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MINIREVIEW

Virulence-Associated Enzymes of *Cryptococcus neoformans*

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Enzymes play key roles in fungal pathogenesis. Manipulation of enzyme expression or activity can significantly alter the infection process, and enzyme expression profiles can be a hallmark of disease. Hence, enzymes are worthy targets for better understanding pathogenesis and identifying new options for combatting fungal infections. Advances in genomics, proteomics, transcriptomics, and mass spectrometry have enabled the identification and characterization of new fungal enzymes. This review focuses on recent developments in the virulence-associated enzymes from *Cryptococcus neoformans*. The enzymatic suite of *C. neoformans* has evolved for environmental survival, but several of these enzymes play a dual role in colonizing the mammalian host. We also discuss new therapeutic and diagnostic strategies that could be based on the underlying enzymology.

The facultative intracellular fungal pathogen *Cryptococcus neoformans* is the causative agent of cryptococcosis, a disease that primarily affects individuals with impaired immunity, such as those with advanced HIV infection (1, 2). *C. neoformans* is a ubiquitous environmental fungus associated with both pigeon guano and eucalyptus trees, and its environmental niche ranges from the tropical to the temperate (3). *C. neoformans* infection is acquired from the environment via inhalation, after which it forms a local infection in the lungs. This infection may be cleared, may be contained as a granuloma, or may disseminate from this initial site, leading to pneumonia and/or meningoencephalitis, the latter being uniformly fatal if untreated. Despite the availability of antifungal therapy, more than 650,000 people die each year from *C. neoformans* infection (1, 2, 4). The principal virulence factors of *C. neoformans* are a polysaccharide capsule, melanin production (5, 6), the ability to grow at body temperature (7), and the secretion of extracellular enzymes (7). These virulence factors confer a selective advantage to *C. neoformans* for both residing in the environment and in a mammalian host. Tightly controlled regulation leads to expression of enzymes required for fungal survival and host damage once inside its mammalian host (8).

Many enzymes contribute to the composite cryptococcal virulence phenotype. Dissection of the pathogenic role of these enzymes will enhance our understanding of cryptococcal pathogenic mechanisms and facilitate directed inhibitor development and/or vaccine discovery. We have included a table summarizing basic information regarding global *C. neoformans* enzymology (Table 1) and a schematic displaying localization of most of the highlighted enzymes discussed (Fig. 1). In this review, we discuss in detail the most important virulence-associated enzymes (Table 2), as well as additional target enzymes with potential for rational antifungal drug design (Table 3). We examine this information in the context of infection and analyze candidate target enzymes for drug inhibition and vaccine discovery.

POLYSACCHARIDE CAPSULE

C. neoformans is the only fungal pathogen with a polysaccharide capsule, an outermost polysaccharide structure located just outside the cell wall. The two major polysaccharide capsule constituents are glucuronoxylomannan (GXM) and glucuroxylomannogalactan (GXMGal) (9–11). GXM is the major component of *C. neoformans*, a compound of α-1,3-linked mannose residues with xylosyl and glucuronyl side groups (12), whereas GXMGal is made of α-1,6-linked galactose residues with xylose, mannose, and glucuronic acid (13). The capsule also contains nonpolysaccharide components, such as mannoprotein (MP) (10, 14, 15), although these MP components may represent transient components destined for cellular export.

The role of capsule in environmental growth is unknown, although speculations have been made that the capsule protects the fungus from desiccation or acts as a food source (16). During mammalian infection, the capsule participates in resisting phagocytosis and modulating the immune response (17–21). Not only protective against phagocytosis in both mammalian and lepidopteran hosts (22, 23), the capsule also protects the fungus after ingestion by serving as a free radical sink that can shield the cell from oxidative bursts (24). Hence, while the capsule is not part of the enzymatic microbial arsenal, the machinery responsible for capsule synthesis and assembly does directly contribute to cryptococcal virulence. The primary structures of GXM and GXMGal subunits have been defined, but the mechanisms of subunit assembly into $>10^6$-Da branched structures have not (25, 26). The degree of branching and conformation of polysaccharides imply an elaborate assembly and regulatory enzymatic machinery (27).

The subunits of GXM and GXMGal are large glycans that require several glycosyltransferases for synthesis. Both xylosyltransferase and glucuronyltransferase activities are involved in capsular polysaccharide biosynthesis (28–31). A xylosyltransferase, Cxt1, was the first glycosyltransferase identified with a defined role in capsule synthesis (31). It is a large transmembrane protein with β-1,2-xylosyltransferase activity (31), and deletion of the corresponding gene (*CXT1*) decreased capsular β-1,2-xylose linkages and fungal growth in the lung in a mouse model of infection (30).

Several acapsular mutants were obtained through identifica-

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TABLE 1 Described enzymes in *Cryptococcus neoformans*

Enzyme	Function(s)[a]	EC no.	Reference(s)
Localized on capsule and/or cell wall			
1,3-β-Glucan synthase	Involved in β-glucan synthesis	2.4.1.34	135
Acid phosphatase	Involved in fungal cell adhesion to host tissues, localized in lysosomes, and related to virulence (Table 2)	3.1.3.2	106, 136, 137
Cas1 glycosyltransferase	Participates in *O*-acetylation	2.4.1.X	138
Chitin deacetylase	Involved in chitin metabolism	3.5.1.41	139
Chitin synthase	Involved in chitin synthesis	2.4.1.16	140
Chitinase	Involved in chitin degradation	3.2.1.14	141
Creatinine deaminase	Involved in arginine and proline metabolism	3.5.4.21	142
Esterase lipase	Catalyzes hydrolysis of fatty acids	3.1.1.3	136
GDP-mannose pyrophosphorylase	Involved in GDP-mannose synthesis	2.7.7.13	143
Glucan 1,3-β-glucosidase	Involved in glucan synthesis	3.2.1.58	16
Glucan 1,4-α-glucosidase	Involved in glucan synthesis	3.2.1.3	16
Gmt1 GDP-mannose	Transport of GDP-mannose	2.7.7.22	144
Lactonohydrolase	Deficient strains show larger capsule size and facilitated immune evasion	3.1.1.15	37
N-Acetylgalactosaminoglycan deacetylase	Involved in polysaccharide metabolism	3.1.1.58	145
Phosphoaminase	Involved in amino acid synthesis		136
Phosphomannomutase	Involved in GDP-mannose synthesis	5.4.2.8	143
Phosphomannose isomerase	Involved in GDP-mannose synthesis	5.3.1.8	143
Uph1 ATPase	Required for vesicle acidification		146
Uxs1 decarboxylase	Converts UDP-glucuronic acid to UDP-xylose		147
α-1,3-Glucanase	Involved in glucan synthesis	3.2.1.59	16
α-Amylase	Hydrolyzes alpha bonds of several polysaccharides and involved in cell wall building	3.2.1.1	148
α-Glucosidase	Breaks down disaccharides to glucose and starch and involved in cell wall building	3.2.1.20	136
α-Mannosidase	Involved in cell building through mannose metabolism	3.2.1.24	136
α-Mannosyltransferase	Involved in polysaccharide metabolism	2.4.1.132	38, 149
β-Endoglucanase	Involved in cell wall formation	3.2.1.4	148
β-Glucosidase	Involved in cell wall formation	3.2.1.21	136
β-Glucuronidase	Involved in cell wall formation, catalyzing breakdown of complex carbohydrates	3.2.1.31	136
Secreted/released			
Acyltransferase	Involved in food acquisition	3.1.1.3	92
Alkaline phosphatase	Involved in regulation of signaling cascades and several protein structure and localized in endoplasmic reticulum	3.1.3.1	150
Aspartyl protease	Involved in food acquisition	3.4.23.X	111
Cellulase	Involved in polysaccharide degradation	3.2.1.4	151
DNase	DNA degradation and related to virulence (Table 2)	3.1.21.1	79
Metalloprotease	Catalyzes mechanism that involves a metal and related to virulence (Table 2)	3.4.24.77	113, 152
Phospholipase B	Similar to phospholipase C function, degrades cell membrane components, supports fungal attachment to host cells, localized on cell wall, and related to virulence (Table 2)	3.1.1.5	91, 92
Phospholipase C	Degrades cell membrane components, supports fungal attachment to host cells, and related to virulence (Table 2)	3.1.4.11	93
Protease	Performs proteolysis interfering with host defense response	3.4.21.53	107, 108
S2P endopeptidase	Performs proteolysis	3.4.24.85	153
Serine peptidase	Performs proteolysis, coordinating several physiological functions	3.4.21.X	152
Superoxide dismutase	Catalyzes dismutation of toxic superoxide, converting superoxide to hydrogen peroxide and oxygen and related to virulence (Table 2)	1.15.1.1	83-85
Localized intracellularly			
2-Methylcitrate synthase	Converts acyl groups into alkyl groups on transfer	2.3.3.5	154
3-β-Hydroxysteroid 3-dehydrogenase	Oxidizes a substrate by reduction reaction that transfers 1 or more hydrides to electron acceptor	1.1.1.270	155
6-Phosphogluconate dehydrogenase	Involved in production of ribulose	1.1.1.44	156, 157
Acetate kinase	Catalyzes formation of acetyl-CoA	2.7.2.1	158
Aconitase	Catalyzes isomerization of citrate to isocitrate and involved in response to nitrosative stress	4.2.1.3	159

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TABLE 1 (Continued)

Enzyme	Function(s)[a]	EC no.	Reference(s)
Adenylyl cyclase Cac1	Converts ATP to cAMP	4.6.1.1	160
Alternative oxidase	Part of electron transport chain in mitochondria	1.10.3.11	161
Aminopeptidase	Catalyzes cleavage of amino acids from amino terminus of protein	3.4.11.21	137
C-9-methyltransferase	Involved in glycosphingolipid pathway	2.1.1.129	127
Can2 carbonic anhydrase	Responds directly to intracellular carbon oxide	4.2.1.1	162, 163
Casein kinase 1	Dephosphorylation of Hog1 under stress conditions	2.7.11.1	164
Catalase	Protects cells from oxidative damage by reactive oxygen species	1.11.1.6	137, 150
Cytochrome *c* peroxidase	Takes reduced equivalents from cytochrome *c* and reduces hydrogen peroxide to water	1.11.1.5	165
Deacetylase	Removes acetyl groups from lysine in proteins and is localized in cell wall	3.5.1.108	166
Dolichyl-diphosphooligosaccharide-protein glycotransferase	Participates in *N*-glycan biosynthesis	2.4.99.18	167
Ferrochelatase	Catalyzes final step in heme biosynthesis from highly photoreactive porphyrins	4.99.1.1	168
Flippase	Participates in phospholipid translocation between membrane sides and localized in cell wall	3.6.3.1	169, 170
Glucose-6-phosphate dehydrogenase	Is in pentose phosphate pathway, maintaining the level of coenzyme NADPH	1.1.1.49	171
Glucose-phosphate isomerase	Catalyzes conversion of glucose-6-phosphate into fructose 6-phosphate	5.3.1.9	172
Glucosylceramide synthase	Involved in glucosylceramide synthesis, localized in cell wall, and related to virulence (Table 2)	2.4.1.80	127, 128
Glucuronyltransferase	Involved in biosynthetic pathway of *O*-acetylated mannan	2.4.1.17	28
Glutathione peroxidase	Protects cells from oxidative damage	1.11.1.9	173
Glyoxal oxidase	Copper metalloenzyme that catalyzes oxidation of aldehydes to corresponding carboxylic acids coupled to reduction of dioxygen to H_2O_2	1.2.1.23	148
Homoisocitrate dehydrogenase	Participates in lysine biosynthesis	1.1.1.87	115
Homoserine kinase	Participates in glycine, serine, and threonine metabolism	2.7.1.39	174
Homoserine *O*-acetyltransferase	Participates in methionine and sulfur metabolism	2.3.1.31	175
Hyaluronic synthase	Involved in production of glycosaminoglycan at cell surface	2.4.1.212	176
Imidazole glycerol-phosphate dehydratase	Participates in histidine biosynthesis	4.2.1.19	177
IMP dehydrogenase	Participates in GTP biosynthesis	1.1.1.205	178
Inositol phosphotransferase 1	Involved in glycosphingolipid pathway	2.7.1.X	127
Inositol-phosphorylceramide synthase	Involved in glycosphingolipid pathway	2.7.1.X	179
Ire1 kinase	Involved in cellular response to unfolded proteins	2.7.11.1	180
Isocitrate lyase	Catalyzes cleavage of isocitrate to succinate and glyoxylate	4.1.3.1	181
Laccase	Polyphenol oxidase and copper-containing oxidase enzyme, localized in cell wall, and related to virulence (Table 2)	1.10.3.2	45, 46, 50
Malate dehydrogenase	Catalyzes oxidation of malate to oxaloacetate	1.1.1.37	182
Mannitol-1-phosphate 5-dehydrogenase	Participates in fructose and mannose metabolism	1.1.1.17	183, 184
Mannose-1-phosphate guanylyltransferase (GDP)	Participates in fructose and mannose metabolism	2.7.7.22	144
Mannosyl phosphorylinositol ceramide synthase	Involved in glycosphingolipid pathway	2.4.X.X	127
Mannosyltransferase	Participates in O-mannosylation of proteins and involved in cell wall integrity and morphogenesis	2.4.1.109	185
Myristoyl-CoA: protein *N*-myristoyltransferase	Catalyzes transfer of myristate from CoA to proteins	2.3.1.97	116
Pde1 phosphodiesterase	Modulates cAMP	3.1.4.1	186
Phosphoglucomutase	Participates in interconversion of glucose 1-phosphate and glucose 6-phosphate	5.4.2.2	172
Protein farnesyltransferase	Participates in formation of farnesyl protein and diphosphate	2.5.1.58	187
Rho1 GTPase	Involved in MAPK cascade	3.6.5.2	188
RNase III	Binds and cleaves double-stranded RNA	3.1.26.3	189
Saccharopine dehydrogenase	Participates in lysine metabolism	1.5.1.10	190
Sphingolipid methyltransferase 1	Participates in methylation of glucosylceramide	2.1.1.1	191
Sterol 14α-demethylase	Involved in sterol metabolism	1.14.13.7	192
Sterol 24-C-methyltransferase	Involved in sterol metabolism	1.15.1.1	193
Thiol peroxidase	Reduces peroxides and inhibits hydrogen peroxide response	1.11.1.7	194

(Continued on following page)

TABLE 1 (Continued)

Enzyme	Function(s)[a]	EC no.	Reference(s)
Thioredoxin reductase	Catalyzes reduction of thioredoxin	1.8.1.9	195
Threonine synthase	Participates in glycine, serine, and threonine metabolism	4.2.3.1	174
Thymidylate synthase	Catalyzes conversion of dUMP to deoxythymidine monophosphate	2.1.1.45	196
Transaldolase	Involved in pentose phosphate pathway	2.2.1.2	159
Trehalose-6-phosphate phosphatase	Participates in starch and sucrose metabolism	3.1.3.12	197
Trehalose-6-phosphate synthase	Participates in starch and sucrose metabolism	2.4.1.15	197
UDP-galactopyranose mutase	Catalyzes conversion of UDP-D-galactopyranose in UDP-D-galacto-1,4-furanose	5.4.99.9	198
UDP-glucose dehydrogenase	Participates in conversion of UDP-glucose to UDP-glucuronate, and formation of glycosaminoglycans	1.1.1.22	199
UDP-glucuronate decarboxylase	Participates in nucleotide sugar metabolism	4.1.1.35	147
Urease	Catalyzes hydrolysis of urea into carbono dioxide and ammonia and related to virulence (Table 2)	3.5.1.5	74
Xylosylphosphotransferase	Participates in *O*-glycosylation biosynthesis and related to virulence (Table 2)	2.7.8.32	28, 31, 200
Δ8 desaturase	Involved in glycosphingolipid pathway	1.14.19.4	127

[a] cAMP, cyclic AMP; MAPK, mitogen-activated protein kinase.

tion of rough colonies. This type of screen identified four genes required for capsule formation: *CAP10*, *CAP59*, *CAP60*, and *CAP64*. Although these genes are not essential, their mutation does confer defects in growth and in mouse models of infection (17, 32–35). Cells from these mutant strains lacked or produced extremely reduced capsule, but these mutations did not correlate with enzymatic deficiency in UDP-glucose dehydrogenase, UDP-glucuronate decarboxylase, UDP-glucuronyl:acceptor transferase, UDP-xylosyl:acceptor transferase, or lipid-linked oligosaccharide biosynthetic pathways. *CAP10* is a putative xy-

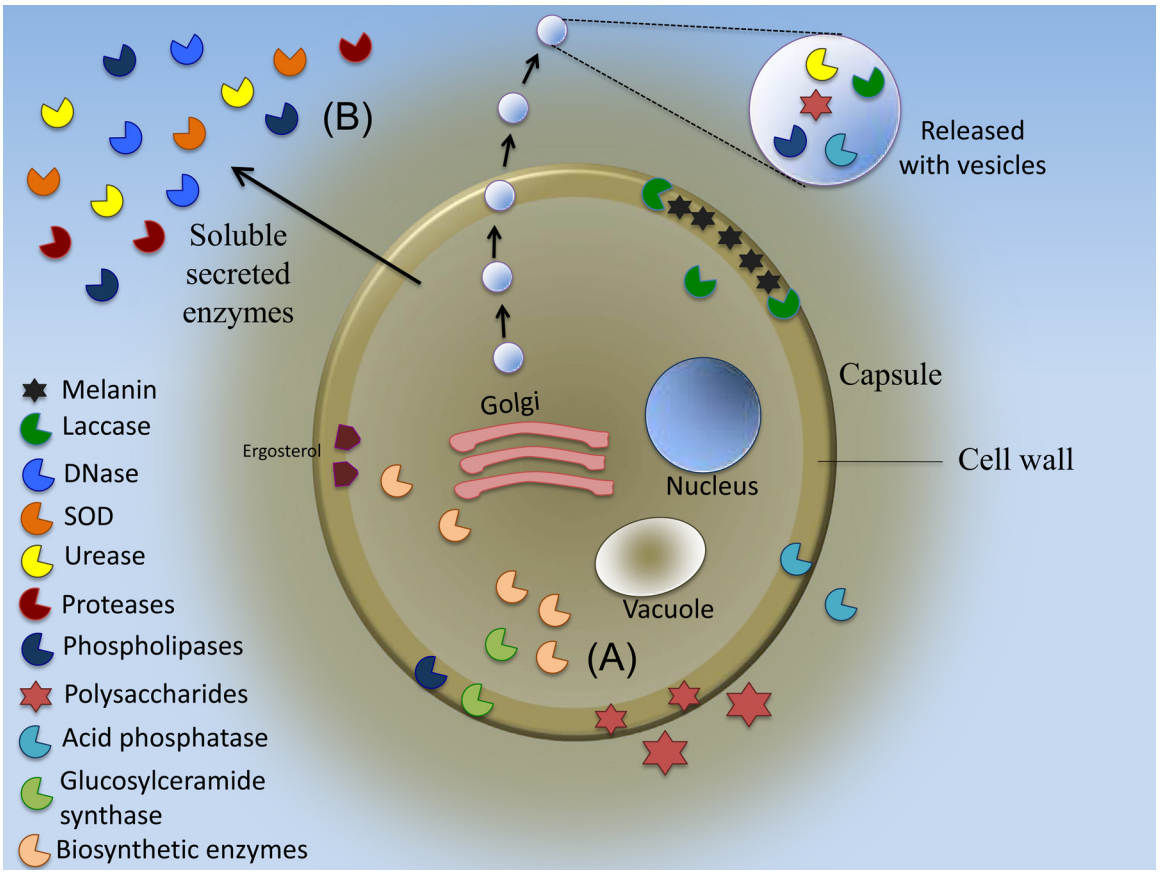

FIG 1 Enzymes are crucial for fungal pathogenesis and can alter the infection process. These enzymes are potential targets for new antifungal agents. (A) Some pathogenesis-related enzymes are retained to be active inside the cell body, while others are secreted. Some, like laccase, are both retained and secreted. (B) Of those released, some are secreted using traditional secretion systems, while others are included as cargo in extracellular vesicles.

TABLE 2 Enzymes related to the virulence in *Cryptococcus neoformans*

Enzyme	Comment(s)	Reference(s)
Acid phosphatase	Deficient strains show affected virulence in mouse and *Galleria mellonella* models of infection	106
DNase	Acts in degrading host DNA and supplies *C. neoformans* with nucleotides	79
Glucosylceramide synthase	Required for virulence in murine model of infection	127, 128
Laccase	Deficient strains show decreased virulence in survival studies with rabbit and mouse models of infection	59
Mannosyltransferase	Required for virulence in murine model of infection	185
Metalloprotease	Deficient strains unable to cross endothelium in *in vitro* model of human blood-brain barrier and is required for invasion of central nervous system	113
Phospholipase B	Required in invasion of host tissue and dissemination in murine model	95
Phospholipase C	Shown to be important for several virulence phenotypes	101, 102
Superoxide dismutase	Attenuated growth of deficient strains within macrophages	89
Urease	Deficient strains less virulent than wild-type strain in mouse model of infection and is involved in fungal escape from lung to cross blood-brain barrier	76
Xylosylphosphotransferase	Deficient strains manifest reduced growth in lung tissue in mouse model of infection	30

losyltransferase gene, and *cap10*Δ mutants show a pleiotropic phenotype, which includes enlarged cell size, smaller extracellular vesicles, and affected expression of some virulence factors (36). *CAP10* therefore is required for both capsule formation and other aspects of fungal virulence.

Capsular lactonohydrolase also affects multiple capsule-related phenotypes (37). A strain lacking lactonohydrolase (*lhc1*Δ) produced capsules with a larger size and altered branching, density, and solvation compared to the parental strain. These capsular structure alterations increased virulence in murine infection (37). Taken together, these results suggest that lactone may be involved in cross-linking of the capsule.

α-1,3-Mannosyltransferase (encoded by *CMT1*) synthesizes the mannose backbone of GXM and thus plays a crucial role in capsule synthesis. However, α-1,3-mannosyltransferase activity is more involved in in serotype A capsule biosynthesis than in the serotype D *C. neoformans* (38, 39). Serotypes A and D represent two of the four *C. neoformans* serotypes: *C. neoformans* var. *neoformans* (serotypes A and D) and *C. neoformans* var. *gattii* (serotypes B and C), which can be distinguished according to their growth differences on diagnostic media (40). The strain-specific capsule synthesis differences, such as the role of *CMT1*, show the importance of studying multiple strain backgrounds.

Much remains to be learned about the enzymatic machinery involved in capsule synthesis, including enzyme localization and kinetics. Detailed studies of capsule structure and the enzymatic machinery involved are critical for a better understanding of the function of the capsule production and regulation.

MELANIN SYNTHESIS

Melanin formation protects *C. neoformans* from oxidative damage as well as from both heat and cold (41, 42). Melanin is synthesized on 2,3- or 3,4-diphenol substrates by a phenoloxidase and accumulates in the *C. neoformans* cell wall (43, 44). The melanin-synthesizing enzyme has two classical laccase characteristics: a glycosylated copper-containing protein with the ability to oxidize diphenolic substrates and the ability to produce decarboxy dopachrome (45, 46). *C. neoformans* melanin synthesis occurs only in the presence of exogenous dihydroxyphenols, since no known *C. neoformans* endogenous substrate exists. Several diphenols can serve as the substrates for pigment synthesis by *C. neoformans* laccase (47), such as the substrates consisting of *para*- and *ortho*-diphenols, monophenols, L-dopa, and esculin, indicating that the enzyme has broad specificity and the ability to generate pigments from different compounds (47–53). Iron increases laccase activity, but hydrogen peroxide has no effect on enzymatic activity, despite the antioxidant properties of melanin (54).

The genes *LAC1* and *LAC2* encode two laccases, but a single deletion in *LAC1* is able to prevent melanin production (55–58). Lac1 localizes in the cell wall, while Lac2 is cytoplasmic, but Lac2 can localize to the cell wall in the absence of Lac1 (55). *lac1*Δ mutants are easily identified as white colonies when cultivated on catecholamine-containing media (59). The *lac1*Δ mutant shows decreased virulence in survival studies with rabbit infection (59), corroborating the important role in the fungal virulence (5, 46). In addition to its cell wall localization, laccase is packaged into extracellular vesicles, a nontraditional mechanism of secretion, and can therefore mediate damage away from the laccase-producing fungal cell (Fig. 1).

Melanin is considered a powerful antioxidant, since it may protect cryptococcal cells against oxygen- and nitrogen-derived oxidants of the type made by host effector cells (5, 60–62). In addition to its capacity to absorb free radical fluxes, melanin can also contribute to acquired resistance against to the antifungals

TABLE 3 Possible target enzymes for rational antifungal drug design

Enzyme(s)	Comment(s)	Reference(s)
14α-Demethylase	A critical enzyme in sterol assembly	119
Glucosylceramide synthase	Glucosylceramide plays critical role in pathogenicity of *C. neoformans*	127, 128
Laccase	Melanization aids virulence	60, 63, 64, 65
Myristoyltransferase	Myristoylation inhibition is fatal for *C. neoformans*	116, 117
Phosphoribosylaminoimidazole carboxylase	Mutants that cannot synthesize adenine have reduced virulence	114
Pyrophosphorylase and cytosine-specific permease	Enzymes are basis of *C. neoformans* flucytosine resistance	201, 202
Sterol synthesis enzymes	Sterol synthesis enzyme mutants show resistance to fluconazole and amphotericin	122-124

amphotericin B and caspofungin, since nonmelanized cryptococcal cells are more susceptible than melanized cells to amphotericin B and caspofugin. Moreover, killing assays demonstrated that addition of melanin particles to amphotericin B or caspofungin significantly reduces their toxicities against *C. neoformans* (63–65). Thus, melanin and laccase are considered promising targets for drugs against *C. neoformans* infection.

EXTRACELLULAR ENZYMES

As nature's "recyclers," environmental fungi secrete a number of degradative enzymes to breakdown macromolecules and obtain nutrients in the environment (7, 66–69). *C. neoformans* is no exception and releases a number of lipases, proteases, and DNases. However, during the infection process, the same degradative enzymes contribute to virulence by destroying tissues, promoting fungal survival, and interfering with effective immune responses.

Urease is almost universally expressed by *C. neoformans* isolates. In the environment, *C. neoformans* is often isolated from avian excreta (70, 71). To survive and grow on this medium, the fungus must metabolize creatinine, xanthines, and uric acid. High urease activity may benefit the fungus under these conditions (72–74), as the enzyme catalyzes the hydrolysis of urea to ammonia and carbamate. Urease is considered a major cryptococcal virulence factor (75). A urease knockout (*URE1*) strain of *C. neoformans* was significantly less virulent than the wild-type strain in a mouse model of infection (76). Urease plays a role in fungal escape from the lung to cross the blood-brain barrier but is not required for fungal growth once inside the brain (76). Urease production varies among clinical isolates; however, the vast majority (99.6%) demonstrate some level of urease activity (74, 77, 78). Nevertheless, occasional urease-negative variants have been isolated in clinical isolates (77), suggesting that this enzyme can be dispensable, provided that there are compensatory virulence mechanisms.

Extracellular DNase is produced by *C. neoformans* in high quantities (79). This DNase may degrade host DNA secreted by neutrophils as part of the innate immune response (80) and additionally may supply *C. neoformans* with nucleotides. A survey of several yeast species, including *C. neoformans*, suggests a correlation between urease activity and extracellular DNase production (79). DNase activity is stronger in clinical strains than in environmental strains, further suggesting DNase may play a role as a virulence factor (81).

Superoxide dismutases (SODs) convert superoxide to hydrogen peroxide and oxygen (82). Two SODs have been described in *C. neoformans* (83–88). SOD contributes to virulence of *C. neoformans* by facilitating growth within macrophages (89), through a mechanism that is likely to involve protection of the fungus against superoxide generated by host immune response (2). In this regard, melanin and SOD may stimulate complementary defenses for the *C. neoformans* cells' protection against oxidative damage. SOD production is regulated by temperature, with increases in expression at 37°C compared to 25°C. Thus, increased SOD production at body temperatures may protect the fungus against oxidizing agents produced from host effector cells (90).

Phospholipases degrade cell membrane phospholipids in an enzyme-dependent mechanism. *C. neoformans* extracellular supernatants contain phospholipase B, phospholipase C, lysophospholipase, and acyltransferase (91–93), and phospholipase activity supports fungal attachment to host cells (94). Phospholipase B promotes fungal invasion of host tissue (95) and hydrolyzes phospholipids in lung surfactant and the plasma membrane (92, 96). Moreover, it contributes to fungal survival by maintaining cell wall integrity (97) and provides nutrients that can be used as sole carbon sources by *C. neoformans* during the infection (98, 99). As described above, it has also been localized to the cell wall (97), and its transport to the plasma membrane and cell wall is *N*-glycan dependent (100). Phospholipase C is crucial for several virulence phenotypes (melanin production, growth at 37°C, phospholipase B secretion, and antifungal drug resistance) and is also involved in homeostasis regulation, cell separation following cytokinesis, and cell wall integrity (101, 102).

Phosphatases remove a phosphate group from their substrates and play important roles in regulating protein structure and signaling cascades (103, 104). A secreted acid phosphatase is involved in fungal cell adhesion to host tissues, suggesting an important role in establishing infection (105). Acid phosphatase is encoded by the gene *APH1* in *C. neoformans*. In both wax worm and murine models of cryptococcosis, *aph1*Δ strain-infected animals survived longer than those in the wild-type-infected model (106), demonstrating the importance of this enzyme during infection.

Proteases break down proteins and are considered important virulence factors, contributing to tissue invasion, colonization, and alteration of the host defense response. Protease activity in *C. neoformans* cultures has been reported by several investigators (107–111). Proteases play important roles in host cell penetration and virulence of *C. neoformans* (112). Recently, a metalloprotease was identified by proteomic analyses of the extracellular proteins from *C. neoformans* and found to be required for invasion of the central nervous system in murine infection of *C. neoformans* (113). Moreover, the metalloprotease knockout (*mpr1*Δ) strain was unable to cross the endothelium in an *in vitro* model of the human blood-brain barrier (113).

DRUG DESIGN AND RESISTANCE

Definition of enzymatic pathways can provide crucial targets for antimicrobial drug design. One way to identify targets is to identify unique metabolic requirements for cryptococcal growth and/or virulence. An example of this is the *C. neoformans* phosphoribosylaminoimidazole carboxylase gene (*ADE2*). Mutants with mutations in this gene lack an enzyme required for adenine synthesis and thus have reduced virulence compared to the wild-type strain (114). This observation suggests potential for rational drug design utilizing differences in adenine synthesis pathways between host and pathogen (as first suggested in reference 7). Several candidate enzymes in *C. neoformans* have been studied regarding fungal amino acid synthesis (e.g., homocitrate synthase, homoisocitrate dehydrogenase, α-aminoadipate reductase, saccharopine reductase, and saccharopine dehydrogenase) (115). However, comparisons between *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* have shown that candidate targets do not necessarily translate across *Cryptococcus* species. Saccharopine reductase, an enzyme involved in lysine synthesis, was not detected in *C. neoformans* var. *gattii* but was detected in *C. neoformans* var. *neoformans*. This *C. neoformans* var. *gattii* strain was able to grow even in the absence of lysine (115), indicating that further research to identify enzymes essential across all *Cryptococcus* species is required.

Another essential process for *C. neoformans* is protein myristoylation. *C. neoformans* myristoyltransferase catalyzes the transfer of myristate from coenzyme A (CoA) to the amino-terminal

glycine residue of a subset of cellular proteins, and this enzyme is essential for *C. neoformans* viability (116, 117). *N*-Myristoyl proteins and myristoylation inhibition by the myristic acid analog 4-oxatetradecanoic acid are crucial for this organism (118). Thus, therapies directed at myristoylation may also be a possible target for rational antifungal drug design.

In some cases, an antifungal target is well defined, but multiple enzymes involved in target synthesis provide several inhibitory strategies. Sterols and their synthetic pathways are major antifungal targets in many fungi, but resistance leads to difficulties in patient treatment. Fluconazole-resistant strains require a 10-fold-higher drug concentration to inhibit sterol 14α-demethylation (119), rendering the drug clinically unfeasible. The molecular basis for differential enzyme function has been identified in several clinical *C. neoformans* strains (120). One documented fluconazole- and amphotericin-resistant *C. neoformans* patient isolate showed reduced relative sterol content and a defect in δ-8-isomerase, depleted ergosterol, and accumulated aberrant δ-8-double-bonded ergosterol precursors (121, 122), suggesting the ability to form membrane pores due to aggregation and formation of amphotericin-ergosterol complexes. Another study evaluating fluconazole- and amphotericin-resistant isolates observed reduced ergosterol content in the isolates, as well as reduced sensitivity of P450 14α-demethylase to inhibition by fluconazole, and a defect in sterol Δ^8-Δ^7 isomerase (123). Another *C. neoformans* strain with defective sterol Δ^8-Δ^7 isomerase was discovered in an amphotericin B-resistant isolate from an AIDS patient (124). These mutations in sterol synthesis enzymes explain resistance evolution and generate targets to fight it with. This information can also help in rational drug design methodologies.

Identification of key virulence-related enzymes is yet another route toward finding an effective drug target. Glycosphingolipids are essential to regulate survival and/or replication of *C. neoformans* in the phagolysosome, as well as in the extracellular environment of the host (125–127). Glucosylceramide plays critical role in pathogenicity of *C. neoformans*, since glucosylceramide synthase (Gcs1) is required for virulence in the murine model of infection (128). *gcs1*Δ mutants corroborate the crucial role of the glycosphingolipid synthesis in regulation of this considerable aspect of *C. neoformans* virulence (127). Thus, the glycosphingolipid pathway may also be a reasonable target for antifungal therapies.

Laccase has been considered a drug target in *C. neoformans* because melanization is critical to virulence. Inhibition of fungal melanization in murine infection using the herbicide glyphosate prolonged average mouse survival. Glyphosate is an inhibitor of both the shikimate acid pathway and L-dopa polymerization (129). Thus, therapies directed at melanization may also be a potential target for antifungal drug design.

Occasionally, a drug proven to work on one microbial pathogen will also be effective against another. This appears to be the case with several viral medications. Drugs such as indinavir and oseltamivir inhibit human immunodeficiency virus (HIV) protease or influenza virus neuraminidase, respectively, and demonstrate the impact an enzymatic inhibitor can have in the clinic (130, 131). The use of protease inhibitors has shown positive effects on *C. neoformans* and *Candida albicans* infections, where drug treatment was associated with inhibition of fungal growth and proliferation *in vitro* (132, 133). These are likely inhibiting the fungal proteases, both cell associated and as part of the fungal secretome.

CONCLUSION

Recent advances in genomics, proteomics, transcriptomics, and mass spectrometry have facilitated the identification and characterization of new fungal enzymes, including those specific to both fungi and *C. neoformans*. These enzymes are required for many important biological processes, including growth and infection. The importance of the secretome in cryptococcal pathogenesis is apparent from the fact that strain differences in secreted enzymes correlate with their virulence (134). Nonetheless, important questions remain. Future research on cryptococcal enzymology will not only identify new enzymes and their roles during infection but also pinpoint enzymatic targets for the development of antifungal agents.

ADDENDUM IN PROOF

There are, of course, many enzymes involved in signaling cascades, most of which were not discussed in this review. One such enzyme is vital to stress response in *C. neoformans* and other pathogenic fungi and thus merits a well-deserved mention: the calcium-dependent phosphatase calcineurin (W. J. Steinbach, J. L. Reedy, R. A. Cramer, Jr., J. R. Perfect, J. Heitman, Nat Rev Microbiol **5**:418–430, 2008). This enzyme is required for growth in a mammalian host and therefore is necessary to cause disease (A. Odom, S. Muir, E. Lim, D. L. Toffaletti, J. Perfect, J. Heitman, EMBO J **16**:2576–2589, 1997). Studies utilizing calcineurin inhibitors for invasive disease in animal models have shown promising results, and this work is now moving into translational stages (D. P. Kontoyiannis, R. E. Lewis, B. D. Alexander, O. Lortholary, F. Dromer, K. L. Gupta, G. T. John, R. del Busto, G. B. Klintmalm, J. Somani, G. M. Lyon, K. Pursell, V. Stosor, P. Munoz, A. P. Limaye, A. C. Kalil, T. L. Pruett, J. Garcia-Diaz, A. Humar, S. Houston, A. A. House, D. Wray, S. Orloff, L. A. Dowdy, R. A. Fisher, J. Heitman, N. D. Albert, M. M. Wagener, N. Singh, Antimicrob Agents Chemother **52**:735–738, 2008, http://dx.doi.org/10.1128/AAC.00990-07). Other enzymes involved in stress responses may similarly be identified and targeted in the future.

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