Scientific Research Publishing

Advances in Microbiology, 2018, 8, 77-99
http://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

Proteomic Differences between Azole-Susceptible and -Resistant *Aspergillus fumigatus* Strains

Edith Vermeulen[1], Sebastien Carpentier[2], Olaf Kniemeyer[3], Machteld Sillen[1], Johan Maertens[1,4], Katrien Lagrou[1,5*]

[1]Department of Microbiology and Immunology, University of Leuven, Leuven, Belgium
[2]Facility for Systems Biology Based Mass Spectrometry, University of Leuven, Leuven, Belgium
[3]Leibniz Institute for Natural Product Research and Infection Biology—Hans Knoell Institute (HKI), Molecular and Applied Microbiology, Jena, Germany
[4]Department of Microbiology and Immunology and UZ Leuven, Clinical Department of Hematology, University of Leuven, Leuven, Belgium
[5]Department of Microbiology and Immunology and UZ Leuven, Clinical Department of Laboratory Medicine and National Reference Center for Mycosis, University of Leuven, Leuven, Belgium
Email: *Katrien.lagrou@uzleuven.be

How to cite this paper: Vermeulen, E., Carpentier, S., Kniemeyer, O., Sillen, M., Maertens, J. and Lagrou, K. (2018) Proteomic Differences between Azole-Susceptible and -Resistant *Aspergillus fumigatus* Strains. *Advances in Microbiology*, **8**, 77-99.
https://doi.org/10.4236/aim.2018.81007

Received: December 11, 2017
Accepted: January 28, 2018
Published: January 31, 2018

Abstract

Background: Azole-resistance is increasingly reported in *Aspergillus fumigatus* infections. It remains challenging to rapidly assess antifungal susceptibility to initiate the appropriate therapy. The aim of this study was to map the proteomic differences of azole-susceptible and -resistant strains. **Methods**: Proteomic studies were performed with ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS). **Results**: UPLC-MS/MS detected 7899 peptides, of which 1792 peptides had a significantly different abundance ($p < 0.05$) between resistant and susceptible strains. The discriminating proteins were identified and provide an interesting tool for future research into *A. fumigatus* resistance. **Conclusions**: UPLC-MS/MS provided proof-of-concept that the proteome of azole-resistant *A. fumigatus* is diverse enough to serve as a diagnostic tool.

Keywords

Aspergillus fumigatus, Triazole Resistance, Proteomic, Mass Spectrometry

1. Introduction

Triazole resistance in *Aspergillus fumigatus* is recognized as a cause of therapy

failure in patients suffering from *Aspergillus* diseases [1]. Azole-resistance can occur primarily, when azole-resistant spores present in environmental air are inhaled, or secondary in a patient on long-term antifungal therapy. *Aspergillus* susceptibility testing in routine laboratory practice is therefore warranted. However, its implementation is cumbersome due to the considerable workload and cost. Another problem microbiologist are facing is the fact that at least 50% of clinical isolates are due to contamination or colonization [2]. The probability that a positive *A. fumigatus* culture represents a case of invasive aspergillosis (IA) was only 22% in a Spanish university hospital [3]. Susceptibility testing by broth microdilution has a slow turn-around-time (48 h after a pure sporulating culture became available, so at least 72 h after sampling the patient). The recognition of azole-resistance is therefore often a late finding in the management of the individual patient, which is especially unfortunate in the setting of IA. As a result, systematic *Aspergillus* susceptibility testing is mainly executed in specialized centers for patient care or for surveillance reasons. Screening techniques to detect azole-resistance rapidly, with minimal effort and cost, are highly sought. Currently described options include the subculture of *Aspergillus* isolates on selective, azole-containing, screening agars [4] or molecular strategies [5] [6] [7]. Subculturing isolates on screening agars achieve a time gain of 24 hours and are less labour intensive compared to conventional broth microdilution. These agars are now commercially available. Molecular techniques have the advantage that resistance detection can theoretically be performed directly on culture-negative samples and is fast, but this is labour intensive and expensive: Batching the samples will be necessary to be feasible, which will also creates longer turn-around times. Real-time PCR approaches will also miss new emerging mutations, or mechanisms not involving the CYP51A gene.

Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) has rapidly gained ground in clinical laboratories as a routine method for microbial species identification. The main advantages of this approach are the simplicity, low cost and speed of analysis (identification in minutes) [8]. MALDI-TOF MS separates the proteome of a microorganism on their mass-charge ratio, disclosing a characteristic spectrum. Species identification is obtained by matching this spectrum to a library of reference spectra. A generated spectrum never "matches" with absolute identity; the software expresses the degree of similarity.

MALDI-TOF MS has a potential use in the subtyping of strains [9] or in microbial resistance detection [10], when distinctive and conserved differences in, respectively, the spectra of the subspecies or in the susceptible and resistant strains can be detected. This is mainly described for beta-lactamase detection in gram-negative bacteria and methicillin resistance in *Staphylococcus aureus*.

The aim of this study was to provide a proof-of-concept that mass spectrometry can be used to differentiate susceptible from resistant *A. fumigatus* strains, the trypsin digested proteome of three azole-resistant *A. fumigatus* strains and of

three susceptible *A. fumigatus* strains, were analyzed in detail via UPLC-MS/MS analysis. This allows quantifying and identifying peptides/proteins specific for resistance or susceptibility.

2. Methods

Fungal Isolates for UPLC-MS/MS analysis—A large *Aspergillus* culture collection is at our disposal at the National Reference Center for Mycosis, University Hospitals Leuven.

UPLC-MS/MS analysis—Three azole-resistant *A. fumigatus* strains (1 with CYP51A genotype TR46/Y121F/T289A, 2 TR34/L98H) and three azole-susceptible *A. fumigatus* strains, randomly chosen from the culture collection, were each subcultured in triplicate on diluted Sabauroud slants, incubated at 37˚C for 48 h and each subculture was extracted independently. Proteins were extracted in acetonitrile (ACN) 50%, formic acid (FA) 35%, as described by Bruker Daltonics (Bremen, Germany) and dried in a vacuum operator until dry. The resulting protein extracts (n = 18) were dissolved in 40 µl 2 M urea, 50 mM ammonium bicarbonate and reduced with 0.020 M dithiotreitol for 15 min and subsequently alkylated with 0.050 M iodoacetamide for 30 min in the dark. Then the sample was digested with 0.01 µg trypsin (Sigma Aldrich) overnight at 37˚C. The digestion was stopped by adding trifluoroacetic acid to a final concentration of 0.5%. Peptides were purified with Pierce C18 Spin Columns (Thermo Scientific), according to the manufacturer, vacuum dried and dissolved in 10 µl of ACN 5%, FA 0.1%. UPLC-MS/MS analysis was performed on a Q Exactive Orbitrap mass spectrometer (Thermo Scientific). Five microliter from each sample was injected and separated on an Ultimate 3000 UPLC system (Dionex, Thermo Scientific). The samples were separated using as buffer A water 99.9%, FA 0.1% and B ACN 80%, water 20%, FA 0.1%, using an EasySpray C18 column (Thermo Scientific) with a gradient of 4% to 10% B (6 min) followed by 10% - 35% B (25 minutes), 35% - 65% B (5 min) and a final elution and re-equilibration step at 95% and 5% B respectively. The flow-rate was set at 300 µL/min. The Q Exactive was operated in positive ion mode (nanospray voltage 1.5 kV, source temperature 250˚C). The instrument was operated in data-dependent acquisition (DDA) mode with a survey MS scan at a resolution of 70,000 for the mass range of m/z 400 - 1600 for precursor ions, followed by MS/MS scans of the top 10 most intense peaks with +2, +3 and +4 charged ions above a threshold ion count of 16,000 at 35,000 resolution using normalized collision energy (NCE) of 25 eV with an isolation window of 3.0 m/z, an apex trigger 5 - 15 sec and a dynamic exclusion of 10 s. All data were acquired with Xcalibur 2.2 software (Thermo Scientific).

Protein identification—The LC-MS raw data were imported to Progenesis Nonlinear software (version 4.1) and peaks were detected on all aligned runs. An mgf file was generated via Progenesis and searched using Mascot (version 2.2.04) in a first round against our in-house database containing all the uniprot sequences of *Neosartorya fumigata* (containing 20,414 accessions) and additionally against the whole fungal database of Swissprot taxonomy fungi (containing

16,473 accessions). Parameters were set at: tryptic digestion, one miscleavage allowed, 10 ppm precursor mass tolerance and 0.02 Da for fragment ion tolerance with a fixed modification of cysteine carbamidomethylation and a variable modification of methionine oxidation. Subsequently files were imported in Scaffold (version 3) combining the Mascot search with Xtandem. Proteins were considered as identified when they met the criteria: min 95% protein, min 1 peptide 95%. FDR at those criteria was calculated as 0.1% at protein level and 0.4% peptide level.

Protein annotation—The fasta files of all the identified proteins (min 95% protein, min 1 peptide 95%) were exported from Scaffold and were subsequently annotated via Blast2go Version 2.7.0 (http://www.blast2go.com/b2ghome). Data containing an Interpro annotation were exported and introduced in cytoscape (version 3.0.2) to visualize related proteins.

Peptide/protein quantification—As indicated above the LC-MS raw data were imported to Progenesis Nonlinear software and normalized. Peptides were considered as significantly different between the resistant and susceptible condition, when ANOVA $p < 0.05$. Protein abundance was calculated via progenesis by considering only the peptides with no conflicts. Proteins were considered as significantly different when ANOVA $p < 0.05$.

Blind clustering of the proteomes—Protein abundances of the ANOVA significant proteins were exported from Progenesis and imported into Statistica 8 (Nine sigma) to perform a Pincipal Component Analysis (PCA) (NonLinear Iterative Partial Least Squares NIPALS algorithm). Scores were exported and visualized using Microsoft Excel.

3. Results

UPLC-MS/MS analysis—A total of 7899 tryptic peptides were detected, of which 22.7% (1792 peptides) had significantly different abundances ($p < 0.050$) between the resistant and susceptible strains. Only 2082/7899 (26.4%) peptides were identified, belonging to 553 proteins when matched against all species in swissprot. A blind clustering of the most important proteins using Principle Component Analysis (PCA) shows both sample groups can be separated (**Figure 1**). Principle component 1 (PC1) explains 44% of the observed variability and PC2 12%. The proteins with confident identification (defined as a confidence score ≥ 40.0) which count at least one peptide with significantly differing abundance between the susceptible and resistant strains (112 proteins) are listed in **Supplemental Table S1**. Among these proteins, 16% (18/112) are ribosomal proteins, 14% (16/112) are involved in stress response or oxidation-reduction, 12% (13/112) in carbohydrate metabolic processes-including four alpha-1,2-mannosidases. Another 5% (6/112) are specifically involved in glucan metabolism. Proteins with uncharacterized function represent 19.6% (22/112). A Pubmed literature search and Aspergillus Genome Database search (AspGD, http://www.aspergillusgenome.org/) was performed for every protein with (a)

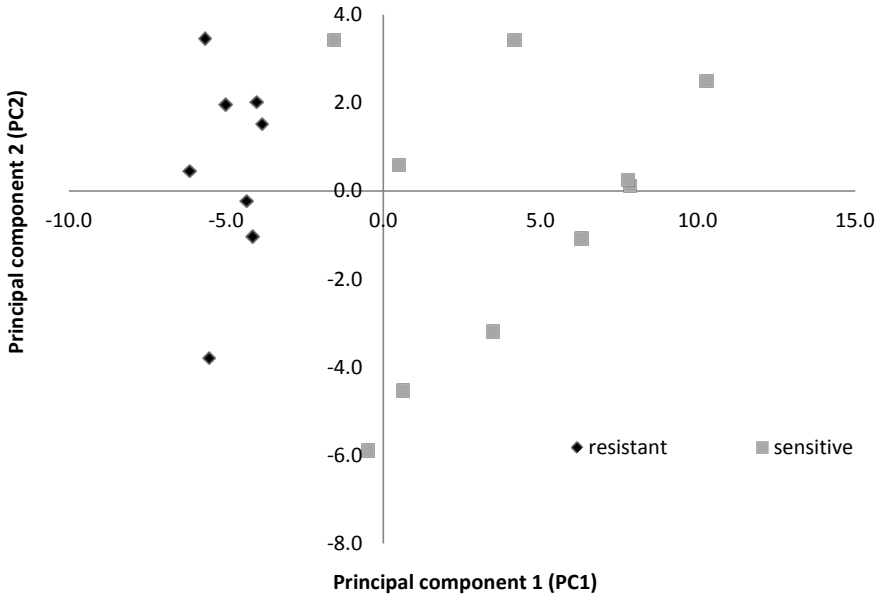

Figure 1. Principal component analysis score plot of the most important proteins of triazole resistant and susceptible *Aspergillus fumigatus* isolates. Black diamonds represent the resistant strains, grey squares the sensitive stains.

significantly different abundant peptide (s) between susceptible and resistant strains (or its orthologs), to evaluate for a known role in virulence, host response, diagnostic properties or antifungal susceptibility. For 29 proteins (26%), relevant information was obtained (Supplemental Table S1); twelve interesting proteins are highlighted in Table 1. No peptides of lanosterol-5α-demethylase, the target protein of azole therapy (encoded by CYP51A), were identified from azole-resistant or–susceptible strains and the known differences in this protein are therefore no contributing factor in the proteomic differences observed here.

4. Discussion

To the best of our knowledge, this is the first study evaluating proteomic differences between triazole susceptible and resistant *A. fumigatus* isolates based on UPLC-MS/MS analysis. Our approach of comparative proteome analysis provided proof-of-concept that significant proteomic differences exist. These differences were larger than expected, which indicates that susceptible and resistant *A. fumigatus* probably accumulated mutations over time. However, only a limited fraction of the differentiating peptides could be identified, which demonstrates the constraints of the current databases. Significant abundancy of a protein in one condition can mean that this protein is indeed less abundant in the other condition, but can also mean that certain peptides of this protein bear mutations/polymorphisms and are therefore not identified in the second condition (independent of their abundancy). Among the proteins which have at least one peptide with significantly different abundance between susceptible and resistant strains, about one out of four proteins (or its orthologs) are known to be relevant in azole resistance, virulence or host response (Supplemental Table S1).

Table 1. Proteins with at least one peptide with significantly different abundance in resistant versus susceptible *A. fumigatus* strains: Highlights.

Systematic name		Protein ID[$]	Condition with abundance (*p*)*	Comment	Ref
Afu5g09580	Hydrophobin RodA	H6SWN9	R (0.003)	Surface proteins important in adhesion to host. RodA is a virulence factor that masks Dectin-1 and Dectin-2 recognition of conidia, resulting in impaired neutrophil recruitment, neutrophil extracellular trap formation, increased fungal survival and clinical disease. RodA plays a role in the transfer of posaconazole to conidia.	[11] [12] [13]
		RODL_ASPFU	R (<0.0001)		
		Q8TFV2	R (0.0001)		
		Q8TFV8	R (0.0002)		
Afu1g17250	Hydrophobin RodB	B0XPA4	R (0.0335)		
Afu8g07060	Hydrophobin, RodC	B0Y9E4	R (0.0004)		
Afu2g09290	HSP60, putative (Chaperonin Cpn60 family)	B0XRX3	S (0.0170)	Immune modulator: vaccine with rHsp60 from *H. capsulatum* and *P. brasiliensis* induces protection. Chaperonins participate in nucleotide excision repair. Other heat shock proteins (Hsp90) are suggested in mediating environmental influence on tandem repeat mutation rates.	[14] [15] [16]
Afu3g10490	DNA damage response protein (Dap1)	B0XXV2	S (0.0229)	*S. cerevisiae* ortholog Dap1p is required for cell cycle progression following damage, is involved in sterol regulation and directs resistance to itraconazole and fluconazole.	[17]
Afu2g01170, Gel1	1,3-Beta-glucanosyl transferase	GEL1	R (0.0144)	GEL1 is the antigen eliciting the highest frequencies of specific T cells producing IFN-c (protective immune response mediating lysis of *Aspergillus* hyphae). Strenghtens cell wall assembly in stress. Expression is induced by voriconazole 0.5 mg/L	[18] [19]
Afu3g12120	Fatty acid oxygenase ppoC	B0XX73	R (0.0011)	The fatty acid oxygenases ppoA, ppoB and ppoC play an important role in host immune recognition and virulence and are important in integrating asexual and sexual spore balance.	[20]
Afu8g01050, Afl1	Fucose-specific lectin	Q8NJT4	S ($<10^{-12}$)	AFL is suggested to be responsible for conidia attachment to the human lung epithelium. AFL is found in sera of ABPA patients, has proinflammatory effects and is a possible virulence factor.	[21]
Afu5g14740, FleA	Fucose-specific lectin	B0Y2N1	S (0.0061)	Genes regulated by SrbA, which plays a role in ergosterol biosynthesis and resistance to azoles. -FleA may enhance attachment of spores to host cell membranes and contribute to pathogenicity. -Idi1 is connected to isoprenoid biosynthesis (precursors for biosynthesis of ergosterol)	[22] [23]
Afu4g09600	GPI anchored protein, putative	B0Y6F1	R (0.0012)		
Afu6g11160, Idi1	Isopentenyl-diphosphate delta-isomerase	B0Y8F7	S (0.0061)		
Afu4g11800, Alp1	Alkaline protease 1	ORYZ_ASPFU	R (0.0001)	Involved in immune evasion: cleaves C3, C4, C5, C1q and IgG, leading to down-regulation of complement activation at hyphal surface. Alp-deficient *A. fumigatus* caused drastically reduced mortality in an IA murine model, compared to the wild-type isolate (not confirmed in other reports).	[24] [25]

[$]Phylome DB database identification [15]. *Condition (susceptible (S) or resistant (R)) with significant abundance of at least one peptide ($p < 0.05$). The *p*-values express the minimal level of significance for abundance at the peptide level.

This illustrates the power of comparative proteome analysis to identify interesting targets for research into antimicrobial resistance. Among the differing components, several mitochondrial proteins were detected, involved in stress response (e.g. antigenic mitochondrial protein HSP60, a mitochondrial superoxide dismutase) and also cofilin, which is suggested to play a role in the regulation of mitochondrial function and stress responses and is linked to multi-drug resistance [26]. These data support the hypothesis that mitochondrial activity effects triazole tolerance [27] [28]. Secondly, several conidial proteins (e.g. RodA, RodB, FleA, Arb2, Con-10) and cell-wall modifying enzymes (e.g. glucanases Exg9, EgIC) were also found with significantly different abundances between susceptible and resistant strains. Overall, the identification of many conidial proteins is to be expected as proteomic studies were performed on sporulating strains. A different sporulation rate between resistant or susceptible strains could be an explanation for these different abundances, but could not be objectified visually. A third interesting observation is that many ribosomal proteins are present among the differentiating proteins, which are considered highly conserved intraspecies. This could indicate that the proteome differences reflect a common genomic background of the strains which evolved to azole-resistance. MALDI-TOF MS instruments in clinical laboratories detect proteins in the range of 2000 - 14,000 m/A, which is known to correspond largely with the ribosomal protein fraction.

5. Conclusion

In conclusion, we proved the presence of substantial proteomic differences between azole-susceptible and azole-resistant *A. fumigatus* strains. We believe that our data provide interesting new options for research into *A. fumigatus* resistance.

Acknowledgements

JM reports grants and personal fees from Pfizer, grants and personal fees from MSD, personal fees from Astellas, personal fees from Gilead, outside the submitted work. KL reports grants and personal fees from Pfizer, grants and personal fees from Gilead Sciences, grants and personal fees from Merck, outside the submitted work. EV, SC, OK and MS have nothing to disclose.

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Supplemental

Table S1. Proteins with at least one peptide with significantly different abundance in resistant versus susceptible *A. fumigatus* strains.

Abundant condition (S/R)	Reference	protein	Systematic name	Seq. Description	Comment	Peptide count	Peptides used for quantitation	Confidence score	MinimalAnova (p)	Max fold change
Hydrophobins										
R		B0XPA4	Afu1g17250	Conidial hydrophobin Rod B	• Surface proteins that confer hydrophobicity; Immunologically silence conidia • RodA masks Dectin-1 and Dectin-2 recognition of conidia, resulting in impaired neutrophil recruitment, increased fungal survival and clinical disease; RodA strongly inhibits neutrophil extracellular trap formation • Posaconazole concentrates within host cell membranes and rapidly transfers to *A. fumigatus*, where it accumulates. Transfer to conidia does not require phagocytosis, but is markedly enhanced by RodA. • Important in adhesionto host (albumin and collagen)	4	4	336	0.0335	1.55
R		B0Y9E4	Afu8g07060	Hydrophobin, putative Rod C		12	12	1095	0.0004	1.99
R		RODL_ ASPFU		Hydrophobin precursor Rod A (Rodletprotein)		14	3	1785	0.0000	2794
R	[1]-[7]	H6SWN9		Hydrophobinrod A fragment		13	1	1624	0.0033	2.98
R		Q8TFV2	Afu5g09580	Hydrophobin hyp 1 fragment		9	1	1327	0.0001	2.48
R		Q8TFV8		Hydrophobin fragment		9	1	1237	0.0002	3.99
Translation/Ribosomal proteins										
S	[1]	B0XQN8	Afu1g11190	eukaryotic translation elongation factor 1 subunit		7	6	588	0.0011	3.15
R	[1]	B0XR75	Afu1g12890	60s ribosomal protein l5		2	2	120	0.0370	1.21
S	[1]	B0XTE9	Afu2g13530	elongation factor 2		16	13	713	0.0106	1.60
S	[1]	B0XWG9	Afu2g13530	40s ribosomal protein s0		5	5	251	0.0004	3.68
S	[1]	B0Y372	Afu5g05630	60s ribosomal protein l23		3	3	93	0.0001	1.44
R	[1]	B0YBW2	Afu7g01460	40s ribosomal protein s5		3	3	117	0.0440	2.17
S	[1]	B0YD67	Afu6g03830	ribosomal protein l14		6	6	249	0.0062	1.57
S	[1]	B0YEB1	Afu4g04460	60s ribosomal protein l13		4	4	175	0.0312	2.01
S	[8]	Q4WWP9	Afu3g06640	40s ribosomal protein s27		2	2	108	0.0001	2.72
R	[1]	B0XYG2	Afu3g08290	aspartyl aminopeptidase		10	9	481	0.0015	1.12
S	[1]	B0XXQ3	Afu3g10920, stm1	telomere and ribosome associated protein Stm1, putative		3	3	64	0.0030	1.60
R	[8]	Q4WLQ8	Afu6g12660	40s ribosomal protein s10b		3	3	79	0.0410	1.52
S	[8]	Q4WRF2	Afu1g16523	40s ribosomal protein s25		4	4	181	0.0142	1.25

Continued

S	[9]	RL34A_SCHPO	rpl34a	60S ribosomal protein L34-A		3	1	85	0.0408	536,330
R	[8]	RS23_ASPFU	rps23	40S ribosomal protein S23		3	3	48	0.0033	1.85
S	[10]	RS9_PODAN	RPS9	40S ribosomal protein S9 (S7) -		2	1	50	0.0001	89.76
R	[11]	HEX1_EMENI	HexA	Woronin body major protein	Associated with the septal pores, role in translation regulation (ribosome binding)	3	1	97	0.0011	1.15
R	[8]	RL17_ASPFU	rpl17	60S ribosomalprotein L17		7	7	355	0.0409	1.36
				Stress response						
R		B0XRX3	Afu2g09290	antigenic mitochondrial protein HSP60, putative (Chaperonin Cpn60 family)	• Cpn60 molecular sequencing is applied for barcoding • Fungal Hsp60 can act as immunodominant antigens and facilitate powerful immunological properties; Vaccination with recombinant Hsp60 from the dimorphic fungi Histoplasma capsulatum and Paracoccidioides brasiliensis induced protection against these mycotic infections • It is suggested that fever can activate Hsp60 gene expression of fungi in the human body and subsequently modify immunoregulatory processes; possible pathogenetic relevance	2	2	60	0.0170	1.11
S	[1] [12] [13] [14] [15] [16]	B0Y8B3	Afu6g10700	Chaperonin, putative (10 kda heat shock mitochondrial, Cpn10, Hsp60 family)	• Molecular chaperonins participate in nucleotide excision repair by maintaining repair proteins in their properly folded state. For another chaperonin, Hsp90, it has been demonstrated that CAG repeat stability is modulated by this chaperone protein. A decrease in Hsp90 levels results in nearly tenfold increases in the rate of contraction of a CAG repeat tract, while not affecting the rate of point mutations. As severe environmental stresses can overwhelm Hsp90 function, Hsp90 may be playing a role in mediating an influence by the environment on TR mutation rates.	2	2	66	0.0330	3.98
S	[1] [16] [17]	B0XXV2	Afu3g10490	DNA damage response protein (Dap1)	S. cerevisiae ortholog Dap1p is required for cell cycle progression following damage and is involved in sterol regulation. Dap1p directs resistancetoitraconazole and fluconazole.	2	2	112	0.0229	10.26

Continued

S/R	[1] [16] [18]	B0YBR6	AFUB_087580, aldA	aldehyde dehydrogenase	• A whole-genome comparison performed on serial isolates from an aspergilloma patient revealed one itraconazole-resistant isolate which carried a mutation in aldA, together with five other nonsynonymous mutations, including the cyp51A mutation P216L • Induced in neutrophil-exposed conidia; repressed by gliotoxin exposure	12	9	569	0.0108	1.53
S		B0Y8I3	AFUB_077440, aldA	aldehyde dehydrogenase AldA, putative		10	8	422	0.0385	3.35
R	[1] [8] [19] [20] [21]	SODC_ASPFL	sodC	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1) - Aspergillus flavus	• Superoxide dismutases destroy toxic radicals which are normally produced within the cells • Role in the resistance to phagocytic killing, but no decrease in virulence could be demonstrated for strains with mutations in three SOD genes in a murine IA model	2	2	82	0.0497	2.17
S	[1]	B0Y6Y9	Afu4g11580, sod2	Mn superoxide dismutase		4	4	137	0.0227	3.93
S	[8]	SODM_ASPFU	sodB	Superoxide dismutase [Mn], mitochondrial precursor (AllergenAsp f 6)		6	6	273	0.0232	4.33
S	[1] [20] [22] [23] [24] [25]	B0YAQ9	Afu5g11320	thioredoxin	• *A. fumigatus* thioredoxin proteins function as antioxidants during infection; impair neutrophil-mediated fungal killing; are required for fungal growth during infection of the cornea • Thioredoxin reductase, a different protein involved in the thioredoxin pathway is named as a possible new target for antifungal therapy, and as a potential biomarker for serological diagnosis • Thioredoxins are cross-reactive allergens involved in the pathogenesis of atopic eczema and asthma • Thioredoxin and thioredoxin reductase are regulated by the AP-1 like bZip transcription factor Yap1, which is involved in the antioxidative stress response and antifungal drug resistance in C. albicans	3	2	58	0.0447	3.92
S	[8]	Q4WQZ2	Afu4g14530	glutathione s-transferase ure2-like protein, gstC		9	2	406	0.0011	349.13
				Carbohydrate metabolic process						
R	[1] [16]	B0XM55	Afu6g13760	alpha-1,2-mannosidase subfamily	Transcript up-regulated in hyphae upon exposure to neutrophils	7	7	257	0.0078	4.16
S	[1]	B0XQJ8	Afu1g10790	alpha-1,2-mannosidase family		14	14	674	0.0000	2.84
R	[1]	B0YCI0	Afu7g04720	alpha-1,2-mannosidase, putative		4	4	190	0.0105	2.52

Continued

R	[8]	Q4WV22	Afu5g10520	alpha-1,2-mannosidase family protein		5	5	291	0.0090	8.29
R	[1] [16]	B0XU55	Afu2g14750	endo-arabinase	Transcript downregulated in response to voriconazole	6	5	203	0.0332	1.57
R	[1]	B0XV35	Afu2g14750	endo-arabinase		3	3	110	0.0055	1.72
S	[1] [16]	B0Y9W3	Afu8g05020, nagA	beta-n-acetylhexosaminidase	Highlyexpressed in biofilm	24	23	1188	0.0011	1.14
S	[1]	B0XYS2	AFUB_ 041890	putativeun characterized class v		40	36	2142	0.0000	1.46
R	[26]	Q875I9	csn	Endo-chitosanase	Sera from aspergilloma and IA patients recognized recombinant chitosanase in immunoblot, indicating expression during infection	7	7	463	0.0001	1.43
R	[27]	CHI1_ COCIM	cts1	Endochitinase 1 (complement-fixation antigen)		4	3	132	0.0027	4.31
R	[28]	F1DGF3_ ASPFM	bgl3	Extracellular beta-glucosidase/cellulase BGL3		21	21	1142	0.0061	1.35
S	[31]	MDHM_ YEAST	mdh1	Malate dehydrogenase, mitochondrial		14	13	553	0.0120	3.17
S	[8] [16]	ENO_ ASPFU	enoA	Enolase (AllergenAsp f 22)	Transcript induced by growth on hydrogen peroxide; hypoxia induced protein; transcript induced by exposure to human airway epithelial cells	6	6	294	0.0100	4.10
				Glucanmetabolism						
R	[1]	B0XYP2	Afu2g00430, exg9	Exo-beta-1,3-glucanase, putative		13	13	511	0.0000	2.25
R	[1]	B0XMS4	Afu1g14450, exgO	Exo-beta-1,3-glucanase Exg0		14	13	1284	0.0006	1.58
R	[1]	B0XND8	Afu1g04260, engl1	endo-1,3-beta-glucanase engl1	Glucanases play a role in cell expansion during growth, in cell-cell fusion during mating, and in spore release during sporulation	12	12	447	0.0041	1.14
R	[1]	B0XSV7	Afu2g00690	Glucoamylase		5	4	210	0.0018	3.94
R	[1]	B0XXF8	eglC	Probable glucan endo-1,3-beta-glucosidase eglC		2	2	55	0.0178	6.96
R	[1]	B0YDR8	Afu6g01800	endoglucanase i		3	3	125	0.0039	1.90
R	[8] [29] [30]	GEL1_ ASPFU	gel1	1,3-beta-glucanosyltransferase gel1	• The antigens eliciting the highest frequencies of specific T cells producing IFN-c (protective immune response which mediates lysis of Aspergillus hyphae) is GEL1. • Strenghtens cell wall assembly in stress conditions • gel1 expression is markedly induced by exposure to voriconazole 0.5 mg/L	3	3	111	0.0144	1.01

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				Extracellular proteins						
R	[1] [32] [33]	B0XPZ1	mep1	metalloprotease mep1	Secreted metalloproteinase that allows assimilation of proteinaceous substrates. Plays a pivotal role as a pathogenicity determinant during infections and contributes to the ability of the pathogen to persist within the mammalian host in dermatophytes and dimorpic fungi.	3	3	143	0.0124	1.26
R	[1]	B0Y1L0	Afu5g01200, cp6	carboxypeptidase S1		3	3	153	0.0014	2.81
R	[1]	B0XYA1	Afu3g08930, sedC	tripeptidylpeptidase sed3		17	17	891	0.0133	1.29
S	[1]	B0Y226	pre6	proteasome component pre6		3	2	42	0.0103	9.30
S	[1] [25] [34] [35]	B0Y5S8	metH	5-methyltetrahydropteroyl-triglutamate-homocysteine s-methyltransferase	• Putative cobalamin-independent methionine synthase; protein induced by heat shock • Yap1-dependent induction in response to hydrogen peroxide (Yap1 is involved in the antioxidative stress response and antifungal drug resistance in C. albicans) • Reacts with rabbit immunosera exposed to *A. fumigatus* conidia	15	14	624	0.0001	2.57
R	[1] [36]	B0XYT5	gta1	glutaminase (nitrogenmetabolism)	Suggested as potential diagnostic target	10	10	701	0.0063	1.94
R	[1]	B0XPL9	Afu1g06470	neutral alkaline nonlysosomal ceramidase, putative		7	6	323	0.0358	1.88
R	[1]	B0XTM3	fmdS	formamidase		2	2	71	0.0021	1.85
R	[1]	B0XVH5	Afu2g05240	cytochrome cd1-nitrite reductase-c-terminal haem d1		8	8	474	0.0281	1.32
				Oxidation-reduction						
S	[1]	B0XV36	Afu2g04490	D-3-phosphoglycerate dehydrogenase		1	0	51	0.0123	
S	[1] [16] [37]	B0Y871	Afu6g10260, akr1	aldehyde reductase (akr1)	Transcript up-regulated in conidia exposed to neutrophils and to human airway epithelial cells	2	2	42	0.0455	2.10
S	[1] [38]	B0Y8P4	fmqD	fad binding domain protein	Downregulated in mycelia exposed to voriconazole	11	11	520	0.0035	1.67
S	[1]	B0Y9I5	AFUB_081170	oxidoreductase, putative		2	1	112	0.0015	Infinity
S	[8]	Q4WWK4	Afu_3g06190	cytochrome c oxidase subunit		2	2	102	0.0065	2.84
R	[1]	B0XYI5	Afu3g08070	GMC oxidoreductase, putative		2	2	102	0.0065	2.52
				Protein binding						
S	[1] [39]	B0XPX0	Afu1g07540	26s proteasome regulatory particle subunit, Rpn8	Mutation confers hypersensitivity to amphotericin B in C. albicans RMN8	3	3	124	0.0121	3.49

Continued

S	[1]	B0XWB1	Afu2g08190	tubulin-specificchaperone Rbl2		1	1	51	0.0007	2.46
				Sporulation						
R	[1]	B0XVP9	Afu2g17530, abr2	conidial pigment biosynthesis oxidase abr2		15	14	981	0.0040	1.70
R	[1] [16] [40]	B0XX73	Afu3g12120, ppoC	fatty acid oxygenase ppoC	• Important in integrating asexual and sexual spore balance. PpoC: positive regulation of sexual sporulation resulting in formation of a cellular spore, negative regulation of asexual sporulation resulting in formation of a cellular spore • The fatty acid oxygenases PpoA, ppoB and ppoC play an important role in host immune recognition and virulence. PpoA is found to be increased in exposure to voriconazole in A. nidulans. • Ppo enzymes are putative cyclooxygenase-like enzymes generating different oxylipin species: oxylipins are proposed to regulate developmental and virulence pathways in the fungal cell	12	10	533	0.0011	72.51
R	[1]	B0XYH2	ams1	alpha-mannosidase		6	6	212	0.0139	1.89
S	[1]	B0YDC6	conJ	conidiation-specificprotein (con-10)		11	11	1026	0.0082	2.18
R	[41]	Q6PWQ1	man70	mannosidase		17	4	1005	0.0027	1.15
				Uncharacterized						
S	[1]	B0YBK6	Afu2g12680	conserved hypothetical protein		2	2	121	0.0424	7.63
R	[1]	B0XRM1	Afu1g13670	conserved hypothetical protein		15	15	1167	0.0000	2.64
R	[1]	B0Y1T8	Afu5g13100	conserved hypothetical protein		2	2	91	0.0130	1.69
S	[1]	B0Y889	Afu6g10450	conserved hypothetical protein		3	3	78	0.0009	5.75
R	[1]	B0Y6B4	Afu4g09220	Uncharacterized protein		1	1	41	0.0001	Infinity
S	[1]	B0YB04	AFUB_080030	Putative uncharacterized protein		3	3	94	0.0012	440551
S	[1]	B0YF63	Afu4g01180	Uncharacterized protein		3	3	68	0.0062	21.98
S	[1]	B0XTF5	AFUB_029230	Putative uncharacterized protein		1	1	50	0.0327	1.95
S	[1]	B0XX21	Afu3g00960	Putative uncharacterized-protein		1	1	47	0.0061	Infinity
S	[1]	B0XZW1	Afu3g14660	Putative uncharacterized protein		1	1	27	0.0004	5.74

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S	[1,38]	B0Y209	Afu5g01990	BYS1 domain protein, putative	Increased expression after voriconazole exposure	2	2	86	0.0393	10.53
R	[1]	B0XVT1	Afu2g05635	Putativeuncharacterized, hypotheticalprotein		5	5	491	0.0037	1.46
S	[1]	B0XT52	Afu7g00610	Putative uncharacterized, cupin domain protein		3	1	113	0.0459	1.09
S	[1] [42]	B0XWT8	Afu3g12790	Conserved glutamic acid-rich protein	Transcript up-regulated in conidia exposed to neutrophils	1	1	51	0.0041	4.59
R	[1]	B0Y1N8	Afu5g01420	Putative uncharacterized protein (secreted protein)		7	7	255	0.0008	1.99
R	[1]	B0XWK2	Afu3g12982	Putative uncharacterized protein (extracellular serine rich protein)		2	2	74	0.0009	1.94
S	[1]	B0Y6P7	Afu4g10610	Uncharacterized protein (stress responsive a b barrel domain protein)		4	4	126	0.0015	1351
S	[8]	Q4WFD3	Afu3g02600	Uncharacterizedprotein (putrescine oxidase)		2	1	79	0.0101	3.02
R	[1]	B0YAV2	Afu8g00630	Putative, uncharacterized (f5 8 type c domain) protein		9	9	816	0.0001	1.70
S	[1]	B0YCY3	Afu6g04690	Putative characterized (conserved hypothetical) protein		14	14	873	0.0027	1.09
S	[8]	Q4WM72	Afu6g10930	Putative, extracellular protein (DNA-directed RNA polymerase activity)		11	11	513	0.0238	1.69
S	[1]	B0Y1Q6	Afu5g12780	Uncharacterized (Kelchrepeat) protein		5	5	176	0.0133	8.79
				Other						
S	[1]	B0XQ18	Afu1g08960	cAMP-regulated phosphoprotein family protein igo1		1	1	52	0.0044	4.33
S	[1]	B0XMN4	Afu1g02980	6-phosphogluconolactonase		2	2	107	0.0444	3.31
S	[1]	B0XZJ8	Afu3g15090	adenosine deaminase family protein (secreted protein)		18	17	1029	0.0006	1.23
R	[1]	B0Y214	Afu5g02040	lipase (secretedprotein)		6	6	290	0.0337	1.19
S	[1] [43] [44]	B0Y2N1	fleA	fucose-specific lectin FleA	• One of the genes regulated by SrbA, which plays a role in ergosterol biosynthesis and mediates resistance to azole antifungals (fluconazole susceptibility, lower voriconazole MIC in SrbA null mutant) • Strong hemagglutinin activity. This lectin may enhance attachment of fungal spores to mammalian cell membranes and contribute to the pathogenicity.	5	2	220	0.0061	Infinity

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S	[45] [46]	Q8NJT4	afl1	Fucose-specificlectin AFL 1	AFL is suggested to be the protein responsible for conidia attachment to the human lung epithelium. AFL is found from the sera of ABPA patients, has proinflammatory effect and is a possible virulence factor.	4	1	81	0.0000	Infinity
S	[1] [38] [47]	B0Y0Q7	Afu5g10570	cofilin	• Cofilin family proteins are essential regulators of actin cytoskeletal dynamics. Recent evidence also implicates cofilin in the regulation of mitochondrial function and stress responses. Charge alterations to conserved surfaces of cofilin that do not interfere with its actin regulatory activity lead to a dramatic increase in respiratory function that triggers upregulation of ABC transporters and metabolic changes that support multi-drug resistance • Increased expression after voriconazole exposure	2	2	96	0.0101	24.71
R	[1] [43] [48]	B0Y6F1	Afu4g09600	GPI anchoredprotein, putative	• Genes regulated by SrbA, plays a role in ergosterol biosynthesis and which mediates resistance to azole antifungals (fluconazole susceptibility, lover voriconazole MIC in SrbA null mutant)	13	12	1105	0.0012	1.96
S		B0Y8F7	idi1	isopentenyl-diphosphate delta-isomerase	• Idi1 is connected to isoprenoid biosynthesis (precursors for biosynthesis of ergosterol)	4	4	162	0.0061	6.33
S	[1]	B0Y6V6	cafA	carbonicanhydrase		6	6	304	0.0316	7.84
R	[1]	B0YAM7	chiB1	class v chitinase 1, ChiB1		4	3	132	0.0033	4.31
R	[8]	DPP5	Afu2g09030	Dipeptidyl-peptidase 5 precursor (Dipeptidyl-peptidase V) (DppV)		8	8	301	0.0306	1.97
R	[49]	H31_ DEBHA	hht1	Histone H3.1/H3.2		4	1	127	0.0067	3.01
S	[1]	B0Y3U1	Afu5g07890	ssdna binding		4	3	116	0.0258	2.34
S	[1]	B0YC37	csx1	mRNA binding post-transcriptional regulator (Csx1), putative		1	1	52	0.0062	Infinity
S	[8] [50]	NDK	ndk1	Nucleoside diphosphate kinase	S. cerevisiae ortholog (YNK1) plays a role in cellular response to DNA damage	8	8	363	0.0376	3.43

Continued

R	[8] [51] [52]	ORYZ_ASPFU	alp1	Alkaline protease 1	• Allows assimilation of proteinaceous substrates. • Significant virulence factor in invasive aspergillosis. Involved in immune : efficiently cleaves C3, C4, C5, and C1q, as well as IgG, which leads to down-regulation of complement activation at the hyphal surface • The role of Alp in the virulence of *A. fumigatus* is described contradictory: an Alp deficient *A. fumigatus* caused drastically reduced mortality in mice compared to the wild-type isolate, but this is not confurmed in other reports	8	8	512	0.0001	3.69
S	[1]	B0YB44	mepB	metallopeptidase MepB	Transcript induced by exposure to human airway epithelial cells and by growth on BSA as a sole nitrogen source	4	4	183	0.0040	1.80

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