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Flavofun: Exploration of fungal flavoproteomes

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Fungi produce a plethora of natural products exhibiting a fascinating diversity of chemical structures with an enormous potential for medical applications. Despite the importance of understanding the scope of natural products and their biosynthetic pathways, a systematic analysis of the involved enzymes has not been undertaken. In our previous studies, we examined the flavoprotein encoding gene pool in archaea, eubacteria, the yeast *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, and *Homo sapiens*. In the present survey, we have selected the model fungus *Neurospora crassa* as a starting point to investigate the flavoproteomes in the fungal kingdom. Our analysis showed that *N. crassa* harbors 201 flavoprotein-encoding genes amounting to 2% of the total protein-encoding genome. The majority of these flavoproteins (133) could be assigned to primary metabolism, termed the "core flavoproteome", with the remainder of flavoproteins (68) serving in, as yet unidentified, reactions. The latter group of "accessory flavoproteins" is dominated by monooxygenases, berberine bridge enzyme-like enzymes, and glucose-methanol-choline-oxidoreductases. Although the exact biochemical role of most of these enzymes remains undetermined, we propose that they are involved in activities closely associated with fungi, such as the degradation of lignocellulose, the biosynthesis of natural products, and the detoxification of harmful compounds in the environment. Based on this assumption, we have analyzed the accessory flavoproteomes in the fungal kingdom using the MycoCosm database. This revealed large differences among fungal divisions, with Ascomycota, Basidiomycota, and Mucoromycota featuring the highest average number of genes encoding accessory flavoproteins. Moreover, a more detailed analysis showed a massive accumulation of accessory flavoproteins in Sordariomycetes, Agaricomycetes, and Glomeromycotina. In our view, this indicates that these fungal classes are proliferative producers of natural products and also interesting sources for flavoproteins with potentially useful catalytic properties in biocatalytic applications.

KEYWORDS

dehydrogenase, flavin, halogenase, monooxygenase, natural product (bio)synthesis, *Neurospora crassa*, oxidase, reductase

Abbreviations: AA3, Auxiliary Activity three; BBE, berberine bridge enzyme; BGC, biosynthetic gene cluster; CDH, cellobiose dehydrogenase; FMO, flavin-containing monooxygenase; FPMO, flavoprotein monooxygenase; GMC, oxidoreductase, glucose-methanol-choline-oxidoreductase; LPMO, lytic polysaccharide monooxygenase; MAK1, maackiain detoxification enzyme one; *N. crassa*, *Neurospora crassa*; PCMH, *para*-cresolmethyl hydroxylase; PFam, Protein Family; *S. cerevisiae*, *Saccharomyces cerevisiae*; VAO, vanillyl alcohol oxidase.

Introduction

Fungi are known to produce a large number of natural products for an array of functions, such as communication, detoxification, and defense. Typically, simple activated structures constitute the basis for the generation of natural products, and various enzymatic modifications of these compounds eventually lead to structurally diverse and highly complicated organic molecules. In addition to acylation, alkylation, and glycosylation, redox reactions are pivotal to defining the bioactivity of the final products. In this regard, oxidoreductases like cytochrome P450- and flavin-dependent enzymes (containing either flavin mononucleotide, FMN, and/or flavin adenine dinucleotide, FAD) play a major role (Toplak and Teufel, 2022). In order to analyze the involvement of flavoenzymes in the biosynthesis of natural products, we chose the saprotrophic filamentous fungus *Neurospora crassa* as a starting point. This fungus served as an important experimental model organism for almost a century culminating in the one-gene-one-enzyme hypothesis by Beadle and Tatum (Beadle and Tatum, 1941). Furthermore, *N. crassa* played a central role in advancing our knowledge of the molecular mechanisms of physiological and genetic processes such as the circadian rhythm and genome defense mechanisms (Galagan et al., 2003). In the same vein, *N. crassa* was also employed as a source for several flavin-containing enzymes and proteins in order to gain insight into central biochemical processes, for example, the isolation and characterization of the assimilatory nitrite and nitrate reductase by Garrett and coworkers (Garrett and Nason, 1967; Lafferty and Garrett, 1974; Colandene and Garrett, 1996). Another prominent example was the isolation and characterization of chorismate synthase, the last enzyme of the common shikimate pathway, by Gaertner and coworkers who demonstrated the requirement for (reduced) FMN to sustain enzymatic activity (Welch et al., 1974). Other examples include L-amino acid oxidase, which is induced in response to the addition of L-amino acids after nitrogen starvation (Sikora and Marzluf, 1982; Niedermann and Lerch, 1990), and 2-nitropropane oxygenase. The latter enzyme produces nitrite and acetone, and thus, enables the utilization of nitro-organic compounds as nitrogen and carbon source (Gorlatova et al., 1998). Finally, the discovery and characterization of cellobiose dehydrogenase (CDH) is worth mentioning (Westermark and Eriksson, 1974). As a saprotrophic fungus, *N. crassa* accommodates lignocellulosic biomass-degrading enzymes, including CDH. Two active CDHs of *N. crassa* (IIA and IIB) were expressed and characterized (Zhang et al., 2011; Sygmund et al., 2012). Recently, they have been shown to play a crucial role in lignocellulosic material degradation by providing electrons to lytic polysaccharide monooxygenase (LPMO) (Kracher et al., 2016). To date, CDHs are classified in the Carbohydrate-Active enZyme (CAZy) database and are assigned to the group AA3 (Auxiliary Activity 3). All enzymes in this group, which show a wide range of functional diversity, belong to the glucose-methanol-choline (GMC) family, which was first outlined by Cavener, 1992.

N. crassa also played a central role in the discovery of the enigmatic blue light receptor. The early work by Muñoz and Butler in 1974 provided the first experimental evidence that a flavin cofactor might serve as the light-sensing chromophore (Muñoz et al., 1974). This was eventually confirmed independently by Liu and Dunlap and their coworkers (Froehlich et al., 2002; He et al., 2002). In the case of the fungal blue light receptor, FAD is bound to the LOV domain of the protein called "white collar" (WC)-1, which forms a heterodimer with WC-2 and acts as a regulator for light-dependent processes such as the circadian rhythm and carotenoid biosynthesis. The discovery of the flavin-dependent blue light receptor WC-1, as well as other flavin-dependent proteins such as VIVID (VVD) and cryptochrome (CRY), have established *N. crassa* as a paradigm for the study of light-regulated developmental processes and responses in fungi (Corrochano, 2007).

On the other hand, the number of reported secondary metabolites produced by *N. crassa* is rather limited, comprising the histidine-derived ergothioneine, the non-ribosomal peptide coprogen, and the polyketide oxoalkylresorcylic acid (Huschka et al., 1985; Funa et al., 2007; Bello et al., 2012).

In this study, we first defined a basic ("core") flavoproteome for *N. crassa* comprising flavoenzymes operating in energy metabolism and degradation of general metabolites (catabolism), biosynthesis of amino acids, cofactors and pigments, acquisition of nutrients (e. g., metal ions), redox homeostasis, RNA/DNA modifications, and light regulation. In the course of our analysis, we also found a large number of predicted flavoenzymes with an undefined function. Interestingly, these flavoenzymes belong mainly to three families: flavin-dependent monooxygenases, berberine bridge enzyme-like enzymes (BBEs), and GMC oxidoreductase. Since members of these families are frequently involved in the biosynthesis of secondary metabolites, we assume that these flavoenzymes play a role in the generation of compounds with functions that are relevant to the ecological niche as well as the life cycle of *N. crassa*. The discovery of this accessory flavoproteome in *N. crassa* has prompted us to analyze the occurrence of the above-mentioned flavoenzyme families in the fungal kingdom. This led to interesting insights into the distribution of certain flavoenzyme families, indicating important contributions to the chemodiversity in fungal metabolism.

Results

The *N. crassa* flavoproteome

We identified a total of 201 flavoproteins encoded in the *N. crassa* genome, amounting to 2% of the protein-encoding genes (Galagan et al., 2003). The functional analysis of the flavoenzymes revealed that 133 are associated with principal metabolic functions such as energy production, degradation, and biosynthesis of primary metabolites. These flavoenzymes were assigned to a core flavoproteome, summarized in Table 1. In addition to the core flavoproteome, we identified 68 flavoenzymes with mostly unknown functions. These flavoenzymes were assigned to the accessory flavoproteome of *N. crassa* (Table 2). We propose that these flavoenzymes operate

TABLE 1 The core fungal flavoproteome of *Neurospora crassa* (tricarboxlic acid cycle, β-oxidation, amino acid metabolism, cofactor biosynthesis, carotenogenesis, metal uptake, tRNA-modifications, light regulation, pyrimidine and purine metabolism).

E.C.	Enzyme/Protein	Gene ID	PFam	Identifier*/**	PDB code***
Energy metabolism and catabolism					
1.1.1.204	xanthine dehydrogenase	NCU03350T0	00941/03450	FAD_binding_5/Co_deh_flav_C	6abu/3amz
1.1.2.3	D-lactate dehydrogenase (cytochrome)	NCU07362T0	00173/01070	Cyt-b5/FMN_dh	1ltd
		NCU08272T0	00173/01070	Cyt-b5/FMN_dh	1kb1
1.1.2.4	D-lactate dehydrogenase	NCU00904T0	01565/02913	FAD_binding_4/FAD-oxidase_C	6lpn
		NCU02179T0	01565/02913	FAD_binding_4/FAD-oxidase_C	3p.m.9
		NCU08126T0	01565/02913	FAD_binding_4	6lpn
1.1.3.15	glycolate oxidase	NCU11278T0	01070	FMN_dh	6gmb
		NCU04539T0	01070	FMN_dh	6gme
1.1.5.3	glycerol 3-phosphate dehydrogenase	NCU05454T0	01266	DAO	2rgo
1.1.99.39	D-hydroxyglutarate dehydrogenase	NCU06441T0	01565/02913	FAD_binding_4/FAD-oxidase_C	6lpn
1.2.3.3	pyruvate oxidase	NCU08771T0	02775	TPP_enzyme_M	4fee
1.3.1.42	NADH:FMN dehydrogenase (OYE)	NCU08900T0	00724L	Oxidored_FMN	4jic
		NCU07452T0	007241	Oxidored_FMN	5nux
		NCU04452T0	00724	Oxidored_FMN	4qnw
1.3.3.12	L-arabino-1,4-lactone oxidase	NCU03188T0	01565/04030	FAD_binding_4/ALO	2vfr/7a24
1.3.5.1	succinate dehydrogenase (ubiquinone)	NCU08336T0	00890/02910	FAD-binding_2/Succ_DH_flav_C	lyq3
1.3.8.4	isovaleryl-CoA dehydrogenase	NCU09885T0/1	00441/02770	Acyl-CoA_dh_1/Acyl-CoA_dh_M	2cx9
			02771	Acyl-CoA_dh_N	
		NCU02126T0	00441/02770	Acyl-CoA_dh_1/Acyl-CoA_dh_M	4kto/1ivh
			02771	Acyl-CoA_dh_N	
1.3.8.6	glutaryl-CoA dehydrogenae	NCU02291T0	00441/02770	Acyl-CoA_dh_1/Acyl-CoA_dh_M	2r0m
			02771	Acyl-CoA_dh_N	
1.3.8.7	(medium-chain) acyl-CoA dehydrogenase	NCU01181T0	00441/02770	Acyl-CoA_dh_1/Acyl-CoA_dh_M	2wbi
	(medium-chain) acyl-CoA dehydrogenase	NCU02287T0	00441/02770	Acyl-CoA_dh_1/Acyl-CoA_dh_M	2pg0
	(medium-chain) acyl-CoA dehydrogenase	NCU06543T0	00441/02770	Acyl-CoA_dh_1/Acyl-CoA_dh_M	2jif
			02771	Acyl-CoA_dh_N	
	(medium-chain) acyl-CoA dehydrogenase	NCU08924T0	00441/02770	Acyl-CoA_dh_1/Acyl-CoA_dh_M	2pg0
1.3.8.9	(very long chain) acyl-CoA dehydrogenase	NCU04231T0	00441	Acyl-CoA_dh_1	4y9j
1.3.99.1	fumarate reductase	NCU02580T0	00890	FAD_binding_2	5glg
		NCU06042T0	00890	FAD_binding_2	5glg
1.3.99.4	3-ketosteroid-delta-1-dehydrogenase	NCU08124T0	00890	FAD_binding_2	1y0p
1.4.3.1	D-amino acid oxidase	NCU03131T0	01266	DAO	1c0k
		NCU06558T0	01266	DAO	7ct4
1.4.3.2	L-amino acid oxidase	NCU01066T0	01593	Amino_oxidase	1reo
1.4.3.4	monoamine oxidase (MaoB)	NCU05933T0	00890/01593	FAD_binding_2/Amino_oxidase	2xfu

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TABLE 1 (*Continued*) The core fungal flavoproteome of *Neurospora crassa* (tricarboxlic acid cycle, β-oxidation, amino acid metabolism, cofactor biosynthesis, carotenogenesis, metal uptake, tRNA-modifications, light regulation, pyrimidine and purine metabolism).

E.C.	Enzyme/Protein	Gene ID	PFam	Identifier*/**	PDB code***
1.4.3.14	L-lysine oxidase	NCU01106T0	01593	Amino_oxidase	7c3i
1.5.3.1	sarcosine oxidase (heterotetrameric)	NCU02019T0	01266	DAO	3ad9
	beta-subunit	NCU09535T0	01266	DAO	1x31
1.5.3.-	proline dehydrogenase/oxidase	NCU02936T0	01619	Pro_dh	5kf6
	electron transfer flavoprotein, α-subunit	NCU08004T0	00766/01012	ETF_alpha/ETF	1efv
	electron transfer flavoprotein, β-subunit	NCU07733T0	01012	ETF	1efv
1.5.5.1	ETF:ubiquinone oxidoreductase	NCU04768T0	05187/07992	ETF_QO/Pyr_redox_2	2gmj
1.5.8.4	dimethylglycine dehydrogenase	NCU04641T0	01266/16350	DAO/FAO_M	4pab
		NCU05500T0/1	01266	DAO	1x31
1.6.5.9	NADH quinone reductase	NCU05225T0	00070/07992	Pyr_redox/Pyr_redox_2	5jwa
		NCU08980T0	07992	Pyr_redox_2	5yjw
	NADPH dehydrogenase 3	NCU09447T0	07992	Pyr_redox_2/DAO	5yjw
1.6.99.1	NAD(P)H dehydrogenase/OYE	NCU04452T0	00724	oxidored_FMN	4qwn
1.6.-.-	pyridine nucleotide-disulfide oxidoreductase	NCU00153T0	00070	Pyr_redox_2	5jwa
1.8.3.5	prenylcysteine lyase	NCU09710T0	01593	Amino_oxidase	2bk5
1.14.13.9	kynurenine 3-monooxygenase	NCU06924T0	01494	FAD_binding_3	6lke
1.14.99.46	pyrimidine monooxygenase	NCU10015T0/1	00296	Bac_luciferase	5wan
	methane sulfonate monooxygenase	NCU10015T2	00296	Bac_luciferase	5wan
	alkanesulfonate monooxygenase	NCU05340T0	00296	Bac_luciferase	6ask
7.1.1.2	NADH:ubiquinone oxidoreductase	NCU04044T0	01512	Complex 1_51K	6yj4
Biosynthesis of amino acids, carotenoids, carbohydrates, cofactors, and nucleotides (anabolism)					
1.3.3.1	dihydroorotate dehydrogenase	NCU06532T0	01180	DHO_dh	4jgd
		NCU04318T0	01180	DHO_dh	2e6a
1.3.3.4	protoporphyrinogen IX oxidase	NCU16396T0	01266	DAO	3lov/3nks
1.3.99.23	15-*cis*-phytoene dehydrogenase	NCU00552T0	01593	Amino_oxidase	4rep/4dgk
1.4.1.13	glutamate synthase (NADPH-dependent)	NCU01744T0/1	04898	Glu_syn_central	1ofd
1.4.3.1	D-aspartate/amino acid oxidase	NCU03131T0	01266	DAO	1c0k
		NCU06558T0	01266	DAO	7ct4
1.4.3.5	pyridoxamine 5′-phosphate oxidase	NCU00590T0	01243	Putative_PNPOx	5bnc
		NCU05165T0	01243	Putative_PNPOx	5jab
		NCU07267T0	01243	Putative_PNPOx	2i02
		NCU08269T0	01243	Putative_PNPOx	1ci0
1.5.1.20	methylenetetrahydrofolate reductase	NCU09545T0	02219	MTHFR	6fcx
		NCU07690T0	02219	MTHFR	6fcx
1.14.13.132	squalene monooxygenase	NCU08280T0	01266/08491	DAO/SE	6c6n
1.16.1.8	methionine synthase reductase	NCU03697T0	00667	FAD_binding_1	2qtl

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TABLE 1 (*Continued*) The core fungal flavoproteome of *Neurospora crassa* (tricarboxlic acid cycle, β-oxidation, amino acid metabolism, cofactor biosynthesis, carotenogenesis, metal uptake, tRNA-modifications, light regulation, pyrimidine and purine metabolism).

E.C.	Enzyme/Protein	Gene ID	PFam	Identifier*/**	PDB code***
1.18.1.2	$NADP^+$-ferredoxin reductase	NCU08005T0	07992	Pyr_redox_2	1e1l
2.2.1.6	acetohydroxyacid synthase	NCU07982T0/1	00205	TPP_enzyme_M	7egv
4.1.1.36	4′-phosphopantothenoylcysteine	NCU04042T0	02441	Flavoprotein	6mol
	decarboxylase (HAL3)	NCU04237T0	02441	Flavoprotein	1mvl
4.2.3.5	chorismate synthase	NCU05420T0	01264	Chorismate_synt	7rca
5.4.99.9	UDP-galactopyranose mutase	NCU01824T0	03275	GLF	3ukl
Metal ion uptake and assimilation					
1.7.1.1	nitrate reductase (NADH/NADPH)	NCU05298T0	00970	FAD_binding_6	2bih
1.7.1.4	nitrite reductase	NCU04720T0	00070/07992	Pyr_redox/Pyr_redox_2	6ruz
		NCU05850T0	00070/07992	Pyr_redox/Pyr_redox_2	3lxd
		NCU06061T0	00070	Pyr_redox	4nwz
1.8.5.8	sulfide:quinone oxidoreductase	NCU07112T0	07992	Pyr_redox_2	6mo6
1.13.12.16	nitronate monooxygenase	NCU01275T0	03060	NMO	5ghv
		NCU03949T0	03060	NMO	6e2a
		NCU04803T0	03060	NMO	6e2a
		NCU09931T0	03060	NMO	5ghv
1.14.13.196	L-ornithine N (5)-monooxygenase	NCU07117T0/1	13434	K_oxygenase	7jvk
1.16.1.7	metallo (ferric) reductase	NCU00023T0	08022	FAD_binding_8	7d3f
		NCU00829T0	08022	FAD_binding_8	7d3f
		NCU00876T0	08022	FAD_binding_8	7d3e
		NCU02009T0	08022	FAD_binding_8	7d3e
		NCU02020T0	08022	FAD_binding_8	7d3e
		NCU02110T0	08022	FAD_binding_8	7d3f
		NCU02278T0	08022	FAD_binding_8	7d3f
		NCU03868T0	08022	FAD_binding_8	7d3e
		NCU08194T0	08022	FAD_binding_8	6wxr
		NCU10775T0/1	08022	FAD_binding_8	7d3e
1.-.-.-	nitroreductase	NCU03696T0	00881	Nitroreductase	1ywq
Redox homeostasis					
1.5.1.30	flavin reductase	NCU04139T0	01613	Flavin_Reduct	3nfw
1.6.2.2	NADH:cytochrome-b5 reductase	NCU00216T0	00970	FAD_binding_6	2eix
		NCU03112T0	00970	FAD_binding_6	2eix
		NCU06518T0	00970	FAD_binding_6	2eix
		NCU08060T0	00970	FAD_binding_6	6mv1/3w2e
		NCU16968T0	00970	FAD_binding_6	6mv1/3w2e
1.6.2.4	cytochrome P450 reductase	NCU05185T0	00258/00667	Flavodoxin_1/FAD_binding_1	3qfc
		NCU09727T0	00258/00667	Flavodoxin_1/FAD_binding_1	3qe2

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TABLE 1 (*Continued*) The core fungal flavoproteome of *Neurospora crassa* (tricarboxlic acid cycle, β-oxidation, amino acid metabolism, cofactor biosynthesis, carotenogenesis, metal uptake, tRNA-modifications, light regulation, pyrimidine and purine metabolism).

E.C.	Enzyme/Protein	Gene ID	PFam	Identifier*/**	PDB code***
		NCU09741T0	00258/00667	Flavodoxin_1/FAD_binding_1	6tlt
1.6.5.2	NAD(P)H quinone dehydrogenase	NCU02948T0	03358	FMN_red	5mp4
1.8.1.4	dihydrolipoyl dehydrogenase	NCU02407T0	00070	Pyr_redox	1jeh
1.8.1.7	glutathione-disulfide reductase	NCU03339T0/1	00070	Pyr_redox	2hqm
1.8.1.9	thioredoxin-disulfide reductase	NCU08352T0/1	00070	Pyr_redox	5w4c
1.8.3.2	sulfhydryl oxidase	NCU09291T0	04777	Erv1_Alr	1jra
		NCU02396T0	04777	Erv1_Alr	4e0i
1.8.4.-	endoplasmic reticulum oxidoreductin	NCU02074T0	04137	ERO1	1rp4/3m31
Detoxification					
1.14.12.10 3	chloro-benzoate-3,4-dioxygenase/ reductase	NCU04575T0	07992	Pyr_redox_2	5yjw
1.14.12.17	nitric oxide dioxygenase	NCU02096T0	00970	FAD_binding_6	3ozv
		NCU10051T0	00970	FAD_binding_6	3ozv
RNA/DNA-modifying enzymes					
1.3.1.91	tRNA-dihydrouridine synthase	NCU02619T0	01207	DUS	1vhn
		NCU00065T0	01207	DUS	3b0v
		NCU00950T0	01207	DUS	6eza
		NCU03195T0	01207	DUS	1vhn
1.14.99.66	lysine-specific histone demethylase	NCU09120T0	01593	Amino_oxidase	513c
		NCU08925T0	01593	Amino_oxidase	4fwe
2.4.2.29	tRNA-uridine modifying enzyme (GidA)	NCU00136T0	01134	GIDA	2zxh
-.-.-.-	wybutosine biosynthesis protein (TYW1)	NCU16769T0	00258	Flavodoxin_1	6djt
Light-dependent enzymes and photosensors					
4.1.99.3	DNA-photolyase/cryptochrome	NCU00582T0	00875/03441	DNA_photolyase/FAD_binding_7	6kii
		NCU08626T0	00875/03441	DNA_photolyase/FAD_binding_7	1dnp
2.7.13.3	Sensory transduction histidine kinase	NCU00939T0	00989	PAS	6qrj
		NCU03164T0	00989	PAS	4q20
		NCU07221T0	00989	PAS	6qrj
		NCU00902T0	08447	PAS_3	5y7y
		NCU01833T0/1	08447	PAS_3	6dk8
		NCU02057T0/1	08447	PAS_3	6qrj
	white collar-1	NCU02356T0/1/2	08447	PAS_3	6pps
		NCU06390T0	08447	PAS_3	5sy5
	VIVID	NCU03967T0	13426	PAS_9	3hji/3is2
Predicted flavoenzymes with unclear function					
	putative sulfite reductase	NCU05238T0	00258	Flavodoxin_1	6c3z

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TABLE 1 (*Continued*) The core fungal flavoproteome of *Neurospora crassa* (tricarboxlic acid cycle, β-oxidation, amino acid metabolism, cofactor biosynthesis, carotenogenesis, metal uptake, tRNA-modifications, light regulation, pyrimidine and purine metabolism).

E.C.	Enzyme/Protein	Gene ID	PFam	Identifier*/**	PDB code***
	FAD-dependent oxidoreductase	NCU04077T0	00677/01266	FAD_binding_1/DAO	2c42
	putative sulfite reductase	NCU05069T0	01266	DAO	4pab
	putative dibenzothiophene monooxygenase	NCU10016T0	08028	Acyl-CoA_dh_2	5xde

*Acyl-CoA_dh_1, acyl-CoA, dehydrogenase C-terminal domain; Acyl-CoA_dh_2, acyl-CoA dehydrogenase C-terminal domain; Acyl-CoA_dh_M, acyl-CoA, dehydrogenase middle domain; Acyl-CoA_dh_N, acyl-CoA, dehydrogenase N-terminal domain; ALO, D-arabino-1, 4-lactone oxidase; Amino_oxidase, flavin-containing amine oxidoreductase; Bac_luciferase, luciferase-like monooxygenase; Chorismate_synt; chorismate synthase; CO_deh_flav_C, CO, dehydrogenase flavoprotein C-terminal domain; Complex1_51K, respiratory-chain NADH, dehydrogenase 51 kDa subunit; Cyt-b5, cytochrome b5-like heme/steroid-binding domain; DAO, FAD-dependent oxidoreductase; DHO_dh, dihydroorotate dehydrogenase; DNA_photolyase, DNA-photolyase; DFP, DNA/pantothenate metabolism flavoprotein; DUS, dihydrouridine synthase; ERO1, endoplasmic reticulum oxidoreductin 1; Erv1_Alr, Erv1/Alr family; ETF, electron transfer flavoprotein domain; ETF_QO, ETF:ubiquinone oxidoreductase, 4Fe-4S; FAD_binding_1, FAD_binding_3, FAD_binding_4, and FAD-binding_8, FAD-binding domains; FAD_binding_6, oxidoreductase FAD-binding domain; FAD_binding_5, FAD-binding domain in molybdopterin dehydrogenases; FAD_binding_7, FAD-binding domain of DNA-photolyases; FAD-oxidase_C, FAD-linked oxidase; FAO_M, FAD-dependent oxidoreductase central domain; Flavin_Reduct, flavin reductase-like domain; Flavodoxin_1, flavodoxin; Flavoprotein, flavoprotein; FMN_dh, FMN-dependent dehydrogenase; GIDA, glucose inhibited division protein A; GLF, UDP-galactopyranose mutase; Glu_syn_central, glutamate synthase central domain; K_oxygenase, L-lysine 6-monooxygenase (NADP- requiring); MTHFR, methylene tetrahydrofolate reductase; Nitroreductase, nitroreductase family; NMO, nitronate monooxygenase; Oxidored_FMN, NADH:flavin oxidoreductase/NADH, oxidase; PAS, PAS_3, and PAS_9, PAS, folds/domains; Pro_dh, proline dehydrogenase; Putative_PNPOx, pyridoxamine 5′- phosphate oxidase; Pyr_redox and Pyr_redox_2, pyridine nucleotide-disulphide oxidoreductase; SE, squalene epoxidase; Succ-DH_flav_C, fumarate reductase flavoprotein C-term; TPP_enzyme_M, thiamine pyrophosphate enzyme central domain.

**Only flavin-containing/binding domains are listed in the table, i.e., domains lacking the flavin cofactor are not mentioned.

***PDB codes in plain and bold faces refer to the best model used for structure prediction and structures solved by X-ray crystallography, respectively.

in secondary pathways, for example, the biosynthesis of natural products that serve various crucial roles in adaptation and defense as well as the detoxification of harmful compounds, such as plant phytoalexins. The saprotrophic lifestyle of *N. crassa* also suggests that some of the flavoenzymes listed in Table 2 are involved in lignin degradation. We assume that the accessory flavoproteome varies substantially among fungal species depending on the ecological niche inhabited by the fungus and its survival strategy, for example, with respect to nutrient acquisition and defense requirements. We have also noticed that the accessory flavoproteome is mainly made up of a few protein families (PFam): 00732 (GMC_oxred_N), 05199 (GMC_oxred_C), 00743 (FMO-like), 01494 (FAD_binding_3), 01565 (FAD_binding_4), and 08031 (BBE). Flavoenzymes belonging to PFam01494 and 00743 catalyze monooxygenation reactions of aromatic ring systems (*e. g.,* salicylate hydroxylase) and Baeyer-Villiger-type reactions, respectively (*e. g.,* cyclohexanone monooxygenase). In addition, flavin-containing monooxygenase (FMO)-like enzymes (PFam00743) are known to catalyze the monooxygenation of heteroatoms such as nitrogen and sulfur. Unfortunately, reliable biochemical data on the function of these monooxygenases is currently not available. In the case of the predicted monooxygenase encoded by NCU07224T0, our analysis indicated a close relationship to FqzB (37% sequence identity), a group A FPMO epoxidase from *Aspergillus fumigatus* involved in the biosynthesis of fumiquinazoline (Matsushita et al., 2020; Westphal et al., 2021). Moreover, structural superposition of the FqzB crystal structure (PDB code: 7cp6) with the AlphaFold model generated for the *N. crassa* protein encoded by NCU07224T0 shows a Cα-RMSD of 1.7 Å, confirming a close structural relationship. Interestingly, FqzB itself was reported as a monooxygenase similar to the maackiain detoxification enzyme (MAK1) discovered in *Nectria haematococca* (Covert et al., 1996). MAK1 reduces the toxicity of the phytoalexin maackiain by hydroxylation of one of its aromatic rings, thus contributing to the virulence of the phytopathogenic fungus. It is thus conceivable that the protein encoded by NCU07224T0 assumes a similar role, although the producer, as well as the chemical structure of the potentially harmful substance, remain unknown.

The second-largest group of accessory flavoenzymes are the BBE-like enzymes. They belong to the superfamily of FAD-linked oxidases and are closely related to the VAO/PCMH family (vanillyl alcohol oxidase/*para*-cresol methylhydroxylase). They share common structural features such as the conserved flavin binding (FAD_binding_4) and substrate binding domain (cap domain). In addition to these shared elements, BBE-like enzymes exhibit a unique C-terminal structural element preceding the substrate binding region (Daniel et al., 2017; Ewing et al., 2020). Another salient characteristic of the BBE-like enzymes is their propensity to bind FAD bicovalently, whereas

TABLE 2 The accessory flavoproteome of *N. crassa*. The Table comprises flavoenzymes potentially involved in secondary metabolism (for example encoded in biosynthetic gene clusters) or catalyzing unidentified reactions. The PDB codes indicate the closest structural model.

Flavoenzyme family***	Gene ID	PFam	Identifier**	pdb code
Monooxygenases (*34*)				
monooxygenase	NCU02536T0	01494	FAD_binding_3	4n9x
monooxygenase (BGC #13)	NCU02925T0	01494	FAD_binding_3	5eow
phenol 2-monooxygenase	NCU03023T0	01494	FAD_binding_3	1pn0
phenol hydroxylase	NCU04591T0	01494	FAD_binding_3	2qa1
p-nitrophenol 4-monooxygenase	NCU05059T0	01494	FAD_binding_3	6ain
phenol 2-monooxygenase	NCU05188T0	01494	FAD_binding_3	1foh
monooxygenase	NCU05643T0	01494/05834	FAD_binding_3/Lycopene_cycl	6pvf
3-hydroxybenzoate hydroxylase	NCU05708T0	01494	FAD_binding_3	2dki
phenol 2-monooxygenase	NCU06804T0	01494	FAD_binding_3	1pn0
FAD-dependent monooxygenase	NCU07055T0	01494	FAD_binding_3	7kpt
FAD-dependent monooxygenase	NCU07094T0	01494	FAD_binding_3	7kpt
FAD-dependent monooxygenase	NCU07162T0	01494	FAD_binding_3	5x6q
6-hydroxynicotinate 3-monooxygenase	NCU07194T0	01494	FAD_binding_3	4bk1
putative hydroxylase similar to maackiain detoxification enzyme (MAK1)	NCU07224T0	01494	FAD_binding_3	7cp6
salicylate hydroxylase	NCU07737T0	01494	FAD_binding_3	6pvg/6pvf
phenol 2-monooxygenase	NCU07916T0	01494	FAD_binding_3	1pn0
salicylate monooxygenase	NCU08050T0	01494	FAD_binding_3	4bk3
FAD-dependent monooxygenase	NCU08173T0	01494	FAD_binding_3	4hb9
salicylate monooxygenase	NCU08315T0	01494	FAD_binding_3	4bk3
salicylate monooxygenase	NCU08393T0	01494	FAD_binding_3	6bz5
2-hydroxybiphenyl 3-monooxygenase	NCU09050T0	01494	FAD_binding_3	5brt
salicylate monooxygenase	NCU09669T0	01494	FAD_binding_3	6bjy
FAD-dependent monooxygenase	NCU11363T0	01494	FAD_binding_3	6nes
flavin-dependent monooxygenase	NCU00784T0	00743	FMO-like	6sf0
cyclohexanone monooxygenase	NCU02894T0	00743	FMO-like	6gqi
phenylacetone monooxygenase	NCU03049T0	00743	FMO-like	4c74
flavin-containing monooxygenase	NCU03755T0	00743	FMO-like	2gv8
cyclohexanone monooxygenase	NCU05885T0	00743	FMO-like	6er9
flavin-containing monooxygenase	NCU06296T0	00743	FMO-like	6sem
flavin-dependent monooxygenase	NCU07332T0	00743	FMO-like	6sek
(dimethylaniline) monooxygenase	NCU07821T0	00743	FMO-like	1vqw
cyclohexanone monooxygenase	NCU08747T0	00743	FMO-like	6er9
flavin-containing monooxygenase	NCU08749T0	00743	FMO-like	6se3
cyclohexanone monooxygenase	NCU09429T0	00743	FMO-like	6sek
(dimethylaniline) monooxygenase	NCU09456T0	00743	FMO-like	1vqw
BBE-like enzymes (*17*)				
(isoamyl) alcohol oxidase	NCU01094T0	01565/08031	FAD_binding_4/BBE	6f73
EncM (SrdI, BGC #13)*	NCU02926T0	01565/08031	FAD_binding_4/BBE	6fyd
EncM (BGC #13)*	NCU02927T0	01565/08031	FAD_binding_4/BBE	6fyf
(isoamyl) alcohol oxidase	NCU04108T0	01565/08031	FAD_binding_4/BBE	6f73
alcohol oxidase (C-terminus)	NCU07029T0	01565/08031	FAD_binding_4/BBE	6f73
alcohol oxidase	NCU07619T0	01565/08031	FAD_binding_4/BBE	6f73
Physcomitrella patens BBE1	NCU07970T0	01565/08031	FAD_binding_4/BBE	3fwa
carbohydrate oxidase	NCU08199T0	01565/08031	FAD_binding_4/BBE	6y01
(isoamyl) alcohol oxidase	NCU08750T0	01565/08031	FAD_binding_4/BBE	6f73

(Continued on following page)

TABLE 2 (*Continued*) The accessory flavoproteome of *N. crassa*. The Table comprises flavoenzymes potentially involved in secondary metabolism (for example encoded in biosynthetic gene clusters) or catalyzing unidentified reactions. The PDB codes indicate the closest structural model.

Flavoenzyme family***	Gene ID	PFam	Identifier**	pdb code
xylooligosaccharide oxidase	NCU09518T0	01565/08031	FAD_binding_4/BBE	5k8e
EncM (BGC #2)*	NCU09635T0	01565/08031	FAD_binding_4/BBE	6fyg
cytokinin oxidase/dehydrogenase	NCU07072T0	01565/09265	FAD_binding_4/BBE	4ml8
EncM/*Physcomitrella patens* BBE1	NCU01123T0	01565/08031	FAD_binding_4/BBE	6eo5
EncM	NCU04084T0	01565/08031	FAD_binding_4/BBE	6fyg
EncM	NCU09163T0	01565/08031	FAD_binding_4/BBE	3w8w
EncM	NCU09165T0	01565/08031	FAD_binding_4/BBE	6fyb
EncM	NCU09273T0	01565/08031	FAD_binding_4/BBE	6fyg
GMC oxidoreductases (9)				
cellobiose dehydrogenase[2]	NCU00206T0	05199/07992	GMC_oxred_C/Pyr_redox_2	4qi7
alcohol oxidase/choline dehydrogenase	NCU01853T0	00732/05199	GMC_oxred_N/GMC_oxred_C	5hsa
aryl-alcohol oxidase/choline dehydrogenase	NCU04938T	00732/05199	GMC_oxred_N/GMC_oxred_C	6zh7
cellobiose dehydrogenasc	NCU05923T0	05199	GMC_oxred_C	4qi6
5-hydroxymethyl furfural oxidase	NCU07113T0	00732/05199	GMC_oxred_N/GMC_oxred_C	6f97
nitrile lyase (HNL)	NCU08977T0	00732/05199/00890	GMC_oxred_N/GMC_oxred_C/FAD_binding_2	6jby
choline oxidase	NCU09024T0	00732/05199/07992	GMC_oxred_N/GMC_oxred_C/Pyr_redox_2	4mjw
glucose oxidase	NCU09798T0	00732/05199	GMC_oxred_N/GMC_oxred_C	5oc1
cholesterol oxidase	NCU01193T0	05199/07992	GMC_oxred_C/Pyr_redox_2	1kdg
FAD-dependent oxidoreductases (3)				
putative monoamine oxidase	NCU0167T0/1/2	01266	DAO	6f32
fructosyl amine:oxygen oxidoreductase	NCU04771T0/1	01266	DAO	4wct
FAD-dependent oxidoreductase	NCU08048T0	01266	DAO	3dme
Amine oxidases (2)				
amine oxidase, homolog of LkcE[1]	NCU01089T0/1	01593	Amino_oxidase	6f7l
amine oxidase	NCU07234T0	01593	Amino_oxidase	2bk

Amino_oxidase, flavin-containing amine oxidoreductase; Bac_luciferase, luciferase-like monooxygenase; BBE, berberine and berberine-like; DAO, FAD-dependent oxidoreductase; FAD_binding_1, FAD_binding_2, FAD_binding_3, and FAD_binding_4, FAD-binding domains; FAO_M, FAD-dependent central domain; FMO-like, flavin-binding monooxygenase-like; GMC_oxred_N/C, GMC, oxidoreductase; K_oxygenase, L-lysine 6-monooxygenase (NADPH- requiring); Lycopene_cycl, lycopene cyclase protein; Pyr_redox_2, pyridine nucleotide-disulphide oxidoreductase.

*These enzymes are part of the biosynthetic gene cluster (BGC) #2 and #13 (see also Supplementary Table 2).

**Only flavin-containing/binding domains are listed in the table, domains lacking the flavin cofactor are not mentioned.

***The classification of the flavoenzymes into different flavoenzyme families was solely based on structural and topological features. Thus, entries in Table 2 do not represent a functional assignment.

[1](Dorival et al. 2018).

[2](Tan et al. 2015).

VAO-like enzymes tend to attach their flavin cofactors either mono- or non-covalently (Figure 1).

In the simplest cases, the BBE-like enzymes perform the oxidation of alcohol groups, for example, at the anomeric center of mono-, di-, or oligosaccharides, leading to the corresponding lactones (reviewed by Daniel et al., 2017). On the other hand, BBE-like enzymes also carry out complex cyclization reactions that are part of the biosynthesis of many important natural products such as alkaloids and terpenes (Sirikantaramas et al., 2004; Winkler et al., 2008). In order to evaluate the catalytic capacities, we generated structural models of the seventeen putative BBE-like enzymes in *N. crassa* using AlphaFold (Jumper et al., 2021). As shown in Figure 1, the models feature similar structural domains as identified in our reference model BBE from *Eschscholzia californica*. Intriguingly, this analysis revealed a close relationship to EncM, carbohydrate and alcohol oxidases. EncM is a bacterial flavoenzyme involved in the biosynthesis of enterocin in *Streptomyces maritima* and catalyzes a complex dual oxidation reaction (Teufel et al., 2013). Moreover, this enzyme was shown to form an unusual flavin N-5 oxide species during the catalytic cycle, and therefore, it is tempting to speculate that the structural relatives from *N. crassa* share the propensity to generate this catalytic intermediate for yet unknown (biosynthetic) transformations. The remainder of the BBE-like enzymes in *N. crassa* are putative oxidases/dehydrogenases of carbohydrates (*e.g.*, cellobiose and oligosaccharides) and alcohols. Regarding the putative carbohydrate oxidases/

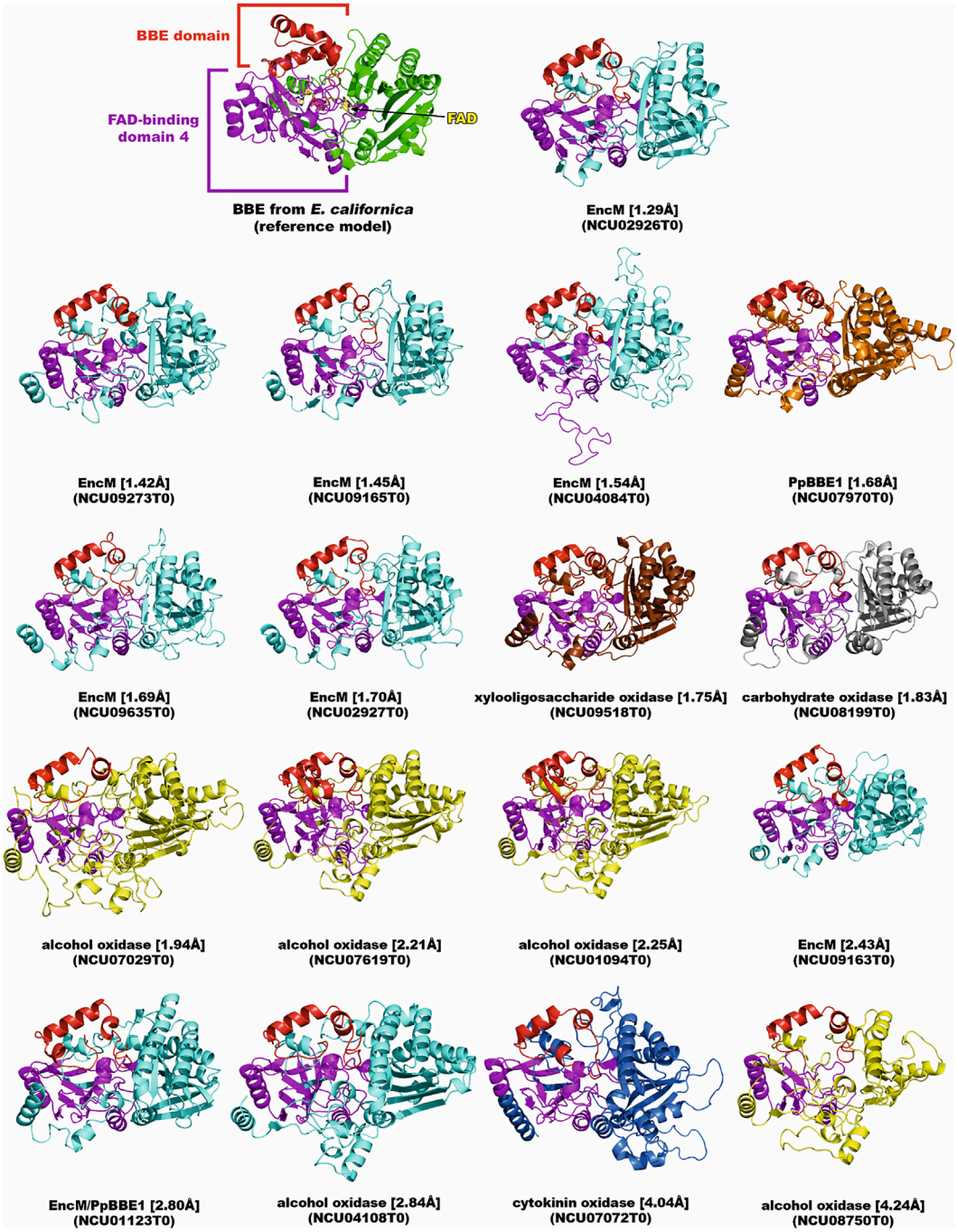

FIGURE 1
BBE-like enzymes in *N. crassa*. The crystal structure of BBE from *Eschscholzia californica* (PDB entry: 3d2d) was used as a reference (upper left). The AlphaFold models of the BBE-like enzymes from *N. crassa* are shown according to their structural deviation (expressed as Cα-RMSD) from the reference model. The BBE domain and the FAD-binding domain 4 of all enzymes are colored in red and magenta, respectively. The domains were assigned and colored according to their PFam annotation (Mistry et al., 2021). The remaining structure of each enzyme, including the substrate-binding domain, was colored according to the putative enzymatic function (color code: BBE, green; alcohol oxidase, yellow; carbohydrate oxidase, grey; cytokinin oxidase, blue; EncM, cyan; *Pp*BBE1, orange; xylooligosaccharide oxidase, brown). The Cα-RMSD value to the reference model and the gene ID for each BBE-like enzyme are given in square and round brackets, respectively.

TABLE 3 Accessory flavoproteomes in the fungal kingdom. The Table provides the total number of genes encoding for flavin-dependent enzymes that belong to the family of GMC oxidoreductases (GMC_oxred_N and GMC_oxred_C), FMN-dependent monooxygenases (FMO-like), FAD-dependent monooxygenases (FAD_binding_3), BBE-like enzymes (FAD_binding_4 and BBE), and the FAD-dependent monooxygenases (Trp_halogenases). The numbers in parentheses indicate the average number of genes found in each division and class. The highest average numbers found in the fungal divisions are highlighted in red; the highest average numbers within a division are highlighted in green (last updated on 15 August 2022).

Division/Class	# Species	PFam00732	PFam05199	PFam00743	PFam01494	PFam01565	PFam08031	PFam04820
		GMC_oxred_N	GMC_oxred_C	FMO-like	FAD_binding_3	FAD_binding_4	BBE	Trp halogenase
Basidiomycota	**631**	**16,960 (26.9)**	**17,032 (27.0)**	**2,934 (4.7)**	**17,069 (27.1)**	**12,774 (20.2)**	**7,017 (11.1)**	**1,345 (2.1)**
Pucciniomycotina	64	528 (8.3)	658 (10.3)	130 (2.0)	706 (11.0)	703 (11.0)	315 (4.9)	1 (0.0)
Ustilagomycotina	39	377 (9.7)	372 (9.5)	78 (2.0)	264 (6.8)	355 (9.1)	145 (3.7)	0
Agaricomycetes	483	15,757 (32.6)	15,704 (32.5)	2,575 (5.3)	15,413 (31.9)	11,354 (23.5)	6,435 (13.3)	1,329 (2.8)
Dacrymycetes	9	141 (15.7)	145 (16.1)	42 (4.7)	352 (39.1)	142 (15.8)	78 (8.7)	15 (1.7)
Tremellomycetes	33	135 (4.1)	132 (4.0)	105 (3.2)	323 (9.8)	200 (6.1)	37 (1.1)	0
Wallemiomycetes	2	12 (6)	13 (6.5)	0	4 (2)	11 (5.5)	3 (1.5)	0
Ascomycota	**1,474**	**32,751 (22.2)**	**33,376 (22.6)**	**8,368 (5.7)**	**60,422 (41.0)**	**54,102 (36.7)**	**22,235 (15.1)**	**690 (0.5)**
Pezizomycetes	73	793 (10.9)	813 (11.1)	196 (2.7)	991 (13.6)	1,381 (18.9)	466 (6.4)	19 (0.3)
Orbiliomycetes	3	25 (8.3)	27 (9.0)	6 (2.0)	21 (7.0)	59 (19.7)	11 (3.7)	0
Eurotiomycetes	406	9,615 (23.7)	9,680 (23.8)	2,203 (5.4)	21,570 (53.1)	15,671 (38.6)	6,987 (17.2)	346 (0.9)
Dothideomycetes	220	5,811 (26.4)	5,934 (27.0)	822 (3.7)	7,818 (35.5)	8,930 (40.6)	3,500 (15.9)	17 (0.1)
Lecanoromycetes	12	245 (20.4)	240 (20.0)	32 (2.7)	392 (32.7)	329 (27.4)	119 (9.9)	12 (1.0)
Leotiomycetes	80	1,873 (23.4)	1,916 (24.0)	401 (5.0)	3,060 (38.3)	2,557 (32.0)	932 (11.7)	41 (0.5)
Sordariomycetes	508	14,211 (28.0)	14,598 (28.7)	4,284 (8.4)	26,032 (51.2)	24,410 (48.1)	10,206 (20.1)	255 (0.5)
Xylonomycetes	2	7 (3.5)	7 (3.5)	0	11 (5.5)	18 (9.0)	6 (3.0)	0
Saccharomycotina	155	144 (0.9)	135 (0.9)	411 (2.7)	465 (3.0)	675 (4.4)	3 (0.0)	0
Taphrinomycotina	13	14 (1.1)	14 (1.1)	10 (0.8)	24 (1.8)	48 (3.7)	2 (0.2)	0
Mucoromycota	**111**	**605 (5.5)**	**574 (5.2)**	**270 (2.4)**	**3,100 (27.9)**	**1,089 (9.8)**	**328 (3.0)**	**8 (0.0)**
Glomeromycotina	13	144 (11.1)	126 (9.7)	43 (3.3)	250 (19.2)	279 (21.5)	117 (9.0)	7 (0.5)
Mortierellomycotina	20	127 (6.4)	131 (6.6)	30 (1.5)	2,083 (104.2)	350 (17.5)	151 (7.6)	0
Mucoromycotina	78	334 (4.3)	317 (4.1)	197 (2.5)	767 (9.8)	460 (5.9)	60 (0.8)	1 (0.0)
Zoopagomycota	**24**	**46 (1.9)**	**53 (2.2)**	**16 (0.7)**	**110 (4.6)**	**134 (5.6)**	**39 (1.6)**	**0**
Zoopagomycotina	6	2 (0.3)	2 (0.3)	5 (0.8)	31 (5.2)	18 (3.0)	1 (0.2)	0
Entomophthoromycotina	5	38 (7.6)	45 (9.0)	3 (0.6)	46 (9.2)	63 (12.6)	38 (7.6)	0
Kickxellomycotina	13	6 (0.5)	6 (0.5)	8 (0.6)	33 (2.5)	53 (4.1)	0	0
Blastocladiomycota	**4**	**7 (1.8)**	**7 (1.8)**	**1 (0.3)**	**27 (6.8)**	**15 (3.8)**	**0**	**0**
Chytridiomycota	**37**	**81 (2.2)**	**79 (2.1)**	**58 (1.6)**	**307 (8.3)**	**182 (4.9)**	**50 (1.4)**	**11 (0.3)**
Chytridiomycetes	24	67 (2.8)	65 (2.7)	50 (2.1)	269 (11.2)	126 (5.3)	36 (1.5)	10 (0.4)
Monoblepharidomycetes	2	14 (7)	14 (7)	8 (4)	38 (19)	42 (21)	14 (7)	1 (0.5)
Neocallimastigomycetes	11	0	0	0	0	14 (1.3)	0	0
Microsporidia	**23**	**0**	**0**	**0**	**3 (0.1)**	**1 (0.0)**	**0**	**0**
Cryptomycota	**3**	**0**	**0**	**0**	**3 (1)**	**3 (1)**	**0**	**0**

dehydrogenases, the recent identification of fungal oligosaccharide-oxidizing flavoenzymes from the auxiliary activity family 7 (AA7) is very intriguing (Haddad Momeni et al., 2021). Apparently, members of this family are capable of donating electrons to the copper-dependent LPMOs and thus boost the oxidative cleavage of recalcitrant polysaccharides such as lignocellulose (Vaaje-Kolstad et al., 2010). Two of the BBE-like enzymes discovered in *N. crassa*, namely NCU08199T0 (putative carbohydrate oxidase) and NCU09518T0 (putative xylooligosaccharide oxidase), share high structural similarity (Cα-RMSD of 1.6 and 0.8 Å, respectively) with an oligosaccharide dehydrogenase from *Fusarium graminearum* (FgChi7B, (Haddad Momeni et al., 2021), and therefore, may constitute a relevant enzymatic system to fuel LPMOs. A hallmark of the BBE-like enzyme family is the bicovalent attachment of the FAD cofactor by a histidine and cysteine residue to the 8α- and 6-position, respectively, of the isoalloxazine ring system (Winkler et al., 2008). In fact, eleven of the BBE-like enzymes of *N. crassa* (Figure 1) feature these two residues according to the generated AlphaFold models at the appropriate positions, and thus, we predict that a bicovalent linkage is formed in these proteins.

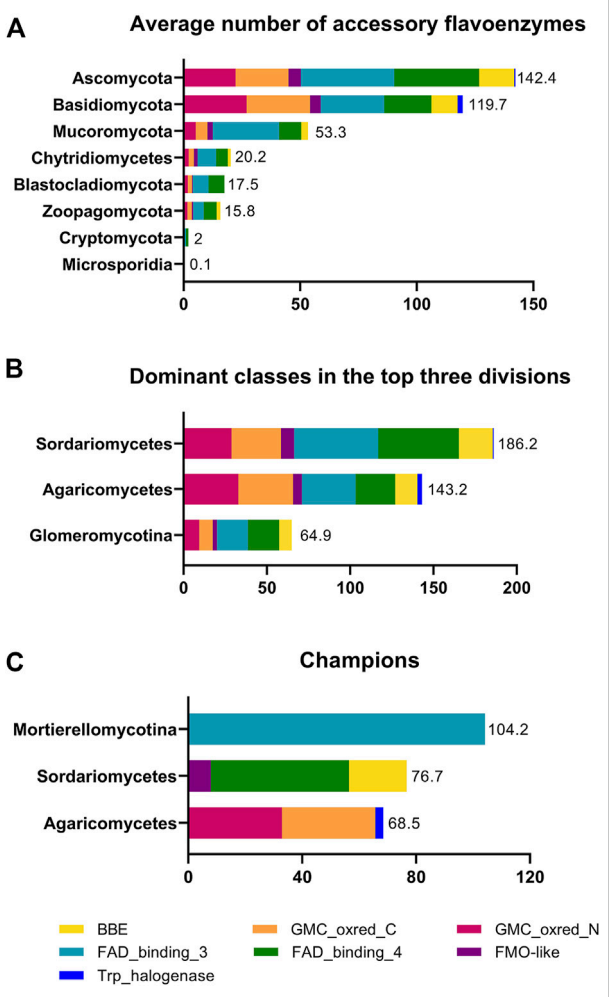

FIGURE 2
Distribution of accessory flavoenzymes in fungi. Panel **(A)** shows the average number of accessory flavoenzymes in the eight fungal divisions. Panel **(B)** shows the classes with the highest average number of accessory flavoenzymes for the divisions Ascomycota, Basidiomycota, and Mucoromycota, i.e., Sordariomycetes, Agaricomycetes, and Glomeromycotina, respectively. Panel **(C)** highlights the classes with the highest average number of accessory flavoenzymes in fungi. The data used in the figure was extracted from Table 3.

GMC-oxidoreductases (represented by PFam00732 and PFam05199) predominantly catalyze the oxidation of hydroxyl groups in a large variety of substrates (e.g., carbohydrates and alcohols) but also the oxidative deamination of *N*-methyl groups (e.g., choline) (see Table 2). Recently, it could be demonstrated that the proteins encoded by NCU00206T0 and NCU05923T0 act as cellobiose dehydrogenases (part of the AA3_1 family in the CAZy database) and deliver electrons *via* a cytochrome domain to LPMOs (Tan et al., 2015; Kracher et al., 2016). Apparently, flavoenzymes from the BBE-like (part of the AA4 family in the CAZy database) enzyme as well as the GMC-oxidoreductase family participate in the enzymatic degradation of extracellular lignocellulose by providing electrons to LPMOs (AA7 family in the CAZy database).

Although not very numerous, amine oxidases are also an interesting group of accessory flavoenzymes (PFam01593, Table 2) with respect to their potential role in the biosynthesis of natural products. In fact, *N. crassa* harbors a gene (NCU01089T0/1) that encodes an amine oxidase homologous to LkcE from the lankacidin polyketide biosynthetic pathway of the bacterium *Streptomyces rochei* (Dorival et al., 2018). Since the biosynthesis of polyketide macrocycles in *N. crassa* has not been described, the role of the *N. crassa* homolog is currently unknown.

The genes encoding enzymes for the biosynthesis of natural products are frequently organized in so-called biosynthetic gene clusters (BGCs). The genome of *N. crassa* harbors sixteen BGCs, but only two of them ($^{\#}$2 and $^{\#}$13) contain one and three flavoenzymes, respectively (see Supplementary Table S1). In the latter case, it has been suggested that this BGC synthesizes the phytotoxic salicylaldehyde sordarial known from the fungus *Magnaporthe oryzae* (Zhao et al., 2019). The role of the BBE-like enzyme (NCU02926T0, termed *SrdI*) appears to be the oxidation of an alcohol group to the corresponding aldehyde yielding a salicylaldehyde derivative. The role of the other BBE-like enzyme (NCU02927T0), however, is unclear, as is the case for the (aromatic-ring) hydroxylase found in the same BGC (NCU02925T0). Thus, only four out of 68 flavoenzymes listed as part of the accessory flavoproteome (see Table 2) are related to BGCs, whereas the majority (>90%) are not organized within BGCs.

Despite its importance as a model organism, the number of elucidated three-dimensional structures of *N. crassa* proteins is surprisingly low. In fact, only 127 PDB entries (as of 15 August 2022) describe structures of *N. crassa* proteins, with 10 entries related to flavoproteins. However, these PDB entries cover only two flavoproteins, VIVID (9 entries!) and cellobiose dehydrogenase (see Tables 1, 2; Kracher et al., 2016). This large bias toward VIVID reflects the importance of *N. crassa* as a model organism to investigate light-dependent processes. In contrast to *N. crassa*, 5,371 entries (as of 15 August 2022) presently relate to proteins from the yeast *Saccharomyces cerevisiae* (5,885 protein-encoding genes, (Goffeau et al., 1996), and 101 PDB entries to yeast flavoproteins (68 genes encode flavoproteins, (Gudipati et al., 2014).

Occurrence and distribution of accessory flavoenzymes in fungi

The large number of accessory flavoenzymes in *N. crassa* prompted us to conduct a global analysis of the fungal kingdom based on the information available at the MycoCosm resource database (www.mycocosm.jgi.doe.gov). Since the analysis of the *N. crassa* genome indicates that the accessory flavoproteome

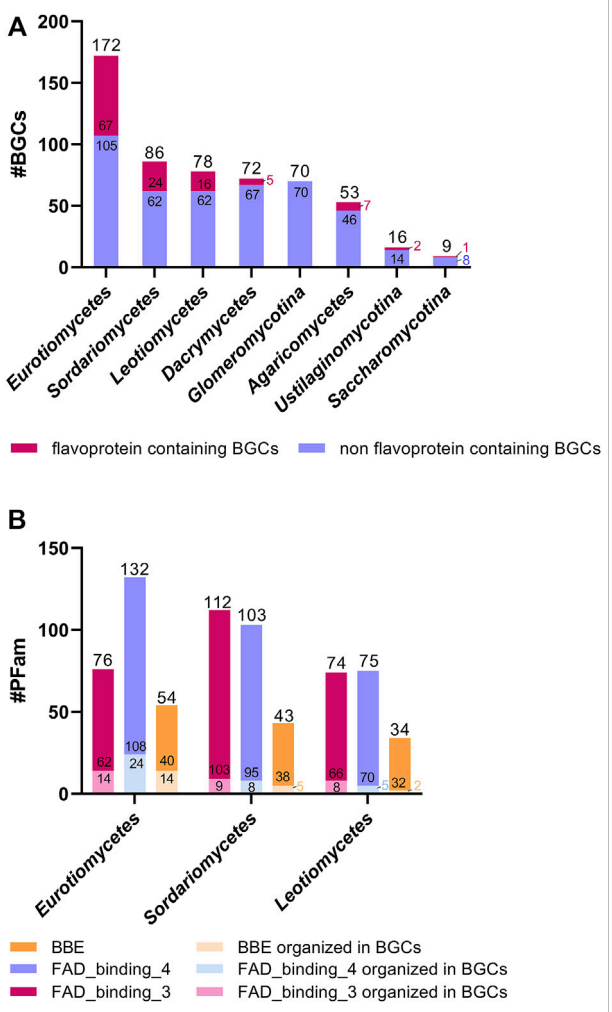

FIGURE 3
Occurrence of flavoenzymes in BGCs. Three species were analyzed for each class. Panel **(A)** shows the total number of BGCs, with the top part (in red) representing the number of BGCs containing at least one flavoenzyme. Panel **(B)** shows the distribution of the three most common families of flavoenzymes, FAD_binding_3, FAD_binding_4, and BBE, in Eurotiomycetes, Sordariomycetes, and Leotiomycetes. The analysis is based on the data compiled in Supplementary Table S2.

is dominated by flavin-dependent (aromatic) monooxygenases (represented by PFam01494), FMO-like monooxygenases (represented by PFam00743), FAD-dependent oxidoreductases/BBE-like enzymes (represented by PFam01565 and PFam08031), and GMC-oxidoreductases (represented by PFam00732 and PFam05199), we focused on these flavoenzyme families. This analysis revealed large differences between the fungal divisions and also within the classes. As shown in Table 3, Basidiomycota and Ascomycota are very rich in all of the flavoenzymes characteristic of the accessory flavoproteome, whereas Mucoromycota, Zoopagomycota, Blastocladiomycota, and Chytridiomycetes feature only a few accessory flavoenzymes. In two divisions, *i. e.,* Microsporidia and Cryptomycota, accessory flavoenzymes appear to be completely absent (Table 3 and Figure 2, panel A).

Interestingly, the accessory flavoenzymes are most prevalent in certain classes such as the Agaricomycetes, Sordariomycetes, and Mortierellomycotina for the divisions Basidiomycota, Ascomycota, and Mucoromycota, respectively (Figure 2, panel B). As a matter of fact, Agaricomycetes have the highest content of GMC-oxidoreductases (PFam00732/05199), whereas Sordariomycetes harbor the largest number of FMO-like (PFam00743) and BBE-like enzymes (PFam01565 and PFam08031). Remarkably, in the class Mortierellomycotina, an exceptionally large number of FAD-dependent monooxygenases (PFam01494, FAD_binding_3) was found. In fact, the average occurrence in this genus (104.2/species) is more than twice as high as in Eurotiomycetes (52.4/species). On the other hand, the occurrence of all other accessory flavoenzymes in Mortierellomycotina, as well as in the entire division Mucoromycota, is rather low (see Table 3). In Mortierellomycotina, the highest number of FAD-dependent monooxygenases was found in *Mortierella elongata* NVP64 (encoded by 215 genes). This fungus is known to host bacterial endosymbionts, such as *Mycoavidus cysteinexigens* (Uehling et al., 2017). However, it is unclear whether the large number of FAD-dependent monooxygenases is connected to this distinct lifestyle. Interestingly, in the case of *Mortierella verticillata* (152 genes encoding FAD-dependent monooxygenases), it was demonstrated that the bacterial endosymbiont produces protective compounds against fungivorous nematodes (Büttner et al., 2021). These cytotoxic benzolactones (necroximes) feature several groups, such as hydroxyl, epoxy, and oxime, that suggest the involvement of FAD-dependent monooxygenases. In other words, it is conceivable that fungal enzymes might participate in the biosynthesis of these anthelmintic compounds. On the other hand, this does not rationalize the extremely large number of genes encoding FAD-dependent monooxygenases.

Although flavin-dependent halogenases are not very numerous (total number in fungi: 2054 genes as of 15 August 2022), their appearance is strongly linked to the occurrence of the other families of accessory flavoenzymes, and thus, Agaricomycetes (in the division Basidiomycota), as well as Sordariomycetes and Eurotiomycetes (both in the division of Ascomycota), contain by far most of the flavin-dependent halogenases (see Table 3). Since flavin-dependent halogenases (group F of the flavin-dependent monooxygenases, Paul et al., 2021) are known to catalyze the halogenation of aromatic rings such as indole or phenyl rings, it can be expected that fungal halogenases are involved in the halogenation of similar structures en route to species-specific natural products. It should also be noted that flavin-dependent halogenases can utilize chlorine, bromine, and iodine for substrate halogenations, mostly depending on the availability of the halide. In this context, it

is worthwhile mentioning that flavin-dependent halogenases are attractive biocatalytic tools to broaden the scope of accessible compounds in medicinal chemistry (Fraley and Sherman, 2018).

Finally, we have analyzed the occurrence of flavoenzymes in BGCs in various fungal classes. As shown in Figure 3A, the number of BGCs, as well as the number of BGCs containing flavoenzymes, varies considerably. The highest number of BGCs was found in Eurotiomycetes, with 67 BGCs containing at least one flavoenzyme (*i.e.,* 40%, for details, see Table S2). The other fungal classes contain fewer BGCs and also fewer BGCs harboring flavoenzymes (>28%–0%). The most dominant flavoenzyme families occurring in fungal BGCs are FAD-dependent monooxygenases (PFam01494), oxidoreductases (PFam01595), and BBE-like enzymes (PFam08031). However, it should be noted that the majority of genes encoding members of these flavoenzyme families are not part of a BGC, as shown in Figure 3B.

Discussion

The previous analysis of the flavoproteome of *S. cerevisiae* (class Saccharomycotina) has revealed a relatively small number of flavoenzymes (68 out of 5,885 protein-encoding genes, *i.e.,* 1.1% of the proteome) (Gudipati et al., 2014). Moreover, it appears that most, if not all flavoenzymes, are involved in basic metabolic functions, in other words, they belong to the core flavoproteome. In contrast, the flavoproteome of *N. crassa* (class Sordariomycetes) comprises not only a much higher number of core flavoenzymes (201 of 10,082, *i.e.*, 2% of the protein-encoding genes (Galagan et al., 2003) but also a substantial number of accessory flavoenzymes (68 of 201, *i.e.* 33.8% of flavoprotein coding genes). It should be noted that the classification into core and accessory flavoenzymes reflects to some extent the lack of solid biochemical knowledge currently available for these enzymes. Hopefully, future research efforts will allow more accurate functional assignments of the accessory flavoenzymes, leading to a consolidated classification (detoxification, defense, *etc.*). In any case, in the course of further analysis, we noticed that the accessory flavoproteome of *N. crassa* is dominated by two types of reactions: monooxygenation and oxidation. The former reaction is catalyzed by flavoenzymes, which either exhibit the FAD_binding_3 or the FMO-like domain. Oxidation reactions, on the other hand, belong either to the BBE-like enzymes or to the GMC-oxidoreductases (see Table 2). Both types of biochemical transformations are frequently found in natural product biosynthesis, and therefore, we assume that *N. crassa* synthesizes several more yet unidentified compounds. In this context, it is noteworthy that members of the BBE-like enzyme family catalyze rather unusual and complex oxidation reactions that lead, for example, to the formation of additional ring systems in alkaloid, terpene, and polyketide biosynthetic pathways (Kutchan and Dittrich, 1995; Shoyama et al., 2012; Teufel et al., 2013).

Intrigued by the large and unexpected number of monooxygenases and oxidases in *N. crassa*, we expanded our analysis and explored the occurrence of the accessory flavoenzymes in the entire fungal kingdom by using the MycoCosm database. As shown in Table 3, accessory flavoenzymes are predominantly found in Ascomycota and Basidiomycota, with a much lower occurrence in the other fungal divisions. Within the Basidiomycota, the class Agaricomycetes features the highest average number of all accessory flavoprotein families except for the flavin hydroxylases (FAD_binding_3), which show a slightly higher occurrence in Dacrymycetes. Similarly, Sordariomycetes in the division Ascomycota also harbor the highest number of these accessory flavoenzymes. Interestingly, VAO-like enzymes, which are absent in *N. crassa*, were mainly found in the class of Agaricomycetes, Eurotiomycetes, and Sordariomycetes (Gygli et al., 2018). Thus, accessory flavoenzymes are not uniformly distributed but exhibit accumulation in certain fungal classes, reflecting their outstanding chemodiversity. To further substantiate this relationship, we have included the flavin-dependent halogenases in our analysis (Table 3 and Figure 2). In terms of their reaction mechanism, flavin-dependent halogenases are related to the monooxygenases in that they also generate a flavin 4a-hydroperoxide intermediate. However, instead of transferring an oxygen atom to the substrate, this intermediate reacts with a halide (typically chloride or bromide) and subsequently transfers the halogen to the aromatic ring of the substrate. Thus, the halogenation reaction relies on the "invention" of the flavin 4a-hydroperoxide, and therefore, it is tempting to hypothesize that flavin-dependent halogenases have evolved from the flavin-dependent monooxygenases. This notion is also supported by the structural similarity of these enzyme families, as both feature a three-layer ββα sandwich flavin-binding domain (Paul et al., 2021).

The rapidly increasing number of fungi-derived organohalogens suggests an important role for the producing species. As noted recently, 217 organohalogen compounds were described in marine fungi from 1994 to 2019 (Wang et al., 2021). Most of these compounds are produced by Aspergillus and Penicillium species, which belong to the Eurotiomycetes. In terrestrial fungi, organohalogens were isolated from Aspergillus, Malbranchea, and Penicillium species (Eurotiomycetes); Colletotrichum and Diaporte species (Sordariomycetes); and Alternaria, Cochliobolus, and Helminthosporium species (Dothiodeomycetes) (Gribble, 2018). Notably, all of these classes belong to the division Ascomycota (and subdivision Pezizomycotina), which represents the division with the highest average number of accessory flavoenzymes (see Table 3 and Figure 2, panel A). Similarly, Agaricomycetes in the division Basidiomycota feature the highest average number of flavin-dependent halogenases (see Table 3 and Figure 2, panels A–C). Interestingly, the flavin-dependent halogenase described for some Russula species is

similar to CmlS, which carries out the dichlorination of *N*-acetyl-*p*-nitrophenylserinol to chloramphenicol in the bacterium *Streptomyces venezuelae* (Podzelinska et al., 2010). Thus, it is conceivable that Russula species also produce chloramphenicol or a similar organohalogen. This would be in line with the recent proposal that the edible mushroom *Russula nigricans* might be responsible for the production of dichloroacetic acid, which could be released by hydrolysis of the amide bond in chloramphenicol (Lajin et al., 2021). In summary, flavin-dependent halogenases are an interesting addition to expand the scope of modifications in natural product biosynthesis.

As mentioned before, the genes encoding the enzymes of a biosynthetic pathway are frequently organized in so-called BGCs. Much to our surprise, we found that most fungal BGCs do not contain any flavoenzymes, even the classes with the highest average number of accessory flavoenzymes, such as Agaricomycetes and Sordariomycetes (Figure 3A). In line with this, the majority of accessory flavoenzymes are encoded by genes that are not part of BGCs, as shown in Figure 3B. For example, in the case of *N. crassa*, only three out of 68 accessory flavoenzymes are encoded by genes in BGC #2 and #13 (see Table 2). Does this finding contradict the hypothesis that accessory flavoenzymes are largely involved in natural product biosynthesis or does it indicate that many fungal biosynthetic processes involving flavoenzymes are not encoded by BGCs? Although it is too early to make a final judgement about this question, flavoenzymes apparently assume other important roles, such as the degradation and detoxification of harmful compounds released into the environment by other species, as exemplified by the putative maackiain detoxification enzyme in *N. crassa* (Table 2).

Conclusion

Fungi possess a remarkable number of flavoenzymes employed for both basic as well as "secondary" reactions, leading to a plethora of natural products. Most of these reactions and their products remain unidentified, even for *N. crassa*, which has been investigated as a model organism for many decades. Despite substantial progress in accurately predicting protein structures through computational methods, the reliable assignment of biochemical function lacks far behind and remains a major challenge for many years to come. Clearly, the enormous scope of fungal flavoproteomes requires a joint effort employing biochemical and genetic approaches guided by robust predictions of potential substrates and enzyme reactivities. This endeavor will not only pave the way for the discovery of many more natural products and complex biological interactions but will also enable scientists to tap into this vast reservoir of flavoenzymes with exciting perspectives for applications in biocatalysis.

Methods

Identification and annotation of flavoproteins from *N. crassa*

In our previous study of selected pro- and eukaryotic flavoproteomes, 156 genes encoding flavoproteins were identified in *N. crassa* (Supplementary Table S1, (Macheroux et al., 2011). This preliminary list was further refined by a keyword search (dehydrogenase, FAD, flavin, FMN, halogenase, hydroxylase, monooxygenase, oxidase, and reductase) in the MycoCosm database. In addition, we have compiled a list of protein domains associated with FMN or FAD binding (see Supplementary Table S1). These PFam domains were used to search the MycoCosm database to retrieve genes encoding flavoproteins in *N. crassa* and also in the fungal divisions and classes reported in Table 3. A literature search (PubMed) was performed using the same keywords mentioned above as well as the commonly used names of known flavoproteins based on previous analyses (Macheroux et al., 2011; Lienhart et al., 2013; Gudipati et al., 2014; Eggers et al., 2021).

Structure predictions of BBE-like enzymes from *N. crassa*

To confirm and assess the structural features and domains of all identified BBE-like enzymes from *N. crassa* (Table 2), we used AlphaFold2 (Jumper et al., 2021) to predict their 3D structures because of the lack of available crystal structures. We used AlphaFold2 *via* the Collaboratory service from Google Research (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/beta/AlphaFold2_advanced.ipynb). The sequence of each BBE-like enzyme was submitted to AlphaFold2, and as a first step, a multiple sequence alignment was performed employing the mmseq2 method (Steinegger and Söding, 2017). Five models per enzyme were predicted and ranked by pLDDT (predicted local distance difference test), a per-residue confidence score. The five predicted models for each enzyme were superimposed on the crystal structure of BBE from *E. californica* (PDB entry: 3D2D), which was used as a BBE reference model. The model with the lowest C_α-RMSD to the reference model and the highest pLDDT score (i.e., highest confidence) was refined with the Amber-Relax protocol of the AlphaFold pipeline. The resulting model (of each enzyme) was used for structural and visual inspection after trimming regions exhibiting a low pLDDT score (i.e., regions of high uncertainty such as the N- and C-termini). PyMOL (DeLano, 2002) was used for trimming and visual inspection.

Author contributions

BK has retrieved data from the MycoCosm database and generated Figure 3; AB retrieved data from the PDB and generated Figure 1; PM initiated the project, collected data from the MycoCosm database and generated Table 1, Table 2, Table 3, and Figure 2; all three authors have jointly written the paper.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fctls.2022.1021691/full#supplementary-material

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