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Global Transcriptome Changes Underlying Colony Growth in the Opportunistic Human Pathogen *Aspergillus fumigatus*

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Aspergillus fumigatus **is the most common and deadly pulmonary fungal infection worldwide. In the lung, the fungus usually forms a dense colony of filaments embedded in a polymeric extracellular matrix. To identify candidate genes involved in this biofilm (BF) growth, we used RNA-Seq to compare the transcriptomes of BF and liquid plankton (PL) growth. Sequencing and mapping of tens of millions sequence reads against the *A. fumigatus* transcriptome identified 3,728 differentially regulated genes in the two conditions. Although many of these genes, including the ones coding for transcription factors, stress response, the ribosome, and the translation machinery, likely reflect the different growth demands in the two conditions, our experiment also identified hundreds of candidate genes for the observed differences in morphology and pathobiology between BF and PL. We found an overrepresentation of upregulated genes in transport, secondary metabolism, and cell wall and surface functions. Furthermore, upregulated genes showed significant spatial structure across the *A. fumigatus* genome; they were more likely to occur in subtelomeric regions and colocalized in 27 genomic neighborhoods, many of which overlapped with known or candidate secondary metabolism gene clusters. We also identified 1,164 genes that were downregulated. This gene set was not spatially structured across the genome and was overrepresented in genes participating in primary metabolic functions, including carbon and amino acid metabolism. These results add valuable insight into the genetics of biofilm formation in *A. fumigatus* and other filamentous fungi and identify many relevant, in the context of biofilm biology, candidate genes for downstream functional experiments.**

The filamentous fungal genus *Aspergillus* consists of over 250 saprophytic species (19). Although some species, such as *Aspergillus niger*, *A. terreus*, and *A. oryzae*, are exploited commercially for the production of enzymes, pharmaceuticals, and traditional Asian foods and beverages, others are capable of colonizing and infecting immunocompromised individuals (2). The primary opportunistic human pathogen is *A. fumigatus*, the fungus responsible for the highest number of deaths and for the second highest number of infections, behind only *Candida albicans* (33).

A. fumigatus produces an abundance of very small (2- to 3-μm) asexual spores (also known as conidia) that easily disperse throughout the air (31). Their continuous inhalation can lead to the production of a wide spectrum of diseases, which are collectively known as aspergillosis (33). In addition to allergic diseases resulting from conidial inhalation, the growth of *A. fumigatus* hyphae can produce either focal infections in preexisting lung cavities or invasive infections in patients undergoing heavy chemo- and radiotherapies for cancer treatments or organ transplantation.

Historically, *in vitro* studies to understand *A. fumigatus* pathobiology used fungal colonies grown in liquid shake conditions. Although these colonies are not single celled, they deserve the definition of planktonic (PL) colonies since they live in a fluid environment as opposed to the ones attached to a surface. Importantly, these PL colonies have pathological and morphological characteristics vastly different from those observed *in vivo* (35). For example, during mycelial development in the lung, the hyphae form and embed themselves in a dense extracellular matrix (ECM) (35). This ECM, whose function, in part, is to tightly "glue" hyphae together and to protect the fungus from an outside hostile environment, is absent when the fungus is grown under liquid shake conditions (3, 35, 48). In contrast, an ECM is produced when the fungus is grown in aerial static conditions (3). Importantly, in this aerially grown biofilm (BF)-like state, the fungus exhibits reduced susceptibility to antifungal drugs (44, 45, 57, 60) and undergoes major metabolic changes that are thought to be involved in virulence (8, 35).

The differences in pathological and morphological characteristics between PL and *in vivo*-grown *A. fumigatus* suggest that PL is a poor *in vitro* disease model. In contrast, the BF model is phenotypically close to the *A. fumigatus in vivo* growth and thus more appropriate for pathobiology studies (8, 35). To provide a global and accurate profile of *A. fumigatus in vitro* biofilm growth, we utilized RNA-Seq (43) to compare the global gene expression profiles of *A. fumigatus* grown in BF and PL conditions. We identified thousands of differentially regulated genes, whose protein products participate in functions such as transcription, translation, and stress response, that likely account for the different growth demands associated with the two conditions tested. However, we also identified hundreds of differentially regulated genes that constitute candidates for the observed pathobiological and morphological differences between the two conditions. For example, we observed extensive upregulation of genes whose proteins participate in transport, secondary metabolism, and cell wall and surface functions in BF relative to what was seen for PL. Interestingly, whereas genes that were upregulated in BF were significantly overrepresented in subtelomeric regions and localized in genomic

Received 6 May 2011 Accepted 20 June 2011
Published ahead of print 1 July 2011
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Supplemental material for this article may be found at http://ec.asm.org/.

doi:10.1128/EC.05102-11

neighborhoods with similar regulation, BF downregulated genes exhibited neither of these two trends. Together, our results provide a fine-grained transcriptional examination of *A. fumigatus* grown in biofilm conditions and offer numerous candidates for downstream functional experiments.

MATERIALS AND METHODS

Fungal strains and culture conditions. We chose the *A. fumigatus* ATCC 46645 wild-type strain, which has been used previously for biofilm studies (3). For shake cultures (PL), 500 ml of Brian medium was inoculated with 10^7 conidia, and the flask was incubated at 37°C in darkness for 16 h at 150 rpm. For the production of a colony on 2% agar Brian medium (BF), porous cellophane was deposited on the surface of the agar and 50 μl of a conidial suspension (10^7/ml) per 9-cm petri dish was spread onto the cellophane by using an inoculation spreader. The petri dishes were also incubated in the dark at 37°C for 16 h.

RNA isolation, mRNA library construction, and Illumina sequencing. Prior to harvesting, each culture was visually inspected to ensure the absence of conidia. We collected fungal tissue from the PL culture by filtration and from the BF culture by using a sterile spatula. Upon harvesting, the fungal colony from the BF culture was immediately frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle. We extracted total RNA using a previously described phenol-chloroform protocol (30). RNA samples were then treated with DNase and further purified using the Qiagen RNeasy minikit, following manufacturer's instructions (20, 24). mRNA libraries were constructed and sequenced at the Vanderbilt Genome Technology Core following Illumina specifications, generating over 20 million reads for each sample. To establish that the RNA-Seq experiments reported in our study do not show significant variation when replicated, we also generated data from two additional *A. fumigatus* strains (Af293 and CEA10) during PL growth and from one additional technical replicate during BF growth.

Read mapping and gene regulation quantification. For each data set, we converted fastq sequence read files to fasta formatted ones using the fq_all2std.pl script in the Maq software package, version 0.7.1 (http://maq.sourceforge.net/index.shtml). We then mapped each data set to the *A. fumigatus* af293 reference transcriptome (52) by using the SeqMap software, version 1.08 (27; http://www.stanford.edu/group/wonglab/jiangh/seqmap/), allowing two mismatches per read. We mapped 11,842,153 and 15,385,865 reads to the *A. fumigatus* af293 reference transcriptome (52) from BF and PL growths, respectively. To quantify global gene expression levels in BF and PL conditions, we calculated the RPKM value, which stands for reads per kilobase per million mapped reads and is a self-normalized value of absolute transcript abundance, of each reference transcript in the two data sets by using the rSeq software, version 0.0.5 (43; http://www.stanford.edu/group/wonglab/jiangh/rseq/).

Identification of differentially regulated genes. To limit the number of false positives, we employed a conservative approach to identify differentially expressed genes by implementing both biological and statistical cutoffs for significance (56). For our biological cutoff, we compared the fold difference of RPKM values between BF and PL conditions by calculating the relative RPKM (rRPKM = $RPKM_{BF}/RPKM_{PL}$) of each gene. We required a 2-fold difference in relative gene expression between conditions (i.e., rRPKM $\geq$ 2 or rRPKM $\leq$ 0.5). For our statistical cutoff, we compared the proportion of reads that mapped to each gene for the BF and PL conditions via Fisher's exact tests, applying a Bonferroni multiple test-corrected *P* value cutoff of 5.5e−6.

The genome architecture of differentially regulated genes. To test whether differentially regulated genes were represented disproportionately in subtelomeric regions, we compared the proportion of upregulated and downregulated genes in subtelomeric regions to the proportion of upregulated and downregulated genes in the rest of the genome (background), independently for each chromosome and for the entire genome. We defined subtelomeric regions as the 300-kb regions preceding the telomere ends (17, 40). We implemented a Bonferroni multiple test-corrected *P* value cutoff of 0.0028.

To test whether differential gene regulation along the *A. fumigatus* chromosomes was structured spatially into genomic neighborhoods, we analyzed the gene order pattern of upregulated and downregulated genes across each chromosome as well as across the entire genome. To assess statistical significance, we used the Wald-Wolfowitz runs test (63), which compares the expected number of runs, assuming randomness, to the observed number of runs. In our analyses, we coded each gene as "upregulated" or "non-upregulated" and defined "runs" as strings of upregulated genes that were preceded and followed by non-upregulated genes or vice versa. We implemented a Bonferroni multiple test-corrected *P* value cutoff of 0.0028.

To characterize further the gene content of genomic neighborhoods of differential gene regulation, we performed sliding window analysis to identify significant clusters of upregulated or downregulated genes. Each gene was encoded as upregulated or downregulated using the expression cutoffs previously described. For each chromosome, a probability was calculated for each window of 6, 12, or 24 consecutive genes, with a step size of 1 gene. The probability of each window being upregulated (or downregulated) was calculated using a cumulative binomial probability, where the probability of success is the fraction of genes that are upregulated (or downregulated). Thus, the probability of a window with *x* out of *n* genes being upregulated (or downregulated) is given by the following equation:

$$P(x \geq k) = \sum_{i=k}^{n} \binom{n}{k} P^i (1 - P)^{(n-i)}$$

where *k* is the number of upregulated (or downregulated) genes in the window, *i* is the index of summation, and *n* is the number of genes in the window.

To account for the testing of multiple hypotheses, we used a false-discovery rate (FDR) cutoff of 0.15, where the FDR was estimated empirically. We randomly shuffled expression levels across the genome and calculated the probability for each window in permuted data sets. A false positive (FP) is a window from a random set, and a true positive (TP) is a window from the real data. To increase the precision of the estimated FDR, we performed 1,000 permutations; therefore, the false positives were divided by 1,000 to account for the number of random permutations. For each window size, the cutoff probability for significant windows was determined by calculating a cumulative FDR, where FDR = [FP/(TP + FP)], and choosing the probability with an FDR of 0.1. Regions of chromosomes with overlapping significant windows were manually concatenated and trimmed for clarity and discussion. Nonadjusted windows are reported in the supplemental data.

Functional associations of differentially regulated genes. To examine whether differentially regulated genes were preferentially associated with certain functions, we compared the proportion of upregulated and downregulated genes belonging to the 2nd- and 3rd-order FunCat categories (59) and to the 109 *A. fumigatus* annotated KEGG pathways (28) to their corresponding values in the rest of the genome (defined as the number of genes in all other categories). We performed all comparisons using Fisher's exact tests (63). Because the total number of genes in certain FunCat categories and KEGG pathways was small, we did not apply a multiple test-corrected *P* value cutoff and instead used a cutoff of 0.05.

We also examined the functional associations of differentially expressed genes for a number of gene sets with functions relevant to BF or PL growth. These included 409 genes encoding antigens and cell-surface proteins (32; The Glycosylphosphatidylinositol (GPI) Lipid Anchor Project, http://mendel.imp.ac.at/gpi/gpi_genomes.html; and 38; The Allergome Project, http://www.allergome.org), 81 genes encoding allergens (references 38 [The Allergome Project, http://www.allergome.org] and 52), 319 genes encoding the major facilitator superfamily (MFS) and ATP-binding cassette (ABC) membrane transporter proteins (52, 58; http://www.membranetransport.org/), 392 transcription factors (1, 5, 21, 22, 34, 47,

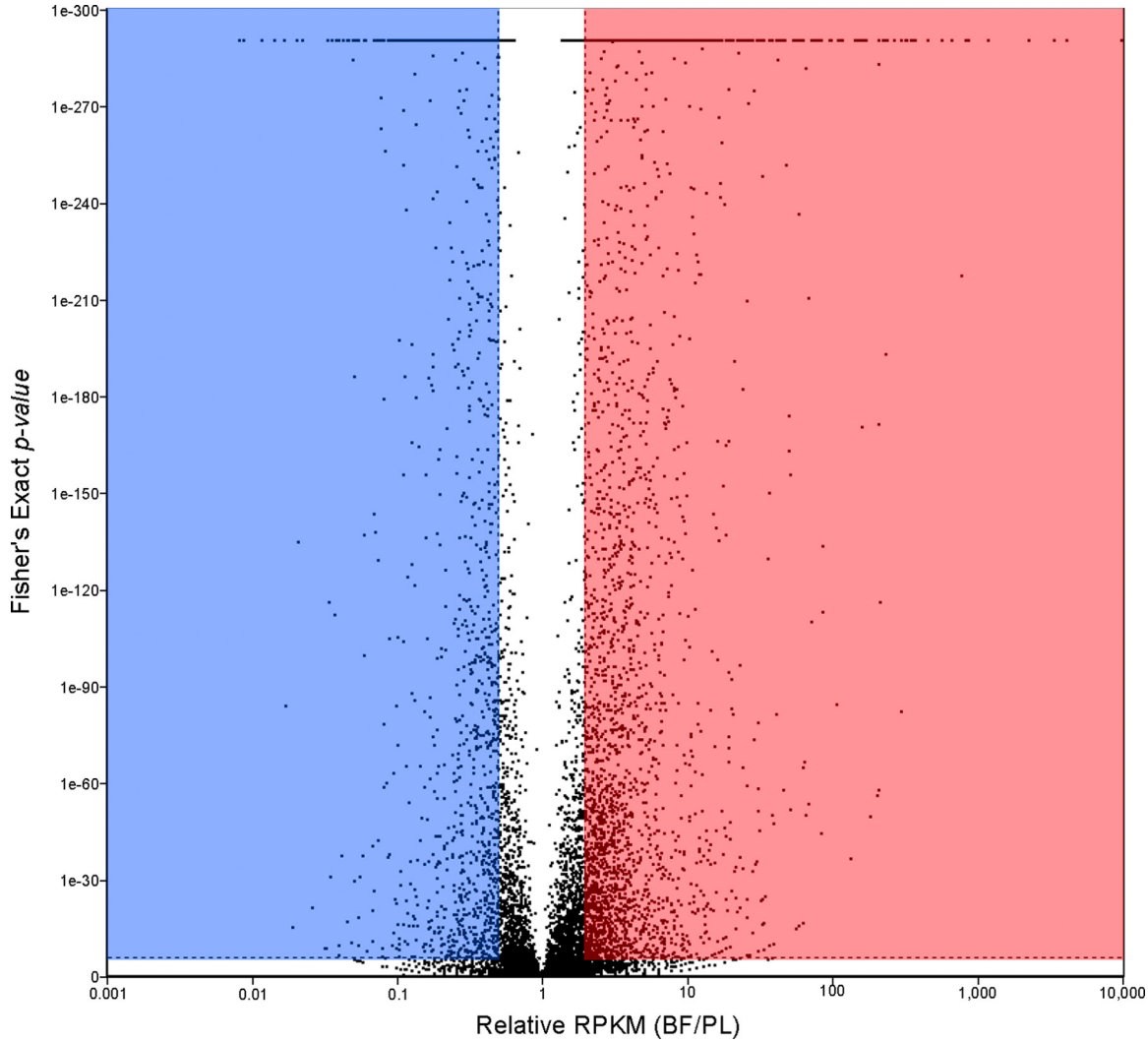

FIG 1 Differentially regulated genes between aerial (BF) and submerged (PL) growth. For each gene, the rRPKM value ($RPKM_{BF}/RPKM_{PL}$) was plotted against its respective Fisher's exact *P* value. *P* values smaller than 1e−290 were reported as 1e−290. The dotted line running parallel to the *x* axis indicates the statistical cutoff ($P < 5.5e{-}6$), whereas the dotted line running parallel to the *y* axis indicates the biological cutoff (2-fold difference in RPKM between BF and PL). The red-shaded and blue-shaded boxes correspond to upregulated and downregulated genes between BF and PL, respectively.

52, 64, 66, 73, 76), and the 383 genes present in 22 secondary metabolism *A. fumigatus* gene clusters (55).

RESULTS

Thousands of genes are differentially regulated in BF relative to PL. Of the 9,887 *A. fumigatus* transcripts (17, 52), 9,525 were expressed in one or both conditions, and 362 genes were not expressed in either condition. Of the 9,525 expressed genes, 251 and 175 genes were uniquely expressed in BF and PL growths, respectively. Of the remaining 9,099 genes that were expressed in both conditions, 5,380 showed uniform expression, 2,565 were upregulated, and 1,164 were downregulated in BF relative to PL (Fig. 1; also see Table S1 in the supplemental material). Remarkably, the range of expression values in both samples ranged over 7 orders of magnitude (see Table S1). Our experiments showed very high levels of correlation for both the biological and technical replication experiments (in all cases examined, Pearson's $r > 0.91$) (see Fig. S1 and Table S2), on par with similar studies in the literature (7), indicating that there is very little technical or biological variation for the conditions tested.

Nonrandom distribution of differentially regulated genes in the *A. fumigatus* genome. The subtelomeric regions of the *A. fumigatus* genome harbor clusters of highly variable and recently evolved genes (17). Considering this, we mapped the chromosomal locations of up- and downregulated genes to examine whether differentially expressed genes were overrepresented in subtelomeric regions and for the presence of physically linked clusters of coregulated genes (Fig. 2). We found significant overrepresentation of upregulated genes ($P = 2.45e{-}6$) and underrepresentation of downregulated genes ($P = 4.5e{-}21$) in subtelomeric regions of the *A. fumigatus* genome. Individual chromosomes showed similar trends (see Table S3 in the supplemental material). For example, chromosome 5 was significantly enriched in upregulated genes ($P = 0.0003$) and significantly un-

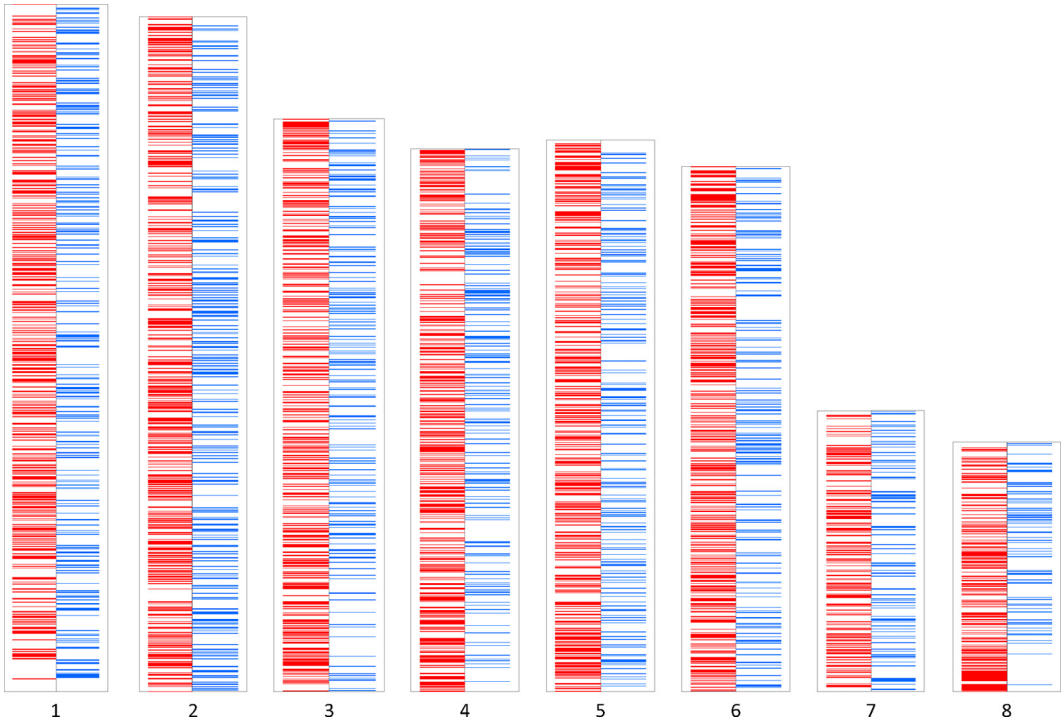

FIG 2 The genome-wide distribution of differentially regulated genes. All differentially regulated genes are plotted against the eight *A. fumigatus* chromosomes. Genes are plotted by order (and not physical distance) on the chromosome. For each chromosome, red bars indicate the positions of upregulated genes, whereas blue bars indicate the positions of downregulated genes. Genes that are not differentially regulated are not shown.

derrepresented in downregulated genes ($P = 0.0005$). The only exception was chromosome 1, in which upregulated genes were significantly underrepresented in its subtelomeric regions ($P = 1.46e{-}8$).

Upregulated and downregulated genes were not randomly distributed across the genome but instead tended to reside within certain genomic neighborhoods ($P_{upregulated} = 1.09e{-}22$, $P_{downregulated} = 6.33e{-}28$). Generally, individual chromosome analyses exhibited the same pattern, with the exception of chromosome 4 for the upregulated gene set ($P = 0.1166$) and chromosomes 2, 4, and 5 for the downregulated gene set ($P = 0.0030$, $P = 1$, and $P = 0.1000$, respectively). To better characterize the gene content of these genomic neighborhoods, we further scanned the genome by using sliding window analyses and identified 27 upregulated gene clusters but no downregulated ones (Fig. 3; also see Table S3 in the supplemental material). The percentage of genes that were upregulated on a typical gene cluster was 72%.

Seven of these 27 upregulated gene clusters overlapped with clusters predicted or known to be involved in secondary metabolism (52, 55) (Fig. 3; also see Table S3 in the supplemental material). Interestingly, 18 of the other 20 clusters contain genes that are hallmarks for secondary metabolism, including polyketide synthases, membrane transporters, transcription factors, cytochrome p450s, reductases, and transferases (see Table S3). For example, a 14-gene cluster (Fig. 3, cluster 1A) on chromosome 1 contains a putative polyketide synthase, an MFS transporter, a flavin adenine dinucleotide (FAD)-dependent oxidase, a short-chain dehydrogenase/reductase, an oxidoreductase, a NACHT domain protein, a transposase, and a reverse transcriptase, whereas a 6-gene cluster (Fig. 3, cluster 1C) also on chromosome 1 contains a cytochrome p450, an MFS transporter, an integral membrane protein, an aldehyde reductase, an oxidoreductase, and a hydrolase. However, our search also identified clusters unlikely to be involved in secondary metabolism, such as a 13-gene cluster (Fig. 3, cluster 8B) on chromosome 8 that contains 3 glycosyl transferases, a β-glucosidase, a glycan biosynthesis protein, two putative sugar transporters, a xylitol dehydrogenase, an extracellular endo-polygalacturonase, and a C6 transcription factor.

Functional classification of differentially regulated genes. To gauge the functions of differentially expressed genes, we compared the proportion of upregulated and downregulated genes in the 2nd- and 3rd-level FunCat categories (59) and the annotated KEGG pathways (28) to the background. We found that 9 and 17 2nd- and 3rd-level FunCat categories were overrepresented in the upregulated gene set, respectively, compared to 6 and 13 in the downregulated gene set (see Table S4 in the supplemental material). Additionally, 10 and 10 KEGG pathways were overrepresented in the upregulated and downregulated gene sets, respectively (see Table S5). Collectively, these results show an overrepresentation of upregulated genes involved in translation and transport in BF relative to PL and an overrepresentation of downregulated genes involved in primary metabolism (see Tables S4 and S5).

Upregulation of secondary metabolic gene clusters. The identification of putative and known secondary metabolic gene clusters in upregulated genomic neighborhoods and the upregulation of the transcription factor *LaeA*, a global regulator of secondary metabolism in *A. fumigatus* (55) (see "Widespread differential regulation of transcription factors" below), prompted us to also examine differential expression in all known *A. fumigatus* secondary metabolism gene

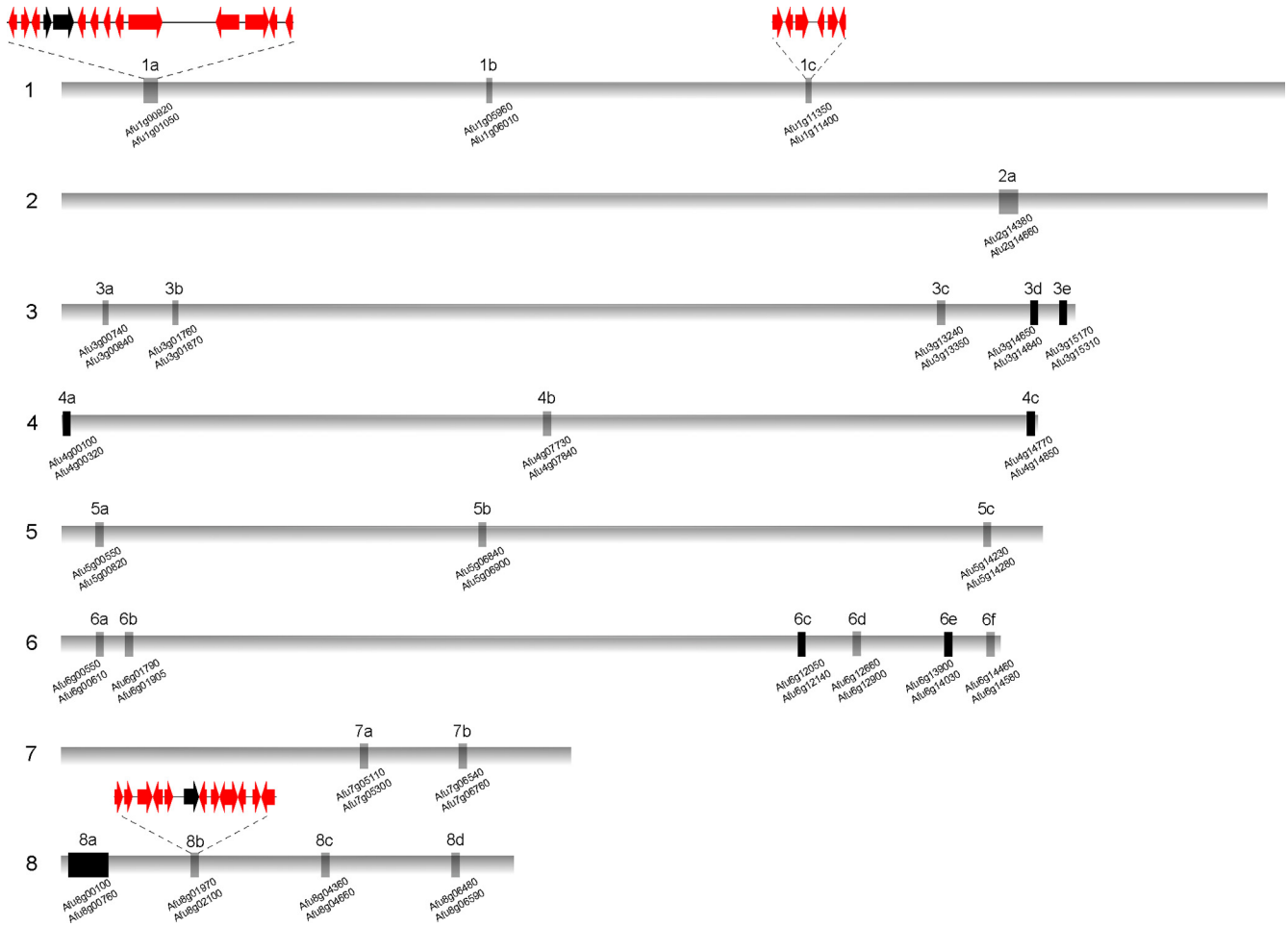

FIG 3 *De novo* identification of gene clusters that are upregulated in BF in the *A. fumigatus* genome. Each bar corresponds to one of the eight *A. fumigatus* chromosomes. Boxes indicate the upregulated gene clusters identified by our sliding window analysis. Boxes colored in black correspond to annotated secondary metabolism gene clusters (55), while gray boxes correspond to novel upregulated clusters of unknown function. The first and last gene names of each cluster are given below each box. Three examples of novel upregulated gene clusters are shown in greater detail. In these clusters, we have indicated the order of gene transcription and color coded upregulated (in red) and nondifferentially regulated (in black) genes (we did not identify any downregulated genes).

clusters. We used the 383 genes in 22 gene clusters described by Perrin and coworkers (55) as the set of known *A. fumigatus* secondary metabolism gene clusters (see Table S3 in the supplemental material). We observed widespread upregulation of secondary metabolism genes and entire clusters. Specifically, 165 of the 383 genes were upregulated (122 genes) or uniquely expressed in BF (43 genes), whereas only 35 of the 383 were downregulated in BF (27 genes) or uniquely expressed in PL (8) (Fig. 4; also see Table S6), suggesting that the upregulated gene set is significantly enriched in genes involved in secondary metabolism relative to background ($P = 2.7e{-}18$). For example, all 8 trichothecene cluster genes (52, 78) were upregulated in BF relative to PL (Fig. 4; also see Table S6). Most noticeably, 44 of the 62 genes in the fumitremorgin supercluster (55), which encodes enzymes that produce fumitremorgin and pseurotin A as well as an unknown compound (36, 37, 55), were upregulated (32 genes) or uniquely expressed (12 genes). Within this supercluster, the putative *O*-methyltransferase CalO6 (Afu8g00200) was the most upregulated gene in the genome, showing a 9,885-fold upregulation in BF relative to PL.

Upregulation of cell wall genes. Because *A. fumigatus* cells are embedded in an ECM during BF formation (3, 35), we investigated the expression patterns of 409 genes coding for enzymes that degrade, modify, or create glycosidic bonds, including those involved in cell wall and ECM biosynthesis. We found that 169 of the 409 genes were upregulated (146 genes) or uniquely expressed (23 genes) in BF, whereas 41 were downregulated in BF (30 genes) or uniquely expressed (11 genes) in PL, suggesting a higher activity for carbohydrate-active enzymes in aerial conditions (see Table S6 in the supplemental material). However, the expression of the synthases responsible for the constitutive synthesis of α- and β-1,3-glucans and chitin did not noticeably change. Among the glycosylhydrolases, the regulation of chitinases was not modified, whereas glucanases and glucosidases were highly expressed when the fungus was grown in an aerial colony; 15 glucanases and 14 glucosidases were upregulated in BF, and only 2 glucanases and none of the glucosidases were downregulated. The great majority of transglycosidases was also upregulated in BF (35 transglycosidases were upregulated and 2 were downregulated) (Table S6). We also observed several significant differences between the two conditions in genes coding for ECM proteins. For example, *RodA*

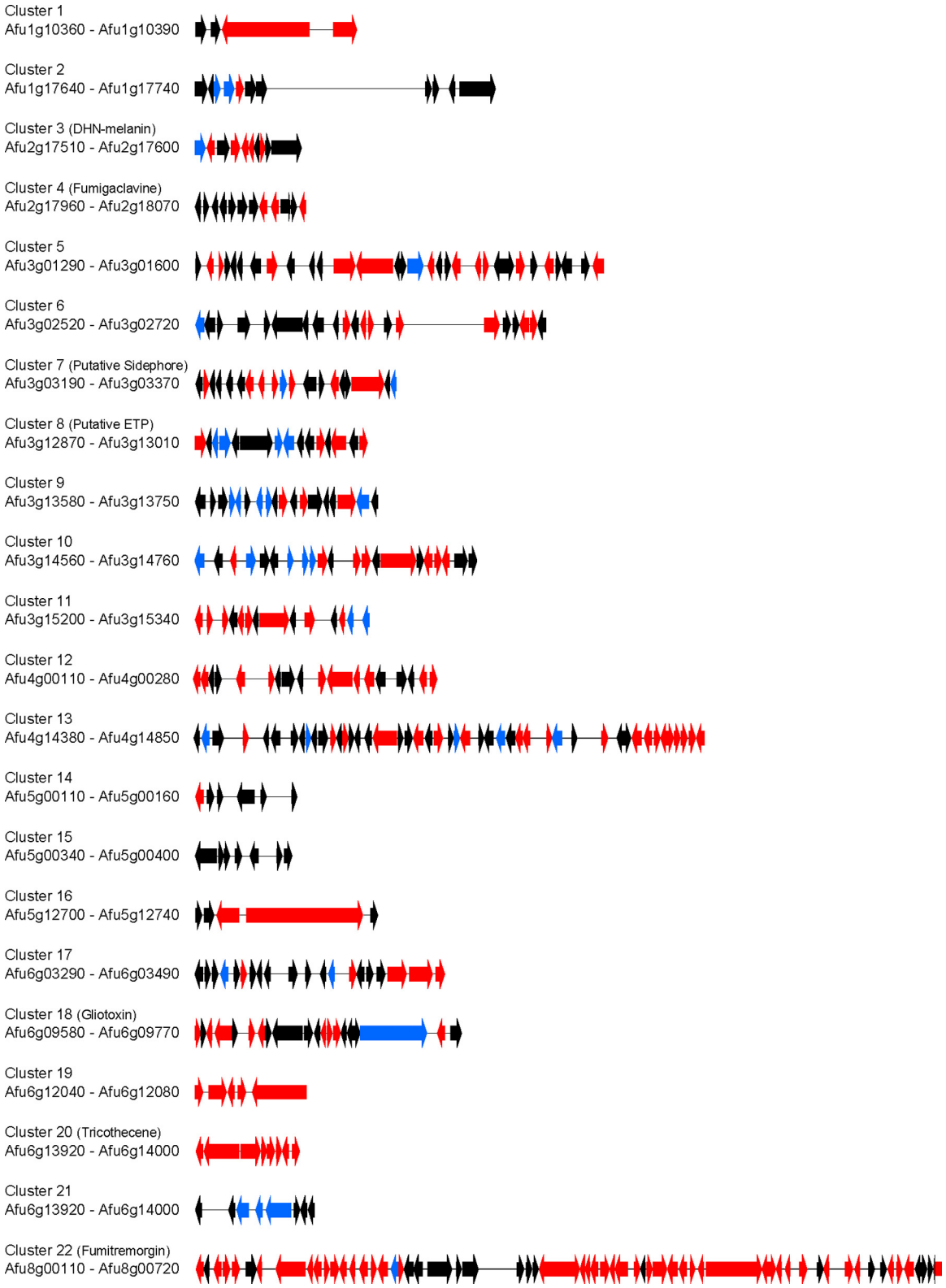

FIG 4 Expression patterns of known secondary metabolism gene clusters. Gene clusters 1 to 22 correspond to the 22 previously characterized secondary metabolism gene clusters in the *A. fumigatus* genome (55). If previously reported, the name of the product produced by the gene cluster is shown in parentheses. The first and last gene names of each cluster are given. For each cluster, we have indicated order of gene transcription and color coded upregulated (in red), downregulated (in blue), and nondifferentially regulated (in black) genes.

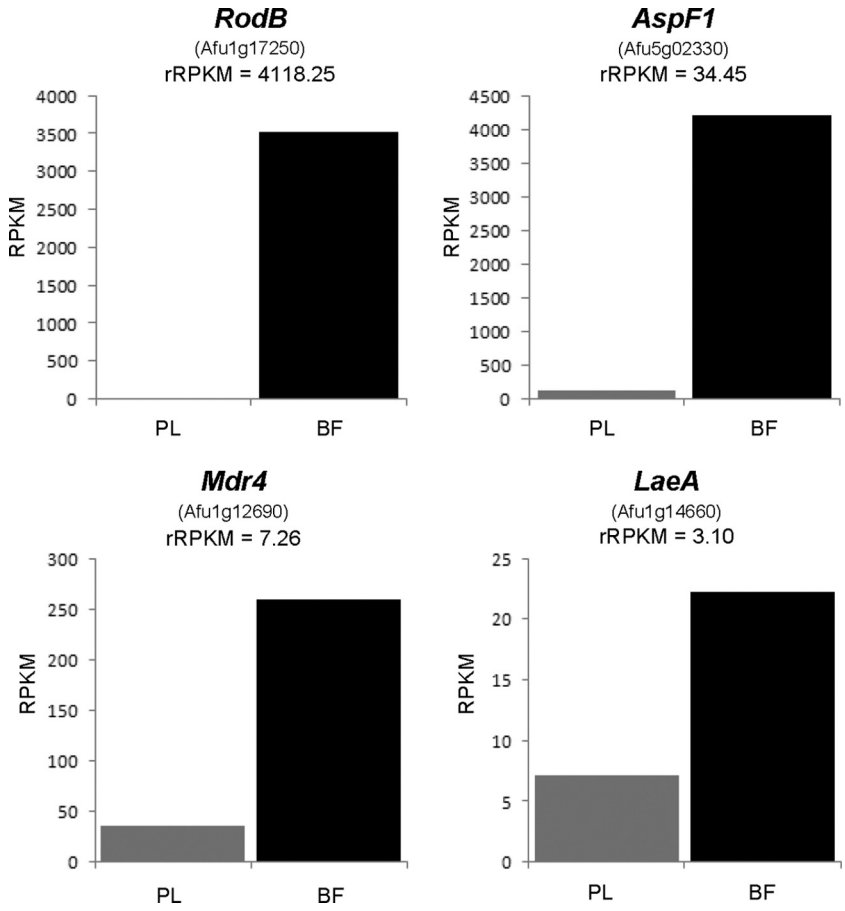

FIG 5 Examples of upregulated genes during biofilm growth. RPKM values (*y* axis) of the cell surface hydrophobin *RodB* (Afu1g17250), the allergen *AspF1* (Afu5g02330), the multidrug transporter *Mdr4*(Afu7g02690), and the global secondary metabolite regulator *LaeA* (Afu1g14660) are shown during PL (gray) and BF (black) growth. For each gene, the rRPKM ($RPKM_{BF}/RPKM_{PL}$) value is reported.

(Afu5g09580), *RodB* (Afu1g17250), and *RodD* (Afu5g01490) were upregulated in BF relative to PL. Interestingly, *RodB* was upregulated by over 4,000-fold and was, overall, the second most upregulated gene (Fig. 5).

Of the 81 allergens examined, 39 genes were upregulated in BF relative to PL and another 2 were uniquely expressed in BF. In contrast, only 12 genes were downregulated in BF relative to PL, and only 1 gene was uniquely expressed in PL (see Table S6 in the supplemental material). Some allergens, such as the galactomannoprotein MP1 (Afu4g03240), the mannosidase MsdS (Afu1g14560), and the RNase AspF1 (Afu5g02330), were expressed >30-fold in BF.

GPI-lation and glycosylation of the proteins were the two major protein modifications that were upregulated in aerially grown mycelia (see Table S6 in the supplemental material). The biosynthesis of the glycosylphosphatidylinositol anchor was upregulated, and so were all 14 genes controlling the steps occurring inside the endoplasmic reticulum (ER), from the palmitoyl coenzyme A (CoA)-dependent inositol acyltransfer, phosphoethanolamine addition, and mannosylation to transfer to proteins. In addition, 38 of the 81 genes coding for putative GPI proteins were upregulated in BF, whereas the genes coding for only 4 of these GPI proteins were downregulated (see Table S6). The other protein posttranslational modification that showed significant upregulation in BF was glycosylation. This is true for both *O* and *N* glycosylation. The genes coding for the four proteins known to be involved in *O* mannosylation—PMT1 (Afu1g07690), PMT2 (Afu3g06450), PMT4 (Afu8g04500), and the ortholog to the *Candida albicans* MNT1 (Afu5g10760)—were upregulated. Finally, 21 of the 25 genes coding for mannosyltransferases located in the ER lumen and in the Golgi apparatus, which are responsible for *N*-glycan biosynthesis, were upregulated in BF relative to PL.

Glycolysis is downregulated in BF. The glycolysis pathway responsible for the anaerobic degradation of glucose to produce ethanol or lactic acid was downregulated in BF (see Tables S4 and S5 in the supplemental material). Specifically, the first step in the glycolysis pathway, resulting in the production of glyceraldehyde-3-phosphate, and the second step, leading to the production of the final product, which collectively account for 21 of the 35 pathway genes, were downregulated. Accordingly, lactate production was reduced in aerial conditions (the lactate dehydrogenase, encoded by Afu5g14800, was downregulated) and so was ethanol production (the pyruvate decarboxylase leading to the production of acetaldehyde was downregulated) in BF. In contrast to glycolysis, respiration bridging the respiratory chain to oxidative phosphorylation was upregulated, including the genes coding for protein complex II, complex III, and complex V, controlling oxidative phosphorylation.

Physiological changes in BF growth can be responsible for drug resistance. Membranes are mainly composed of phospholipids and sterols. Although genes involved in glycerol phospholipid metabolism were not differentially regulated, those involved in the sterol metabolism were upregulated in BF relative to PL. Specifically, 10 out of 20 genes leading to ergosterol from farnesyl diphosphate were upregulated, including the *cyp51A* gene (Afu4g04820), which is targeted by the azole class of drugs and which was 2.3-fold upregulated ($P < 1e{-}300$).

Reduced susceptibility to drugs in BF conditions (60) could also result from an increased activity of efflux pumps and transporter proteins (57). To test this hypothesis, we examined the expression patterns of the 274 genes encoding the major facilitator superfamily (MFS) proteins and the 45 genes encoding the ATP-binding cassette (ABC) proteins (58). We found that 146 of these 319 genes were upregulated (140 genes) or uniquely expressed in BF (6 genes) and 16 were downregulated in BF (11 genes) or uniquely expressed in PL (5 genes) (see Table S6 in the supplemental material). Among the ABC transporters identified were all members of the MDR1 family (MDR1: Afu5g06070, Afu4g14130, Afu6g03470, MDR4: Afu7g02690, Afu6g03080, Afu3g02760, Afu3g03670, Afu1g12690, Afu3g03430, Afu7g00480; MDR2: Afu4g10000) suspected to be associated with drug resistance in *A. fumigatus* (50, 70), and the genes belonging to the *ScYOR1* family (Afu4g09150, Afu5g07970, Afu5g08150, Afu5g10510, Afu1g16880, Afu1g10390, Afu2g01500) associated with drug resistance in yeast (see Table S6) (10).

Widespread differential regulation of transcription factors. The *A. fumigatus* genome contains 392 predicted or experimentally validated transcription factors (1, 5, 21, 22, 34, 47, 52, 64, 66, 73, 76) (see Table S6 in the supplemental material). We found that nearly half of these transcription factors were differentially expressed (124 upregulated [119 genes] or uniquely expressed in BF [5 genes] and 71 downregulated in BF [69 genes] or uniquely expressed in PL [2 genes]), including several that function in asexual and sexual development.

Ribosome and translation. Translation (FunCat ID 12.04) and ribosome biogenesis (FunCat ID 12.01) were upregulated in BF relative to PL (see Table S4 in the supplemental material). Given that much of a typical cell's energy is invested in producing ribosomes, it is not surprising that ribosome biogenesis and translation are upregulated during aerial growth, which is the faster of the two conditions tested (3, 9).

DISCUSSION

New approach to understanding essential changes in *A. fumigatus* lifestyle. In the lung parenchyma, like in the aerial colony on an agar plate (BF), the *A. fumigatus* mycelium is covered by an ECM (3, 8, 35, 46, 48, 57). We reasoned that better understanding the genome-wide transcriptional configuration during BF would provide insight into the genes involved in this process and potentially a better understanding of the establishment of a colony during *in vivo* growth. Our comparative RNA-Seq analysis of growth in these two conditions (BF and PL) shows that this single change in nonnutritional environmental conditions led to the differential expression of thousands of genes (Fig. 1; also see Table S1 in the supplemental material). Considering that a recent comparison of BF and PL growth in *A. fumigatus* by using microarray and two-dimensional (2D) gel electrophoresis technologies identified only ~700 genes and ~40 proteins that were differentially abundant during development (8), RNA-Seq appears to be the most powerful tool for genome-wide functional comparisons of fungal growth to date (7, 49, 77, 79).

Composition and organization of the extracellular matrix. During biofilm growth, fungi produce an ECM that functions in the cohesive linkage of fungal cells with themselves or to the substratum (3, 35, 46). Although little is known about the chemical composition of fungal biofilms, it is well established that ECM composition differs greatly between fungal species. In *C. albicans*, the ECM is mainly composed of β-1,3-glucan (51), whereas the *Cryptococcus* ECM is based on glucuronoxylomannan, the major capsule component (39). In contrast, the *A. fumigatus* ECM is composed of polysaccharides, pigments, hexoses, and proteins (3). One of the three polysaccharides that have been identified so far in the *A. fumigatus* ECM is α-1,3-glucan, a major component of the ECM in an *in vivo* aspergilloma model, where the hyphal network is tightly packed and has binding properties (18, 35). Accordingly, the observed upregulation of the three α-1,3-glucan synthase genes in BF provides indirect support for their function in hyphal adhesion.

Two types of proteins, hydrophobins and adhesins, can help organize the structure of the colony. Hydrophobins are hydrophobic proteins that contain highly conserved cysteine bridges and which are most often responsible for conferring a hydrophobic character to fungal morphotypes (67). For example, the *A. fumigatus* RodAp hydrophobin is a beta-amyloid protein that self organizes into rodlets, confers a hydrophobic character to the conidia, and promotes the adhesion of cells to host proteins (69). Although the RodA and RodB proteins have been purified from conidia (53), our results show that both genes are transcribed prior to the formation of the conidiophore, as it was verified that no conidiophores were present when the RNA was isolated. Furthermore, *RodB* is the second most highly expressed gene in BF, and *RodA*, *RodB*, and *RodD* are all upregulated in BF cells, consistent with recent findings suggesting that several hydrophobins (*RodB*, *RodD*, *RodE*, and *RodF*) are expressed in the *A. fumigatus* ECM (3). The function of hydrophobins during hyphal growth is currently investigated by multiple sequential deletions of all hydrophobin genes.

The other protein family that participates in structuring the colony is the secreted glycosylated adhesins. Adhesins have been characterized in bacteria and yeasts, and their role in cell-to-cell adhesion is well established (11, 16, 65, 75). One of the major changes in BF is an increase in the transcription of genes favoring glycosylation. Glycosylation controls many cell interactions in eukaryotes. For example, in *Candida*, two families of glycosylated adhesins, the Als proteins from *C. albicans* and the Epa proteins from *C. glabrata*, have been shown to mediate adhesion to epithelia and to play a role in biofilm formation (14), whereas in humans, glycan heterogeneity leads to different ligand-receptor complex formations (13).

The upregulation of glycosylation in BF cells was also directly associated to the upregulation of GPI-lation. Although this might have been an expected result, since the vast majority of fungal cell wall proteins is both GPI-lated and *O* and *N* glycosylated, this is the first time this correlation has been observed. Adhesins have not been investigated biochemically in filamentous fungi; however, a number of putative adhesins have been bioinformatically predicted to exist in the *A. fumigatus* genome (72). An upregulation of 11 of the 25 genes with the highest adhesion probability

score (Afu1g09510, Afu1g14430, Afu3g00420, Afu3g01150, Afu3g09690, Afu4g03240, Afu6g13720, Afu7g00580, Afu7g02460, Afu7g05340, and Afu8g01970) was identified. Most of these proteins are also predicted to be GPI anchored. The expression of glycosylated proteins exposed on the hyphal cell wall and present in the extracellular matrix likely results in strong mycelial adhesion between the hyphae of a colony. In addition, increased *N* glycosylation could protect the fungus against stress, in agreement with other data suggesting that the fungus is under higher stress in BF than in PL growth (54).

Fighting antifungal drugs. Whereas most of antifungal drugs are very active *in vitro* against PL cells, one of the major problems in treating aspergillosis is that the same drugs often have very poor *in vivo* impact (12). This is consistent with several reports suggesting that BF cells exhibit higher resistance to drugs than PL cells (44, 45, 57, 60). The RNA-Seq data suggest at least three physiological events that could be responsible for the poor *in vivo* drug efficacy.

First, the presence of an ECM rich in sticky polysaccharides and proteins may reduce the penetration of the drugs. Although this has not been investigated in *A. fumigatus*, work in *Candida* has shown that the extracellular β-1,3-glucan matrix sequesters antifungals (51, 74). Second, sterol metabolism regulation in BF versus PL growth is in the direction expected to counteract the efficacy of azoles. Azoles inhibit the ergosterol biosynthesis pathway by targeting *cyp51A* (Afu4g04820), a gene coding for the 14-α-sterol demethylase enzyme (41). In *A. fumigatus*, it is currently thought that mutations within the *cyp51A* promoter and coding region alter the protein's structure, conferring resistance due to target site alterations (41, 62). *cyp51A* is more than 2-fold upregulated in *A. fumigatus* BF versus PL cells. This higher expression of *cyp51A*, as well as that of other genes responsible for increasing sterol concentration in the membranes of the aerial mycelium, which is expected to lead to an increase in the amount of target sterol, is likely to be associated with azole resistance, as has already been shown in *C. albicans* (15). Finally, azole resistance might be facilitated by multidrug resistance membrane transporters, which play an active role in exporting antimicrobials out of the cytoplasm (42, 50, 61). For example, a recent study showed that *Mdr4* is upregulated in an *in vivo A. fumigatus* biofilm mouse model during voriconazole treatment (57). Consistent with this hypothesis, we identified an overrepresentation of upregulated genes coding for MFS and ABC transporters, including *Mdr1*, *Mdr2*, and *Mdr4*, which are known to be involved in azole resistance (see Table S6 in the supplemental material).

Tolerance to toxic and aggressive environments. The ecological niche for *A. fumigatus* is the soil, where it has to survive in a highly toxic environment and compete with microflora including bacteria, fungi, and protozoa (68). The wide variety of potent mycotoxins produced by *A. fumigatus* helps the fungus survive in this environment, whereas in mammalian hosts, these secondary metabolites have broad virulent and pathogenic effects including cytotoxicity, mutagenicity, carcinogenicity, and immunosuppression (29). Our data show that many genes within secondary metabolite-encoding clusters are upregulated in BF (Fig. 4; also see Table S6 in the supplemental material). For example, similar to the findings of Bruns and coworkers (8), we observed substantial upregulation of the fumitremorgin C supercluster (Figs. 3 and 4). This cluster codes for an unknown compound, as well as the known toxins fumitremorgin (36), a tremorginic mycotoxin capable of inhibiting cell cycle in mammals, and pseurotin A, a neuritogenic compound and chitinase inhibitor (37). Additionally, we observed an upregulation of an entire gene cluster coding for a trichothecene mycotoxin, a compound that can inhibit protein synthesis in eukaryotes (4). Finally, we observed an upregulation of *LaeA* (Fig. 5), a known global regulator of secondary metabolism gene clusters in *A. fumigatus*, which may partially explain the observed widespread upregulation of genes within secondary metabolite-encoding clusters (Fig. 4). Importantly, our comparison also identified several other upregulated gene clusters that are candidates for secondary metabolism involvement but whose function is currently unknown (Fig. 3).

In addition to the protection against other soil microbes conferred by secondary metabolites, *A. fumigatus* is extremely rich in transporters involved in the efflux of toxins that are present in the soil (25, 71). Our results show that the ion transporter genes involved in the efflux of arsenic and copper toxic ions (6/8 genes in the arsenic cluster and 14/15 genes in the copper MIP cluster), two of the most prevalent toxic ions in the soil, are upregulated in BF (see Table S1 in the supplemental material). This suggests that the fungus is well armed to be resistant to toxic ions in the soil. However, and in contrast to plants (6), arsenite efflux does not rely on major intrinsic proteins or aquaporin, which conducts water molecules and selected solutes in and out the cell, as two of the three annotated aquaglyceroporins (Afu4g03390, Afu4g00680, and Afu6g08480) are downregulated in BF relative to PL.

MFS and ABC transporters are significantly upregulated in BF relative to PL. Interestingly, saprophytic fungi, such as *Aspergillus*, typically harbor the highest numbers of transporters (58). For example, the TrasportDB database lists 934 transporters in *A. oryzae* (58; http://www.membranetransport.org/). In contrast, yeasts, which reside in relatively benign environments, have much smaller numbers of transporters; for example, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* contain only 185 and 316 genes, respectively (with 9 and 24 ABC transporters). A similar trend exists in bacteria, where saprotrophs like *Pseudomonas* have at least 3 times as many transporters as do pathogens. Thus, the presence of large numbers of transporters may be a marker for saprotrophism. Given that orthologs of the transcription factors controlling MDR proteins in yeasts (such as *YAP1*, *PDR1*, *PDR3*, and *PDR8*) have been identified by BLAST in *A. fumigatus*, one of the outstanding questions is which are the transcription factors controlling their expression.

Saprophytic microbes not only fight against each other but also compete for food. In contrast to MDR pumps, nutrition-associated transporters usually have specific physiological substrates. We observed that ATP-dependent transporters of the P-type ATP (P-ATPase) superfamily are highly expressed in BF. Specifically, 14 genes, including the gene coding for the major Pma1 protein ATPase, are upregulated, whereas only 5 are downregulated in BF from a set of 23 genes. Similarly, pumps transporting putatively Zn, iron, potassium, sodium, and to a lesser extent, calcium and phosphate are upregulated in BF, and so are amino acid transporters (poorly studied in *A. fumigatus*), peptide transporters, and GABA permeases (FunCat ID 20.01.07). Among the nine upregulated peptide transporters, four are members of the 8-oligopeptide transporter family (23). Monosaccharide/hexose transporter genes are also upregulated. This is in agreement with the release of glucose on the surface of the colony, since transporters can also function as efflux pumps (26) and may be involved in

the active secretion of glucose that was found in high amounts during BF growth (3). Interestingly, the transporters for monocarboxylate (such as lactate and pyruvate—the end products of glycolysis) are highly upregulated in BF. The active transport of nutrient is in agreement with a higher metabolic activity during BF growth.

ACKNOWLEDGMENTS

We thank members of the Rokas lab for valuable comments on this work, Travis Clark and Chelsea Baker for help with Illumina sequencing, and David McCauley for statistical advice. This work was conducted in part using the resources of the Advanced Computing Center for Research and Education at Vanderbilt University.

J.G.G. is funded by the Graduate Program in Biological Sciences at Vanderbilt University and the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH, NIAID: F31AI091343-01). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIAID or the NIH. Work in J.-P.L.'s *Aspergillus* lab is partly funded by the ESF grant Fuminomics and the ALLFUN FP7 project. Research in A.R.'s lab is supported by the Searle Scholars Program and the National Science Foundation (DEB-0844968).

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