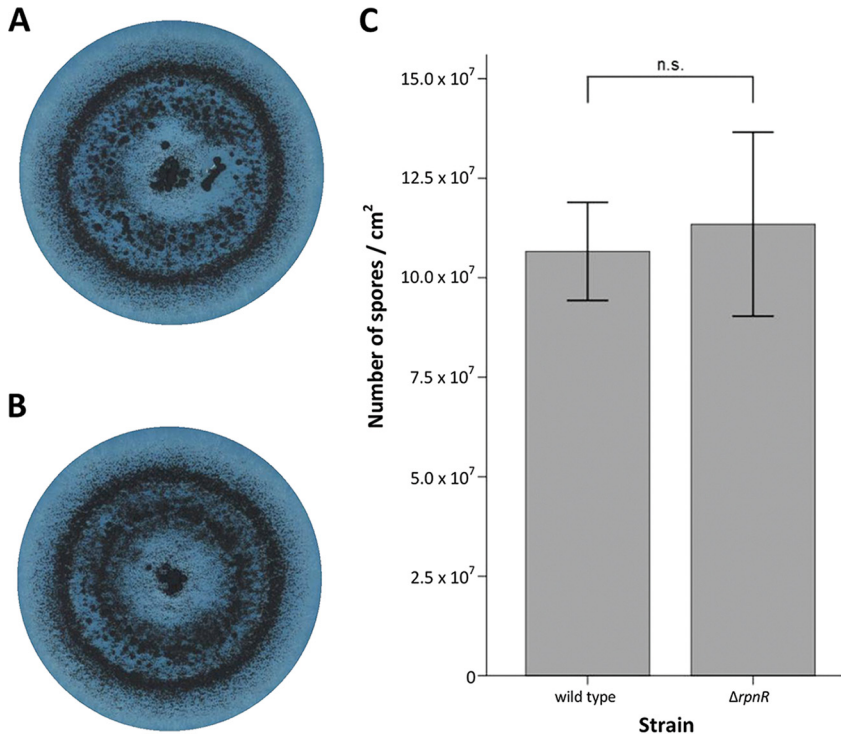

FIG 1 Spatial distribution of sporulation of wild-type strain MA234.1 (A) and the Δ*rpnR* strain (B) and numbers of spores per square centimeter produced by MA234.1 and the Δ*rpnR* strain (C). Localization and quantification of sporulation were monitored 48 h after removal of the upper membrane of sandwiched colonies. n.s., not significant. The error bars indicate standard deviations.

proteotoxic-stress resistance would be affected. Indeed, germination of the Δ*rpnR* strain in MM-X containing 1 mM the unfolded protein response-inducing agent dithiothreitol (DTT) was delayed compared to the wild type, especially at a high concentration of spores (Fig. 2A, D, and G). The growth rate of the Δ*rpnR* strain was also reduced at high spore concentrations but did not differ from that of the wild type at lower spore concentrations (Fig. 2A, D, and G). Germination of the Δ*rpnR* strain in MM-X containing 0.02% H_2O_2 was also delayed at all spore concentrations and was even absent at low spore concentrations. In addition, the growth rate was decreased at intermediate spore concentrations, while the growth rate was not affected at high spore concentrations (Fig. 2B, E, and H). Interestingly, germination of the Δ*rpnR* strain was also delayed when no stressor was present. In fact, the differences between the Δ*rpnR* strain and the wild type in germination timing and growth speed were equal in medium containing DTT or H_2O_2 and in medium without these stressors when a low or high spore concentration, respectively, was examined (Fig. 2C, F, and I).

SDS-PAGE revealed that protein secretion was not affected in shaken liquid cultures of the Δ*rpnR* strain when maltose was used as a carbon source (data not shown). However, it was reduced in the presence of xylose (Fig. 3A). Proteomics was performed to examine whether secretome complexity was also affected. To this end, similar amounts of total secreted protein from MA234.1 and the Δ*rpnR* strain were analyzed by mass spectrometry (MS). A total of 316 proteins were identified in the medium of the Δ*rpnR* strain, 223 of which were detected in at least 3 out of 4 replicates. These numbers were 341 and 257, respectively, for the wild type. A signal peptide for secretion was found in 194 and 222 of the secreted Δ*rpnR* and wild-type proteins, respectively, while 11 and 10 of the proteins that did not have a signal peptide are predicted to have an intracellular localization. The wild-type secretome was relatively enriched for 28 proteins compared to the Δ*rpnR* strain secretome (Table 1; see Table S1 in the supplemental material). Conversely, the Δ*rpnR* secretome was not enriched for proteins

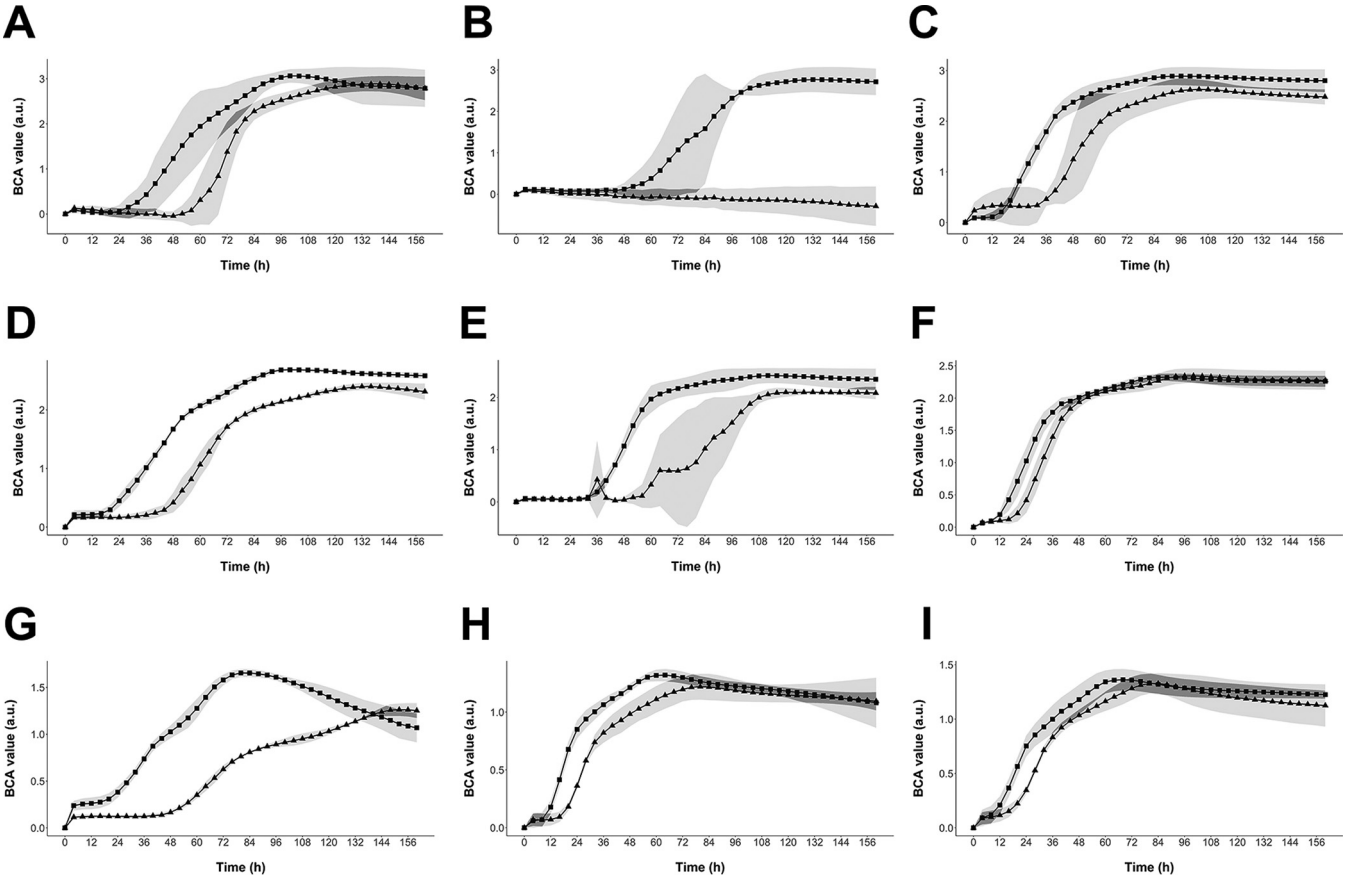

FIG 2 Growth curves of wild-type strain MA234.1 (squares) and the Δ*rpnR* strain (triangles) in wells with MM-X containing 1 mM DTT (A, D, and G), 0.02% H_2O_2 (B, E, and H), or no stressor (C, F, and I) with 10^3 (A, B, and C), 10^4 (D, E, and F), and 10^5 (G, H, and I) conidia per well. The data points and lines for each strain show the average of 3 measurements, while the light-gray shading shows the 95% confidence intervals. Dark-gray shading indicates overlap between the confidence intervals of the two strains. Absence of overlap implies significant differences between the strains.

compared to the wild type. Of the proteins enriched in the wild type (i.e., relatively reduced in the Δ*rpnR* strain), 11 could not be assigned to a Gene Ontology (GO) subset (GO slim) term (process component), and 13 were associated with carbohydrate metabolic process, 2 with developmental process, and 2 with response to stress. The GO slim terms protein catabolic process, cellular amino acid metabolic process, cellular protein modification process, asexual sporulation, and pathogenesis could each be assigned to one protein (Fig. 3B; see Table S1). Closer inspection of the set of proteins without a GO term showed that it contains the β-1,3-glucanase BgxB (An02g13180) (15), the orthologue of an XlnR-regulated lipase of *Aspergillus oryzae* (An10g00790), a putative muconate cycloisomerase (An01g14730), a glutamyl-tRNA^{Gln} amidotransferase (An05g01860), and a putative α-1,6-mannanase (An07g07700). From the 16 proteins involved in carbohydrate metabolism (the 13 associated with carbohydrate metabolic process plus BgxB, An07g07700, and MsdS), 9 are involved in carbon source modification, while 7 are (putatively) involved in remodeling of the cell wall (Table 1).

Gene expression analysis. RNAs of the Δ*rpnR* strain and its progenitor, MA234.1, that had been transferred to MM-X for 4 h after pregrowth in nutrient-rich transformation medium (TM) were sequenced (see Materials and Methods). A total of 505 genes were differentially expressed, 253 and 252 of which were down- and upregulated, respectively, in the Δ*rpnR* strain (see Table S2 in the supplemental material). Expression of *rpnR* was 0, thus confirming its proper deletion. Of the downregulated genes in the Δ*rpnR* strain, 0 and 14 genes were also downregulated in the Δ*flbA* and Δ*fum21* strains, respectively (Table 2). Similarly, 2 and 4 of the upregulated genes in the Δ*rpnR* strain were also upregulated in the Δ*flbA* and Δ*fum21* strains, respectively (Table 2). GO term

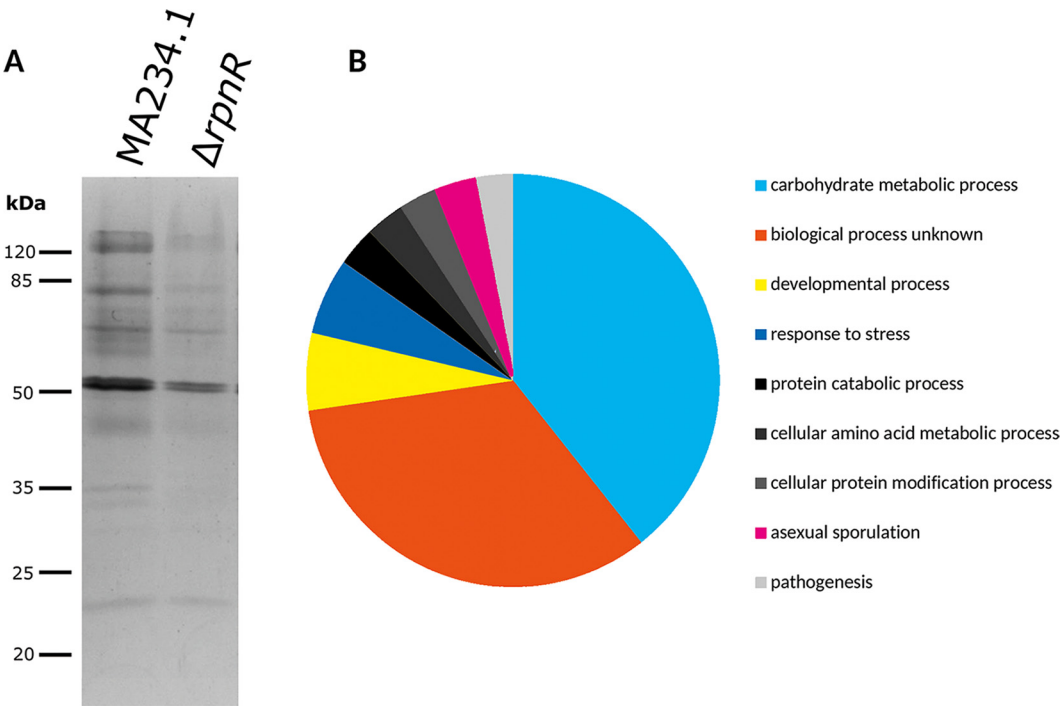

FIG 3 Differences in the secretomes of the Δ*rpnR* strain and the wild type. (A) SDS-PAGE of medium proteins of shaken liquid cultures of the wild type and the Δ*rpnR* strain pregrown in TM-G for 16 h and transferred to MM-X for 24 h. The gel was stained with Coomassie brilliant blue R-250. (B) Pie chart of GO slim terms of the 28 relatively underrepresented secreted proteins of the Δ*rpnR* strain. Proteins PepE, Bgt1, and An01g14960 occur in 2 categories and MsdS in 3.

enrichment analysis of the downregulated genes showed that GO terms related to translation were overrepresented (see Fig. S2 in the supplemental material). In fact, about 20% of the downregulated genes were 60S or 40S ribosomal subunits, while 7 out of 13 subunits of translation initiation factor 3 (eIF3) (16) were also downregulated (see Table S2). This was reflected in the overrepresentation of the KEGG annotation terms ribosome, translation, genetic information processing, nucleotide metabolism, and RNA transport (Table 3). In addition, the GO term alpha amino acid metabolic process was overrepresented (see Fig. S2). Furthermore, small secreted proteins were

TABLE 1 Underrepresented secreted carbohydrate metabolism proteins in the Δ*rpnR* strain medium compared to the wild type

Process	Name or protein ID	Function
Carbon source utilization	AxeA	Acetyl xylan esterase
	Suc1	Invertase
	AguA	α-Glucuronidase
	An11g00390	Rhamnogalacturonan lyase
	An04g03170	β-Glucosidase
	An11g02100	β-Glucosidase
	CbhB	Cellobiohydrolase
	An08g01900	Xylan β-xylosidase
	An08g05230	Endoglucanase 4
Cell wall remodeling	Bgt1	β-1,3-Glucanosyltransferase
	AgnB	α-1,3-Glucanase (53)
	BgxB	β-1,3-Glucanase
	An08g08370[a]	α-1,2-Mannosidase
	An08g03060[a]	α-Mannosidase (54)
	An07g07700[a]	α-1,6-Mannanase
	MsdS[a]	α-1,2-Mannosidase

[a]The protein is likely active on the fungal cell wall (55) and might play a role in glycosylation of proteins like those present in the cell wall.

TABLE 2 Differentially expressed genes in the Δ*rpnR* strain that are also down- and upregulated in the Δ*flbA* or Δ*fum21* strain and fold changes in the strains compared to the wild type[a]

Expression in the Δ*rpnR* strain	Protein ID	Functional annotation[b]	Strain	Fold change	
				Δ*rpnR* strain	Δ*flbA* or Δ*fum21* strain[c]
Downregulated	1142051	No annotation	Δ*fum21*	131.0	192.7
	1142053	Zinc finger transcription factor, C2H2 type	Δ*fum21*	106.7	1312.2
	1166044	No annotation	Δ*fum21*	37.2	126.6
	1087288	Taurine catabolism dioxygenase TauD	Δ*fum21*	16.1	16.5
	1082505	Major facilitator superfamily transporter	Δ*fum21*	15.7	22.3
	1107461	Chitinase	Δ*fum21*	7.9	4.8
	1089440	Major facilitator superfamily transporter	Δ*fum21*	4.9	11.6
	1157348	UDP-glucose 4-epimerase	Δ*fum21*	3.5	5,6
	1150465	No annotation	Δ*fum21*	3.2	4.2
	45784	No annotation	Δ*fum21*	2.8	14.8
	1186369	No annotation	Δ*fum21*	2.8	33.6
	1109756	No annotation	Δ*fum21*	2.7	8.5
	1124492	Phosphoglycerate mutase	Δ*fum21*	2.3	4.4
	1184525	NRPS-like protein	Δ*fum21*	2.1	8.0
Upregulated	1145520	Triacylglycerol lipase	Δ*flbA*	20.5	11.3
	1152605	Acyl-coenzyme A dehydrogenase	Δ*flbA*	2.5	3.5
	1183897	*An*AFP	Δ*fum21*	9.6	6.6
	1184369	Carbohydrate esterase family 16 protein	Δ*fum21*	3.4	4.5
	1156756	No annotation	Δ*fum21*	2.6	5.4
	1184413	No annotation	Δ*fum21*	2.4	4.0

[a]All the strains were grown in TM-G for 16 h, followed by 4 h in MM-X. Differentially expressed genes in the Δ*flbA* and Δ*fum21* strains can be found in references 9 and 10.
[b]When no function of the gene could be inferred from MycoCosm, GO/KEGG terms, and a blastp search, the functional annotation is given as "no annotation." *An*AFP, *Aspergillus niger* antifungal protein.
[c]As indicated in the "Strain" column.

overrepresented, as well as genes of secondary-metabolism cluster 49 (4 out of 17 genes) (Table 3). Terms related to regulation of translation and regulation of the ribosome were underrepresented (see Fig. S3 in the supplemental material). This implies that RpnR specifically regulates constituents of the ribosome but not their regulators. Other underrepresented terms were related to the nucleus, DNA binding, and RNA synthesis (see Fig. S3), indicating that RpnR specifically affects proteins that act outside the nucleus and does not regulate the ribosome at the RNA level. Terms related to zinc ion binding were also underrepresented (see Fig. S3).

Among the upregulated genes in the Δ*rpnR* strain, GO terms related to arabinose metabolism, oxidoreductive activity, and coenzyme binding were overrepresented (see Fig. S4 in the supplemental material). More precisely, genes encoding the arabinofuranosidases AbfB, AbfC, and AbfE were upregulated in the Δ*rpnR* strain (see Table S2). However, since no proteins were overrepresented in the proteome of the Δ*rpnR* strain (see above), these arabinofuranosidases are apparently not upregulated at the protein level. Many KEGG annotation terms were overrepresented (Table 3). Of these, the 2nd-level term amino acid metabolism represented 12% of the upregulated genes, carbohydrate metabolism 11%, xenobiotics biodegradation and metabolism 10%, lipid metabolism 10%, and metabolism of cofactors and vitamins 7%. Furthermore, 5 Pfam domains were overrepresented (Table 3), including domains associated with lipid metabolism (PF13561.1) and oxidoreductive activity (PF00106.20 and PF00107.21). Also, genes encoding proteins with a transmembrane domain and genes belonging to secondary-metabolism cluster 75 (5 out of 18) were overrepresented (Table 3). Together, these data indicate that RpnR deficiency reduces expression of ribosomal subunits, whereas it activates arabinose metabolism, lipid metabolism, coenzyme metabolism, and oxidoreductase activity.

DISCUSSION

The transcription factor Rpn4 of *S. cerevisiae* is involved in regulation of DNA repair (17, 18) and functions as a transcriptional activator of 26S proteasomal subunits (13, 19)

TABLE 3 Overrepresented KEGG pathways, Pfam domains, and secondary-metabolism-related annotation terms among the down- and upregulated genes of the Δ*rpnR* strain compared to the wild type[a]

Gene regulation	Location	Overrepresented term in Δ*rpnR* strain
Downregulated	KEGG pathways	KEGG third level: ribosome
		KEGG second level: translation
		KEGG first level: genetic information processing
		KEGG second level: nucleotide metabolism
		KEGG third level: RNA transport
	Secondary-metabolism clusters	Part of secondary-metabolism cluster 49
Upregulated	KEGG pathways	KEGG first level: metabolism
		KEGG second level: overview
		KEGG third level: valine, leucine and isoleucine degradation
		KEGG second level: xenobiotics biodegradation and metabolism
		KEGG third level: fatty acid metabolism
		KEGG second level: amino acid metabolism
		KEGG third level: fatty acid biosynthesis
		KEGG fourth level: K00059:fabG; 3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)
		KEGG EC: acyl-carrier protein
		KEGG third level: biosynthesis of unsaturated fatty acids
		KEGG second level: lipid metabolism
		KEGG second level: carbohydrate metabolism
		KEGG third level: biotin metabolism
		KEGG third level: propanoate metabolism
		KEGG third level: chlorocyclohexane and chlorobenzene degradation
		KEGG third level: benzoate degradation
		KEGG third level: toluene degradation
		KEGG EC: EC 1.1.1.65
		KEGG fourth level: K05275:E1.1.1.65; pyridoxine 4-dehydrogenase (EC 1.1.1.65)
		KEGG fourth level: K01714:dapA; 4-hydroxy-tetrahydrodipicolinate synthase (EC 4.3.3.7)
		KEGG EC: EC 4.3.3.7
		KEGG second level: metabolism of cofactors and vitamins
		KEGG third level: metabolism of xenobiotics by cytochrome P450
		KEGG third level: aminobenzoate degradation
		KEGG third level: caprolactam degradation
		KEGG third level: fatty acid degradation
		KEGG third level: carbon metabolism
		KEGG third level: vitamin B_6 metabolism
		KEGG third level: PPAR signaling pathway
		KEGG third level: butanoate metabolism
		KEGG third level: chemical carcinogenesis
	Pfam domains	PF13561.1 enoyl-(acyl carrier protein) reductase
		PF08659.5 KR domain
		PF00106.20 short-chain dehydrogenase
		PF00107.21 zinc-binding dehydrogenase
		PF00701.17 dihydrodipicolinate synthetase family
	Secondary-metabolism clusters	Part of secondary-metabolism cluster 75
		Part of secondary-metabolism cluster: decorating
	Other terms	Transmembrane domain

[a]All the strains were grown in TM-G for 16 h, followed by 4 h in MM-X.

by binding to PACE promoter sequences (13, 17, 20). Here, it is shown that the *A. niger* orthologue of Rpn4, RpnR, functions differently than its *S. cerevisiae* counterpart. Deletion of its encoding gene resulted in reduced proteotoxic- and oxidative-stress resistance, which may well explain why expression of the ribosomal subunits and the total amount of secreted proteins were also reduced when *A. niger* was grown on xylose. Expression of genes encoding 26S proteasome subunits remained unchanged (data not shown). This contrasts with the *S. cerevisiae rpn4* deletion strain, in which 26S proteasome subunit expression was decreased while ribosomal subunit genes were unaffected (17, 21). It is, however, in line with results in *N. crassa*, where the majority of proteasome genes do not contain the DNA sequences to which *N. crassa* Rpn4 binds (14). It should be noted that reduced proteotoxic- and oxidative-stress resistance was not found at low and high spore concentrations, respectively. How can this be explained? With increasing spore concentrations, the amounts of stressor and nutrient

per spore decrease. These amounts may impact the phenotype. For instance, the effect of H_2O_2 is expected to be lower at high spore concentrations because its effective amount per spore is less.

There is a link between unfolded-protein levels in the cell and ribosomal gene expression and RNA translation. Proteotoxic stress is counteracted by the unfolded-protein response (UPR), which not only increases the abundance of chaperones and heat shock proteins in the endoplasmic reticulum (ER), but also, importantly, reduces the influx of new proteins into the ER through downregulating translation. Translation attenuation in animals (22) and *S. cerevisiae* (21) is achieved by inhibition of translation initiation factor eIF2α activity by phosphorylation. In addition, *S. cerevisiae* downregulates genes encoding ribosomal subunits (23, 24), which increases resistance to proteotoxic stress (21, 25). Whether these processes also occur in filamentous fungi, such as *A. niger*, is not known. It is known that activation of genes involved in the UPR occurs via transcription factor Hac1 in *S. cerevisiae* (26, 27) and its homologue HacA in *A. niger* (28). Hac1/HacA is activated by Ire1/IreA, a molecule that senses proteotoxic stress in the ER. As a result, it removes an intron from Hac1 pre-mRNA, so that an active Hac1 protein is produced (29). On the other hand, downregulation of *S. cerevisiae* genes encoding ribosomal subunits is regulated by deactivation of protein kinase A (30), which in turn activates the general stress response regulator Msn4.

Colonies of *A. niger* that are transferred from a nutrient-rich medium to a defined medium (i.e., TM to MM) with xylose as a carbon source induce genes encoding secreted enzymes. This induction has been shown to induce a response reminiscent of the UPR in *A. niger* (31), likely due to increased influx of proteins in the ER. The fact that the Δ*rpnR* strain is more sensitive to proteotoxic stress agrees with the finding that genes encoding ribosomal proteins are downregulated in this deletion strain. In addition, 7 out of the 15 subunits of eIF3 (16) were found to be downregulated in the Δ*rpnR* strain. This might represent another way in which translation is inhibited, analogous but not similar to eIF2α activity inhibition in yeast and animals (see above). Decreased translation would reduce the amount of unfolded proteins. This, apparently, is effective, since protein secretion, but not biomass formation, is affected in the Δ*rpnR* strain. The fact that protein secretion was not affected when the Δ*rpnR* strain was grown in maltose-containing medium may be explained by the less complex secretome that is found on this carbon source (32).

The biomass of the Δ*rpnR* strain was higher or not reduced in colonies grown on agar medium. In all cases, the diameter was reduced, implying that the biomass per surface area was increased. This was probably caused by increased hyphal branching, but the underlying mechanism is not known. What also remains unexplained is why the Δ*rpnR* strain is less tolerant of proteotoxic stress. UPR malfunction could be an explanation. However, the fact that chaperones or heat shock proteins are not differentially expressed in the Δ*rpnR* strain suggests that the rest of the UPR is induced equally strongly as in the wild type. This is also reflected by the fact that both HacA and IreA are not differentially expressed. Therefore, other factors make the Δ*rpnR* strain more sensitive to proteotoxic stress. It is also unclear why 28 specific proteins have lower abundance in the secretome of the Δ*rpnR* strain than in the wild type, in addition to the overall secretion reduction. Their corresponding genes are not differentially expressed, so the difference in the secreted protein level might arise from posttranscriptional events, such as high sensitivity to ribosome depletion.

What do these data mean for the function of RpnR in FlbA-mediated sporulation-inhibited protein secretion? In wild-type colonies, FlbA is active in sporulating regions of the colony, where it downregulates *rpnR*. Our results indicate that this leads to less proteotoxic-stress resistance and lower ribosome abundance and thus to lower protein secretion in sporulating regions than in the nonsporulating zones of the colony. Indeed, this has been observed in wild-type colonies. Downregulation of ribosomal protein genes has been shown to occur during sporulation in *Aspergillus fumigatus* (33), also supporting this theory. The facts that this ribosomal protein downregulation is dependent on BrlA (33) and that *rpnR* is also upregulated in zones of maltose-grown colonies

TABLE 4 Primers used in this study

Primer name	5′–3′ sequence
1	AGTATTCCACTCAGCTCTGGACTAACCTCTTTAATGGGTCAGTCAATGCCTGGACTTATC
2	TGATGTGTGTGACGTGATGTGTATATCCTAGCAGGTATAGACACGGAATTTGCCAC
3	TGATGATGGATATATGGAAGCTGGAGGATGTCCAATTGAGAGGTAAGCCAGGG
4	TAGCAGGTGAGAACTCACTTGTACTCGACTCCTGCAGAAACCACAAAGGTACCACTACGTC
5	TAGGATATACACATCACGTC
6	CATCCTCCAGCTTCCATATATC
7	GGTTTAGCGGGTGCAGAAG
8	GGCGTCGGTTTCCACTATC
9	AAAGTTCGACAGCGTCTCC
10	GTTTGGGCTTCAATGTCTTAC
11	ATAGGGAGAGCGGCCCCGGAGATGCAGATCGAGTG
12	GAAGATCTGGCGGCCCCACCGTGACCCAATGAGAA
13	ATCCACTGCACCTCAGAGCC
14	CATGCATGGTTGCCTAGTGAA

lacking *fluG* (34) imply that downregulation of ribosomal proteins can be activated by several genes in the sporulation pathway. Taken together, the data show that the putative transcription factor RpnR of *A. niger* regulates proteotoxic-stress resistance and, possibly as a consequence, protein secretion and may therefore be an important target for improving *A. niger* as a cell factory for enzyme production.

MATERIALS AND METHODS

Strains and culture conditions. *A. niger* strain MA234.1 (transient *kusA*::*amdS*; *pyrG*$^+$) (35) and its derivative Δ*rpnR* strain (see below) were grown at 30°C on MM (36) with 25 mM xylose as a carbon source and either containing (MM-XA) or not containing (MM-X) 1.5% agar. Alternatively, 1% (wt/vol) beechwood xylan was used instead of xylose. Liquid cultures were pregrown in TM (MM containing 0.5% yeast extract and 0.2% Casamino Acids) (37) containing 25 mM glucose (TM-G). To this end, 100 ml medium was inoculated with 5×10^8 freshly harvested conidia from 3-day-old MM-XA cultures and shaken in 250-ml Erlenmeyer flasks at 200 rpm. After 16 h, the mycelium was washed with 0.9% NaCl, and 10 g (wet weight) was transferred to a 1-liter Erlenmeyer flask containing 150 ml MM-X. Growth was prolonged for 4 h (RNA sequencing) or 24 h (SDS-PAGE and proteomics) at 200 rpm. Biomass formation and sporulation were assessed by growing colonies in a 0.45-mm-thick layer of 1.25% agarose between perforated polycarbonate membranes (0.1-μm pores; diameter, 76 mm; Profiltra, Almere, The Netherlands) (38). To this end, the sandwiched cultures were inoculated in the center of the agarose layer with 10^4 conidia contained in 2 μl H_2O. The upper polycarbonate membrane was placed 24 h postinoculation to prevent spreading of conidia. After 7 days, the diameter of the colony was measured and the dry-weight biomass was determined by subtracting the average weight of a noninoculated agarose layer. Alternatively, the upper membrane was taken off to enable the colony to sporulate during a 48-h period. Differences in biomass, colony diameter, or spore production were calculated using an independent-samples *t* test.

Inactivation of *rpnR* and complementation of the deletion strain. The *rpnR* deletion construct was made by amplifying the 5′ and 3′ flanks of *rpnR* (protein ID, An08g06850) (12) from genomic DNA by PCR using primer pairs 1/2 and 3/4, respectively (Table 4). The hygromycin resistance gene *hph* was amplified from the vector pAN7.1 (39) using primer pair 5/6 (Table 4). Split marker fragments of this selection marker fused to the 3′ and 5′ flanks were created by fusion PCR (40) using primer pairs 7/8 and 9/10 (Table 4) for the 5′ and 3′ fragments, respectively.

The *rpnR* complementation construct was made by amplifying the genomic coding sequence of *rpnR* together with its 1,017-bp promoter and 559-bp terminator sequences. To this end, MA234.1 genomic DNA was used in combination with Phusion polymerase (Thermo Scientific, Wilmington, DE, USA) and primer pair 11/12 (Table 4). The resulting fragment was inserted in the NotI site of vector pMA357 (9) using an InFusion HD cloning kit (Clontech, Mountain View, CA, USA). This resulted in vector pDA2, containing the *amdS* selection cassette and the gene *rpnR* under the control of its own promoter and terminator.

The split marker fragments and the complementation construct were transformed to strain MA234.1 and the Δ*rpnR* strain, respectively, as described previously (41). Transformants were purified twice on MM-XA containing 100 μg ml^{-1} hygromycin or 15 mM CsCl and 10 mM acetamide as a nitrogen source for selection of deletion and complemented strains, respectively.

RNA sequencing and analysis. Mycelium from biological triplicates was taken up in TRIzol reagent (Invitrogen, Bleiswijk, The Netherlands) after homogenization in a Tissue Lyzer II (Qiagen, Venlo, The Netherlands). After extraction with chloroform, total RNA was precipitated with isopropanol, washed with ethanol, and resuspended in RNase-free water. RNA was purified using a NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany), and its concentration and purity were checked using a Nanodrop ND-1000 (Thermo Scientific).

Library construction, cluster generation, and sequencing of cDNA were performed by ServiceXS (Leiden, The Netherlands). Library construction and cluster generation were performed with the NEBNext Ultra Directional RNA Library Prep kit for Illumina (New England Biolabs, Ipswich, MA, USA). Sequencing of cDNA was performed using an Illumina NextSeq 500 with NextSeq control software 2.0.2 (Illumina, San Diego, CA, USA), generating single reads of 75 bp. For image analysis, base calling, and quality checking, the Illumina data analysis pipelines RTA v2.4.11 and Bcl2fastq v2.17 were used. The sequences were then aligned to the *A. niger* ATCC 1015 genome assembly (version Aspni7) (42) obtained from MycoCosm (43) using HISAT2 version 2.0.5 (44). Functional annotation of the predicted genes was obtained from MycoCosm as described previously (5), which, in the case of the differentially expressed genes, was manually approved by examining the GO and KEGG terms and by blastp search. However, ribosomal subunits were named by homology with *S. cerevisiae*, as proposed by Nakamura et al. (45).

The expression levels of genes were normalized to fragments per kilobase of exon model per million fragments (FPKM). Cuffdiff version 2.2.1 (46) was used to identify reads mapping to predicted genes and to identify differentially expressed genes. The bias correction method was used while running Cuffdiff (47). In addition to Cuffdiff's requirements for differential expression (including a false-discovery rate [FDR]-adjusted *P* value of <0.05), we selected for a >2-fold change and a minimal expression level of 10 FPKM in at least one of the samples. The quality of the results was analyzed using CummeRbund (48).

Custom scripts were developed in Python and R to analyze over- and underrepresentation of functional annotation terms in sets of differentially regulated genes using the Fisher exact test. The Benjamini-Hochberg correction was used to correct for multiple tests using a *P* value of <0.05. Over- and underrepresented GO terms were visualized using RamiGO (49). GO terms, KEGG pathways, potential protein-degrading activity, presence of a secretion signal or a transmembrane domain, requirements for terming a protein a small secreted protein, and a function in secondary metabolism were predicted as described previously (5).

SDS-PAGE. Proteins were precipitated with acetone and taken up in a 5% volume of sample buffer compared to the original volume of culture medium. Proteins were separated in 12.5% SDS-PAGE gels using a Pierce prestained protein molecular weight marker (Thermo Scientific). Gels were fixed with 50% methanol and 10% acetic acid; stained with 0.1% Coomassie brilliant blue R-250; and destained with 25% methanol, 10% acetic acid. Protein bands were imaged using a Universal Hood III with Image Lab software (Bio-Rad Laboratories BV, Veenendaal, The Netherlands).

Proteomics. Culture medium (4-ml samples of biological quadruplicates) was concentrated 100-fold for 30 min at 4 000 $\times$ *g* and 4°C using an Amicon Ultra-4 centrifugal filter unit with a cutoff of 10 kDa (Millipore, Billerica, MA, USA). The concentrated medium was washed twice by adding 2.5 ml phosphate-buffered saline (PBS), followed by centrifugation steps. The protein concentrations of the samples were estimated by SDS-PAGE analysis. Based on this, sample buffer was added to correct for protein concentrations in the different samples. Samples were separated on a 12% Bis-Tris SDS-PAGE gel (Bio-Rad). The gel was run for 2 to 3 cm and stained with colloidal Coomassie dye G-250 (Gel Code blue stain reagent; Thermo Scientific), after which each lane was cut into two pieces to reduce complexity. The gel pieces were reduced, alkylated, and digested overnight with trypsin at 37°C. The peptides were extracted with 100% acetonitrile, dried in a vacuum concentrator, and resuspended in 10% (vol/vol) formic acid. An ultrahigh-performance liquid chromatography (UHPLC) 1290 system (Agilent) coupled to an Orbitrap Q Exactive HF Biopharma mass spectrometer (Thermo Scientific) was used for MS. Peptides were trapped for 5 min in solvent A (0.1% formic acid in water) on a Dr Maisch Reprosil C_{18} column (3 μm by 2 cm by 100 μm) before being separated on an analytical column (Agilent Poroshell EC-C_{18}; 2.7 μm by 50 cm by 75 μm) using a gradient of solvent A consisting of 13 to 44% in 95 min, 44 to 100% in 3 min, 100% for 1 min, 100 to 0% in 1 min, and finally 0% for 10 min. The flow was passively split to 300 nl min^{-1}. The mass spectrometer was operated in data-dependent mode. Full-scan MS spectra from *m/z* 375 to 1,600 were acquired at a resolution of 60,000 at *m/z* 200 after accumulation to a target value of 3E6. Up to 15 most intense precursor ions were selected for fragmentation. High-energy collisional dissociation fragmentation was performed at a normalized collision energy of 27% after accumulation to a target value of 1E5. Tandem MS (MS-MS) was acquired at a resolution of 15,000. Raw data files were processed using Proteome Discover (version 1.4.1.14). A database search was performed against the *A. niger* CBS 513.88 database (12) using Mascot (version 2.5.1; Matrix Science, London, UK) as a search engine. Cysteine carbamidomethylation was set as a fixed modification and methionine oxidation as a variable modification. Trypsin was specified as the enzyme, and up to two missed cleavages were allowed. Filtering was done at a 1% false-discovery rate and an ion score of >20. For CRAPome analysis (50), proteins with a SAINTexpress (51) probability score of ≥ 0.75 were considered differentially expressed. Differentially expressed proteins were mapped to GO slim terms using the GO slim mapper on AspGD (12). SignalP 4.1 (52) was used to identify signal peptides for secretion.

Stress resistance assays. Resistance to H_2O_2 and DTT was determined in biological triplicates by inoculating 10^3 to 10^5 spores in 96-well plates filled with 200 μl MM-X per well and either supplemented or not with 0.02% H_2O_2 or 1 mM DTT. The spores were incubated at 30°C for 7 days. Growth was monitored using oCelloScope (BioSense Solutions, Farum, Denmark) and UniExplorer (BioSense) software. The bicinchoninic acid (BCA) normalized algorithm was used to determine the total surface area of the fungal mycelium.

Accession number(s). The RNA sequencing data have been deposited in NCBI GEO with accession number GSE102899 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi).

SUPPLEMENTAL MATERIAL

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SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

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We declare no conflict of interest.

D.A., S.G.V.D.B., H.P., M.A., and R.A.O. designed and performed experiments; D.A., H.P., M.A.F.A., M.A., A.F.J.R., R.A.O. and H.A.B.W. interpreted data; D.A. and H.A.B.W. wrote the manuscript.

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