# Expression, Purification, and Characterization of *Aspergillus fumigatus* Sterol 14- $\alpha$ Demethylase (CYP51) Isoenzymes A and B<sup> $\nabla$ </sup>

Andrew G. S. Warrilow,<sup>1</sup> Nadja Melo,<sup>1</sup> Claire M. Martel,<sup>1</sup> Josie E. Parker,<sup>1</sup> W. David Nes,<sup>2</sup> Steven L. Kelly,<sup>1</sup> and Diane E. Kelly<sup>1</sup>\*

Institute of Life Science and School of Medicine, Swansea University, Swansea, Wales SA2 8PP, United Kingdom,<sup>1</sup> and Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409-1061<sup>2</sup>

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Aspergillus fumigatus sterol 14- $\alpha$  demethylase (CYP51) isoenzymes A (AF51A) and B (AF51B) were expressed in *Escherichia coli* and purified. The dithionite-reduced CO-P450 complex for AF51A was unstable, rapidly denaturing to inactive P420, in marked contrast to AF51B, where the CO-P450 complex was stable. Type I substrate binding spectra were obtained with purified AF51B using lanosterol ( $K_s$ , 8.6  $\mu$ M) and eburicol ( $K_s$ , 22.6  $\mu$ M). Membrane suspensions of AF51A bound to both lanosterol ( $K_s$ , 3.1  $\mu$ M) and eburicol ( $K_s$ , 4.1  $\mu$ M). The binding of azoles, with the exception of fluconazole, to AF51B was tight, with the  $K_d$  (dissociation constant) values for clotrimazole, itraconazole, posaconazole, and voriconazole being 0.21, 0.06, 0.12, and 0.42  $\mu$ M, respectively, in comparison with a  $K_d$  value of 4  $\mu$ M for fluconazole. Characteristic type II azole binding spectra were obtained with AF51B, whereas an additional trough and a blue-shifted spectral peak were present in AF51A binding spectra for all azoles except clotrimazole. This suggests two distinct azole binding conformations within the heme prosthetic group of AF51A. All five azoles bound relatively weakly to AF51A, with  $K_d$ values ranging from 1  $\mu$ M for itraconazole to 11.9  $\mu$ M for fluconazole. The azole binding properties of purified AF51A and AF51B suggest an explanation for the intrinsic azole (fluconazole) resistance observed in *Aspergillus fumigatus*.

The sterol pathways of eukaryotes are highly conserved and are part of a larger biosynthetic pathway that includes the formation of dolichols, coenzyme Q, heme A, and isoprenylated proteins. In *Saccharomyces cerevisiae*, the first step exclusively involved in sterol synthesis is the formation of squalene, with the first sterol intermediate in the pathway being lanosterol, culminating in ergosterol some 15 enzymatic steps later. In fungi, these reactions are governed by individual enzymes, but closer examination of other fungal genome sequences has revealed that there is often duplication (29) and, in some instances, triplicate versions of the same gene (1). We are interested as to why fungi have kept multiple copies of these genes, the roles of the proteins *in vivo*, and their contribution to both sterol biosynthesis and fungal resistance.

14- $\alpha$  Demethylase (CYP51) is an ancestral activity of the cytochrome P450 superfamily, which is also the target of azole antifungals (18). The isolation of *CYP51* was initially from *Saccharomyces cerevisiae* (17), and in the fungal pathogen *Aspergillus fumigatus*, cytochrome P450 was first observed in 1990 (5, 6). Evidence that alteration of CYP51 activity might contribute to azole resistance first emerged in 1997 (11). In this particular study, we have looked in detail at the biochemical properties of the two CYP51 forms in *Aspergillus fumigatus* (29) encoded by *CYP51A* (Afu4g06890) and *CYP51B* (Afu7g03740). A comparison of the deduced amino acid sequences show 63% identity between them; and both ortho-

\* Corresponding author. Mailing address: Institute of Life Science and School of Medicine, Swansea University, Swansea SA2 8PP, United Kingdom. Phone: 44 1792 292207. Fax: 44 1792 503430. E-mail: d.kelly@swansea.ac.uk. logues in *A. fumigatus* have been shown to act in a compensatory manner in the ergosterol pathway; i.e., neither is essential individually, but a double knockdown is lethal (13). It is postulated that CYP51A may encode the major sterol 14- $\alpha$  demethylase activity required for growth on the basis of accumulation of multiple missense mutations linked to azole resistance (31), with CYP51B either being functionally redundant or having an alternative function under particular growth conditions still to be defined. We expressed both proteins in *Escherichia coli* to investigate their azole binding properties.

## MATERIALS AND METHODS

**Construction of pSPORT** *AF51A* and pSPORT *AF51B* expression vectors. The coding regions of the *A. fumigatus* (strain Af293 [http://www.aspergillusgenome .org/gbrowse/afum\_af293]) CYP51 isoenzyme A (*AF51A*) and B (*AF51B*) genes (ExPASy protein database accession numbers Q4WNT5 and Q96W81, respectively) were synthesized commercially by GeneCust Europe (Dudelange, Luxembourg) in pUC57 with codon optimization for expression in *E. coli*. NdeI and HindIII sites were included at the 5' and 3' ends, respectively, of the coding sequence with an additional DNA sequence encoding 6 histidine residues prior to the stop codons. The *AF51A* and *AF51B* genes were excised from pUC57 and cloned into the modified pSPORT expression vector (22) using NdeI/HindIII. Positive recombinants were selected by growth on LB agar plates containing 0.1 mg  $\cdot$  ml<sup>-1</sup> sodium ampicillin, and DNA sequencing confirmed the presence of the correct inserts.

Heterologous expression in *E. coli* and isolation of recombinant AF51A and AF51B proteins. Overnight cultures (10 ml) of pSPORT *AF51A* and pSPORT *AF51B* were used to inoculate 1-liter volumes of Terrific broth supplemented with 20 g  $\cdot$  liter<sup>-1</sup> peptone and 0.1 mg  $\cdot$  ml<sup>-1</sup> sodium ampicillin. Cultures were grown at 37°C and 230 rpm for 7 h, prior to induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and expression at 27°C and 170 rpm for 18 h in the presence of 1 mM 5-aminolevulenic acid. The AF51A and AF51B proteins were isolated according to the method of Arase et al. (3), except that 2% (wt/vol) sodium cholate and no Tween 20 were used in the sonication buffer. The solubilized AF51A and AF51B proteins were purified by affinity chromatography using nickel-nitrilotriacetic acid (Ni<sup>2+</sup>-NTA) agarose, as described previously

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		10	20	) 30	40	50	) 60	) 7(	) 80	90	100
C.	albicans			MAIVETVIDG	INYFLSLSVT	QQISILLGVP	FVYNLVWQYL	YS-LRKDRAP	LVFYWIPWFG	SAASYGQQPY	EFFESCRQKY
C.	tropicalis			MAIVDTAIDG	INYFLSLSLT	QQITILVVFP	FIYNIAWQLL	YS-LRKDRVP	MVFYWIPWFG	SAASYGMQPY	EFFEKCRLKY
S.	cerevisiae		-MSAT-KSIV	GEALEYVNIG	LSHFLALPLA	QRISLIIIP	FIYNIVWQLL	YS-LRKDRPP	LVFYWIPWVG	SAVVYGMKPY	EFFEECQKKY
C.	glabrata		-MSTENTSLV	VELLEYVKLG	LSYFQALPLA	QRVSIMVALP	FVYTITWQLL	YS-LRKDRPP	LVFYWIPWVG	SAIPYGTKPY	EFFEDCQKKY
A.	gossypii		-MSESLL	QTVVAYVELV	LHHFMALSWT	QQLSIVIVAP	FIYSLVWQTL	YS-FRKDRVP	LVPFMVPWVG	SALAYGRAPY	EFFGKCQQKY
U.	necator		MYIAD	ILSDLLTQQT	TRYGWIFMVT	SIAFSIILLA	VGLNVLSQLL	FRRPYEPP	VVFHWFPIIG	STISYGIDPY	KFYFDCRAKY
в.	graminis		MGISE	SFMFPYLQPL	LQLGFGIALA	SGIISLLLL	TFLNVLKQLL	FKNPNEPP	IVFHWIPIIG	STISYGMNPY	KFFHESQAKY
М.	fructicola		MGVLE	TIAGPLAQEI	SQRSTGTIIA	AGVAAFVVLA	VVLNVLNQVL	FANPNEPP	VVFHWLPIIG	STITYGIDPY	RFFFDCRAKY
в.	fuckeliana		MGILE	AVTGPLAQEI	SQRSTGVVVA	AGVAAFIVLS	VVLNVLNQVL	FANPNEPP	MVFHWLPVIG	STITYGMDPY	KFFFDCRAKY
о.	yallundae		MGILD	TVTVPLAQQV	SQRGLGVVIA	AGFAAFLVVS	VVLNVLSQIL	FKNPNEPP	VVFHFFPIIG	STVTYGIDPY	KFFFDNKAKY
о.	acuformis		MGILE	AVSVPLAQQV	SQRGLGVVIA	AGFAAFLVVS	VVLNVLSQIL	FKNPNEPP	VVFHFFPIIG	STVTYGIDPY	KFFFDNKAKY
в.	jaapii		MGFFE	AVSVPLNEQI	SQRGLAVVIT	AGFGAFVVVS	IVLNVLSQLL	LKNPNEPP	VVFHWLPIVG	STITYGIDPY	RFFFENKAKY
N.	fischeri B		MGLIA	FILDGICKHC	STQSTWVLVG	VGLLSILAVS	VIANVLQQLL	FKNPHEPP	VVFHWFPFIG	STISYGIDPY	KFFFDCRAKY
A.	fumigatus B		MGLIA	FILDGICKHC	STQSTWVLVG	IGLLSILAVS	VIINVLQQLL	FKNPHEPP	VVFHWFPFIG	STISYGIDPY	KFFFDCRAKY
A.	clavatus B		MGLIT	DILDGVCKYC	STQSIWMLGG	VGLLSLLAVA	VVVNVLRQLL	FKNPNEPP	LVFHWFPFIG	STISYGIDPY	KFFFDCRAKY
A.	flavus B									MDPY	RFFFNCREKY
A.	nidulans B		MGLVS	LVLDNVCERC	SALSVWALSG	LGLLSVIIIA	VVLNVLRQIL	FKNPNEPP	VVFHWFPFIG	STISYGIDPY	KFFFNCRAQY
P.	marneffei B		MGLFA	AALNKICDHS	STQSIYVLAG	LGFAIVIVLS	VVINVLSQLL	FKNPNEPP	VVFHWFPFIG	NTISYGIDPY	KFFFECRKKY
T.	stipitatus B		MGLFA	AALDRICEQC	STQSIYVLAG	LGFATVIVLS	VIINVLSQLL	FKNPNEPP	VVFHWFPFIG	NTISYGMDPY	KFFFDCRNKY
v.	inaequalis		MGLLS	PLLAXLPGSD	RSWLFYTLAS	FGFTVAI	VAANLVKQLL	FSNPNEPP	VVFHWFPFFG	NTVVYGIDPI	KFFAECKEKH
М.	graminicola		MALLQ	EVLAQFDAQF	GQTSLWKLVG	LGFLAFSTLA	ILLNVLSQLL	FRG-KLSDPP	LVFHWVPFIG	STITYGIDPY	KFFFSCREKY
₽.	digitatum			MDLVP	LVTGQIKCIA	YYTTGLVLAS	IVLNVIKQLV	FYNRKEPP	VVFHWIPFIG	STVAYGMDPY	QFFFASRAKY
P.	italicum			MDLVP	LVTGQILGIA	YYTTGLFLVS	IVLNVIKQLI	FYNRKEPP	VVFHWIPFIG	STIAYGMDPY	QFFFASRAKY
N.	fischeri A				MVPMLLLT	AYMAVAMLTA	ILLNVVYQLF	FRLWNRTEPP	MVFHWVPFLG	STISYGIDPY	KFFFACREKY
А.	fumigatus A				MVPMLWLT	AYMAVAVLTA	ILLNVVYQLF	FRLWNRTEPP	MVFHWVPYLG	STISYGIDPY	KFFFACREKY
A.	clavatus A				MLSLTLFG	LYLVSATAVV	ILVNVVYQHL	FRLRNRTEPP	MVFHWIPFIG	STITYGIDPC	KFFFACREKY
A.	flavus A			MIFS	RSMASFTLVS	AYAAAGLLAI	IVLNLLRQLL	FRNKTDPP	LVFHWIPFLG	STVTYGMDPY	AFFFSCRQKY
A.	nidulans A				MLSPTLFS	AYVLVGAIVA	VLVNVIRQIF	FRNKNEPP	MVFHWVPFVG	STISYGMNPY	KFFFSCREKY
s.	pombe				MAFSLV	SILLSIALAW	YVGYIINQLT	SRNSKRPP	IVFHWIPFVG	SAVAYGMDPY	VFFRECRAKY
σ.	maydis	MVA	SSSSATASLL	DQLFALTPLA	DSS-AWIKTI	TVLVLLPLLA	VVLNVASQLL	LAT-PKNHPP	VVFHFVPVIG	SAIYYGIDPY	KFFFECREKY
М.	globosa			MLQEIG	AWP-VWQQAL	TFLVGGLALI	VGINVLVQVL	VPR-NKSLPP	MVFHWVPVVG	SAITYGMDPY	RFFFNCREKY
P.	chrysosporium	MSLSQ	YGPIAGLVG-	QAYDALA	SMS-TSRLVL	FLLINIPILS	VVCNVIYQL-	LPK-DKSLPP	VVWHWFPWFG	SAAAYGEDPI	KFFFDCKEKY
A.	cinnamomea	MSLNM	NVSSSEPSW-	AAQAHAQ	LAS-PSRLIL	LFVVNIPLIA	VVFNVLYQLV	MPR-DRTVPP	VVFHLIPWFG	SAAAYGGDPV	EFFKSCREKY
с.	cinereus				MGL-DLVKLG	FFLAVVPVAT	ILLNVLRQLL	FPT-RAPNPP	EVFHWIPIVG	SALQYGNDPI	NFFFKCREKY
с.	cinerea	MSTLAAQLNG	TLPLSADAWS	GFLAQAKQHV	SAL-DSRTFV	LLLINAPVIA	IVLNVLRQLV	IPK-KATDPP	EVFHWIPIVG	SAIQYGNDPL	NFFFKCREKY
с.	neoformans	MSA	IIPQVQQLLG	QVAQFFPPWF	AALPTSLKVA	IAVVGIPALI	IGLNVFQQLC	LPR-RKDLPP	VVFHYIPWFG	SAAYYGEDPY	KFLFECRDKY
P.	graminis	MSS	LIDP	LIEFI	GSFSTFNQIL	IYFLLSITSI	ISINIFNQLA	IPK-DPTTPP	VVFHLFPFIG	SAVSYGIDPY	AFLESCRKKY
Cl	ustal Consens									G54 *	* :::

SRS-1

		110	) 120	) 130	) 140	) 150	) 160	) 170	180	) 190	) 200
C.	albicans	GDVFSFMLLG	KIMTVYLGPK	GHEFVFNAKL	SDVSAEDAYK	HLTTPVFGKG	VIYDCPNSRL	MEQKKFAKFA	LTTDSFKRYV	PKIREEILNY	FVTDE-SFKL
c.	tropicalis	GDVFSFMLLG	KVMTVYLGPK	GHEFIYNAKL	SDVSAEEAYT	HLTTPVFGKG	VIYDCPNSRL	MEQKKFAKFA	LTTDSFKTYV	PKIREEVLNY	FVNDV-SFKT
s.	cerevisiae	GDIFSFVLLG	RVMTVYLGPK	GHEFVFNAKL	ADVSAEAAYA	HLTTPVFGKG	VIYDCPNSRL	MEQKKFVKGA	LTKEAFKSYV	PLIAEEVYKY	FRDSK-NFRL
C.	glabrata	GDIFSFMLLG	RIMTVYLGPK	GHEFIFNAKL	ADVSAEAAYS	HLTTPVFGKG	VIYDCPNHRL	MEQKKFVKGA	LTKEAFVRYV	PLIAEEIYKY	FRNSK-NFKI
А.	gossypii	GDVFAFMLLG	RVMTVYLGTK	GHEFILNAKL	AEVSAEEAYT	KLTTPVFGEG	VVYDCPNHRL	MEQKKFCKNA	LSTEAFRRYV	PMVMDEVRKY	LRTSK-HFMM
υ.	necator	GDIFTFILLG	KKVTVYLGLQ	GNNFILNGKL	KDVNAEEIYT	NLTTPVFGRD	VVYDCPNSKL	MEQKKFMKTA	LTIEAFHSYV	TIIQNEVEAY	INNCVSF
В.	graminis	GNIFTFILLG	KKTTVYLGRQ	GNNFILNGKL	RDVNAEEIYT	VLTTPVFGTD	VVFDCPNSKL	MEQKKFMKAA	LTTEAFRSYV	PIIQNEVKSF	IEKCDDF
М.	fructicola	GDVFTFILLG	KKTTVYLGRK	GNDFILNGKH	KDLNAEEIYT	VLTTPVFGKD	VVYDCPNAKL	MEQKKFMKIG	LSTEAFRSYV	PIIQMEVENF	MKRSSAF
в.	fuckeliana	GDIFTFVLLG	KKTTVYLGRK	GNDFILNGKL	KDLNAEEIYT	VLTTPVFGKD	VVYDCPNAKL	MEQKKFMKIG	LSTEAFRSYV	PIIQMEVENF	MKRSSAF
о.	yallundae	GNIFTFILLG	KKTTVYLGRD	GNEFILNGKI	KDVNAEEIYT	VLTTPVFGKD	VVYDCPNSKL	MEQKKFMKIG	LTTDAFKTYV	PIIQNEVETF	IKRSPEF
о.	acuformis	GDIFTFILLG	KKTTVYLGRN	GNEFILNGKI	KDVNAEEIYT	VLTTPVFGKD	VVYDCPNSKL	MEQKKFMKIG	LTTEAFKTYV	PIIQDEVETF	IKRSAEL
в.	jaapii	GDCFTFILLG	KKTTVYLGTN	GNEFILNGKI	KDVNAEEIYT	VLTTPVFGKD	VVYDCPNAKL	MEQKKFMKIG	LTTEAFKQYV	PIIQDEVETF	IKRSAAF
N.	fischeri B	GDIFTFILLG	KKTTVYLGTK	GNDFILNGKL	RDVCAEEVYS	PLTTPVFGRH	VVYDCPNAKL	MEQKKFVKYG	LTSDALRSYV	PLITDEVESF	VKNSPAF
A.	fumigatus B	GDIFTFILLG	KKTTVYLGTK	GNDFILNGKL	RDVCAEEVYS	PLTTPVFGRH	VVYDCPNAKL	MEQKKFVKYG	LTSDALRSYV	PLITDEVESF	VKNSPAF
А.	clavatus B	GDIFTFILLG	KKTTVYLGTK	GNDFILNGKL	RDVCAEEVYS	PLTTPVFGRH	VVYDCPNAKL	MEQKKFVKFG	LTSDALRSYV	HLITDELESF	VKSSSAF
А.	flavus B	GDIFTFVLLG	KKTTVYLGTK	GNDFILNGKL	RDVCAEEVYS	PLTTPVFGRH	VVYDCPNAKL	MEQK			
А.	nidulans B	GDIFTFVLLG	KKTTVYLGTK	GNDFILNGKL	KDVCAEEVYS	PLTTPVFGRH	VVYDCPNAKL	MEQKKFVKYG	LTSDALRSYV	QLITAEVEDF	AQKSSVF
Р.	marneffei B	GDIFTFILLG	KKTTVFLGTK	GNDFILNAKL	RDVCAEEVYS	PLTTPVFGKH	VVYDCPNSKL	MEQKKFVKFG	LTSEALRSYV	ELITKEVNEY	VANSNAF
T.	stipitatus B	GDIFTFILLG	KKTTVFLGTK	GNDFILNAKL	RNVCAEEVYS	PLTTPVFGKH	VVYDCPNSKL	MEQKKFVKFG	LTSEALRSYV	ELITKEVNEY	VANADAF
v.	inaequalis	GDIFTFILLG	RKTTVYIGTK	GNEFILNGKQ	SHVNAEEIYS	PLTTPVFGSD	VVYDCPNSKL	MEQKKFVKYG	LTTEALKSYV	TLIQQEVEDY	TKRYPQF
М.	graminicola	GDVFTFILLG	KKTTVCLGTK	GNDFILNGKL	KDVNAEEIYS	PLTTPVFGKD	VVYDCPNSKL	MEQKKFVKYG	LTTSALQSYV	TLIAAETRQF	FDRNNPHKKF
₽.	digitatum	GNIFTFILLG	KKTTVYLGVE	GNEFILNGKL	KDVNAEEIYG	KLTTPVFGSD	VVYDCPNSKL	MEQKKFIKYG	LSQEALESYV	PLIADEISSY	IKSSPSF
₽.	italicum	GDIFTFILLG	KKTTVYLGVE	GNEFILNGKL	KDVNAEEVYG	KLTTPVFGSD	VVYDCPNSKL	MEQKKFIKYG	LSQEALESYV	PLIADETNAY	IKSSPNF
N.	fischeri A	GDIFTFILLG	QKTTVYLGVQ	GNEFILNGKL	KDVNAEEVYS	PLTTPVFGSD	VVYDCPNSKL	MEQKKFIKYG	LTQSALESHV	PLIEKEVLDY	LRNSPNF
Α.	fumigatus A	GDIFTFILLG	QKTTVYLGVQ	GNEFILNGKL	KDVNAEEVYS	PLTTPVFGSD	VVYDCPNSKL	MEQKKFIKYG	LTQSALESHV	PLIEKEVLDY	LRDSPNF
А.	clavatus A	GNIFTFILLG	QKVTVYLGVE	GNEFILNGKL	KDVNAEEVYS	PLTTPVFGSD	VVYDCPNSKF	MEQKKFIKFG	LTQSALEAHV	PLIEKEVLDY	LETSPRF
А.	flavus A	GDIFTFILLG	RKITVYLGIQ	GNEFILNGKL	KDVNAEEIYS	PLTTPVFGSD	IVYDCPNSKL	MEQKKFIKFG	LTQAALESHV	PLIEKEVLDY	LKTSPNF
A.	nidulans A	GDIYTEVMLG	KKMTVYMGVK	GNDFILNGKL	KDLNAEEVYS	PLTTPVFGSD	VVYDCPNSKL	MEQKKFIKFG	LSQSALESHV	PLIEKEVEDI	INTSPRF
s.	pombe	GDVFTFVCMG	REMTAFLOVQ	GNDFLFNGKL	ADLNAEEAYS	HLTTPVFGKD	VVIDIPNHVF	MEAKKFIKSG	LGFSQFRSIV	PLILNEMDAF	LSISPDF
0.	maydis	GDVFTFVLLG	RKITVALGPK	GSNLVFNARH	QQVIAEDAII	RETTPVFGKE	VVIDVPNAVP	MEOKDEWKCC	LONENEDMVU	POINDEVREI	INNDARION
M.	globosa	GDVFTFKLFG	RNVIVALGPK	GSNLVFNGRL	TQVSAEEAIT	SLITPVFGKG	VVIDVPNAVL	MEQKREVING	LOWENERGIV	CMIEDEVIOR	INNDAAF LFL
Ρ.	chrysosporium	GNVETEILMG	REVIVALIPA	GNNF IMGGKH	TTESALEVIG	GLTTPVFGKD	VVIDCPNELL	MEQKKEVKEG	LSIENFRQIV	CMIEREMERE	MKCDDAELIN
A.	cinamomea	COVETEILLC	DDITUNICOV	GNDF VEGGRE	TVIAAEDVIQ	HLITPUECKD	UNADADAEKE	MOOVDETVAC	LIVENTREIV	CMIEREVEDE	IEVDBOENTV
C.	cinereus	COVETEILLG	RRIIVALGPK	GNNF I LGGKS	IVENAEDATI	HLITPVIGKD	UUVDUDNEKE	MEOKDEUKUC	TOTENLEATV	CMIEREVECE	INNDBARGUE
с. С	cinerea	CDIETETIMO	REVIVALOPK	CNNL SLOCKT	LVENAEDAIT	UI TTDUECKC	VVIDVPNEKE	MOOVERTVEC	TAMENTURALA	DMITCECEDE	ETKEVCISDO
D.	arominic	CNUETEVIIN	KKUTUDICIE	CNALTINCKI	COUNDEEDVT	ALNETTO	VVIDCPNEML	TEDUVEEVD_	DITESDQ31P	KTOON	VK
C1	yraminis	*· ··* ·	· * ·	* · · · ·	SQVIMAEEAII ** *	* ·	I	LEDRVEERP-		KIQQA	V 11

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						SRS	-2		SRS-3		
		210	220	230	240	250	260	270	280	290	300
с.	albicans	KEKTHG	VANVMKTOPE	ITIFTASRSL	FGDEMRRIFD	RSFAOLYSDL	DEGETPINEV	FPNLPLPHYW	REDAAOKKIS	ATYMKEIKSR	RE
с.	tropicalis	KERDHG	VASVMKTOPE	ITIFTASRCL	FGDEMRKSFD	RSFAOLYADL	DKGFTPINFV	FPNLPLPHYW	RRDAAORKIS	AHYMKEIKRR	RE
s.	cerevisiae	NERTTG	TIDVMVTOPE	MTIFTASRSL	LGKEMRAKLD	TDFAYLYSDL	DKGFTPINFV	FPNLPLEHYR	KRDHAOKAIS	GTYMSLIKER	RK
C.	glabrata	NENNSG	IVDVMVSOPE	MTIFTASRSL	LGKEMRDKLD	TDFAYLYSDL	DKGFTPINFV	FPNLPLEHYR	KRDHAOOAIS	GTYMSLIKER	RE
А.	gossypii	NERSSG	VVNVMETQPE	MTIFTASRSL	LGAEMHSMLD	ADFAYLYADL	DKGFTPLNFV	FRDLPLDNYR	RRDNAQRTIS	STYMKVIERR	RK
υ.	necator	QGESG	TVNISKVMAE	ITIYTASHAL	QGEEVRENFD	SSFAALYHDL	DMGFTPINFT	FYWAPLPWNR	ARDHAQRTVA	RTYMNIIQAR	REE
в.	graminis	RKSKG	IINIDAVMAE	ITIYTASHTL	QGKEVRDRFD	SSLAVLYHDL	DMGFTPINFM	LHWAPLPHNR	ARDHAQRTVA	KIYMEIINSR	RTQ
М.	fructicola	KGQKG	TANIPPAMAE	ITIYTASHTL	QGKEVRDRFD	TSFASLYHDL	DMGFSPINFM	LHWAPLPHNR	ARDHAQRTVA	STYMDIIQKR	RAQ
в.	fuckeliana	KGPKG	TADIGPAMAE	ITIYTASHTL	QGKEVRDRFD	TSFASLYHDL	DMGFSPINFM	LHWAPLPHNR	ARDHAQRTVA	KTYMDIIQNR	RAQ
о.	yallundae	KGHSG	TVNIPAQMAE	ITIYTASHAL	QGKDCRDKFD	SSFAELYHAL	DMGFSPINFM	LHWAPLPHNR	ARDHAQRTVA	KTYMEIMEAR	RKD
о.	acuformis	KGHSG	TVNIPAQMAE	ITIYTASHAL	QGKDCRDKFD	SSFAELYHAL	DMGFSPINFM	LHWAPLPHNR	ARDHAQRTVA	KTYMEIMEAR	RKD
В.	jaapii	KGQSG	TADIPAQMAE	ITIYTASHCL	QGKDVREQFD	SSFASLYHDL	DMGFSPINFM	LHWAPLPHNR	ARDHAQQTVA	RKYMEIMEMR	RKD
N.	fischeri B	QGHKG	VFDVCKTIAE	ITIYTASRSL	QGKEVRSKFD	STFAELYHNL	DMGFAPINFM	LPWAPLPHNR	KRDAAQRKLT	ETYMEIIKAR	RQT
А.	fumigatus B	QGHKG	VFDVCKTIAE	ITIYTASRSL	QGKEVRSKFD	STFAELYHNL	DMGFAPINFM	LPWAPLPHNR	KRDAAQRKLT	ETYMEIIKAR	RQA
A.	clavatus B	QGPKG	VFDVCKTIAE	ITIYTASRSL	QGKEVRNKFD	STFAELYHDL	DMGFAPINFM	LPWAPLPHNR	KRDAAQRKLT	ETYMEIIKAR	RQA
A.	flavus B	KGPNG	VFDVCKTIAE	ITIYTASRSL	QGKEVRSRFD	STFAELYHDL	DMGFAPINFM	LPWAPLPHNR	KRDAAQKRMT	ETYMEIIKER	RKA
A.	nidulans B	QNAKG	VFDVSRTIAE	ITIYTASRSL	QGKEVRDKFD	STFAELYHDL	DMGFAPINFM	LPYAPLPHNR	KRDAAQRKMA	ETYMEIIKER	RKS
₽.	marneffei B	KQLHG	TIDVGKTMAE	ITIYTASRSL	QGKEVRDKFD	STFAELYHDL	DMGFAPINFM	LPWAPLPHNR	KRDAAQKKMA	ETYMEIIRHR	RQA
T.	stipitatus B	KQPRG	TFDVGKIMAE	ITIYTASRSL	QGKEVRDKFD	STFAELYHDL	DMGFAPINFM	LPWAPLPHNR	KRDAAQKKMA	ETYMEIIRKR	RQA
v.	inaequalis	KGEKG	SFDVCASMAE	ITIFTASRSL	QGKEVRDKFD	ASFADLFHDL	DMGFSPINFM	LPWAPLPHNR	RRDAANKKMT	ETYLEIIQSR	KAE
М.	graminicola	ASTSG	TIDLPPALAE	LTIYTASRSL	QGKEVREGFD	SSFADLYHYL	DMGFTPINFM	LPWAPLPQNR	RRDYAQKKMS	ETYMSIIQKR	RES
₽.	digitatum	KGQSG	TIDLVPAMAE	ITTFTAARTL	QGEEVRSKLT	TEFAKLFHDL	DLGFTPINFM	LPWAPLPQNR	KRDRAHRRMR	EIYVDIIQAR	REAGEEANDN
₽.	italicum	KGQSG	TIDLAAAMAE	ITIFTAARTL	QGEEVRSKLT	SEFADLFHDL	DLGFSPINFM	LPWAPLPHNA	SAIKHTTYAR	DLSGNYPSAT	GSWRRRQR-R
N.	fischeri A	QGSSG	QVDISAAMAE	ITIFTAARAL	QGQEVRSKLT	AEFADLYHDL	DKGFTPINFM	LPWAPLPHNK	KRDAAHARMR	SIYIDIINQR	RLD
A.	fumigatus A	QGSSG	RVDISAAMAE	ITIFTAARAL	QGQEVRSKLT	AEFADLYHDL	DKGFTPINFM	LPWAPLPHNK	KRDAAHARMR	SIYVDIITQR	RLD
А.	clavatus A	QGTSG	LVDIAAAMAE	ITIFTAARAL	QGEEVRSKLT	AEFADLYHDL	DRCFTPVNFM	FPWAPLPRNK	KRDAAHVRMR	AIYVDIINQR	RRD
<b>A</b> .	flavus A	KGTSG	RVEITDAMAE	ITIFTAGRAL	QGEEVRKKLT	AEFADLYHDL	DRGFTPINFM	LPWAPLPRNR	KRDAAHARMR	EIYMDIINER	RKN
A.	nidulans A	KGDSG	VLDAPAAMAE	LTIYTAGSAL	QGKEVRKKLT	AEFADLFHDL	EMGFTPINFI	LPWAPLPQNR	KRDIAHARMR	ETYMEIINQR	RKN
s.	pombe	GPGKEG	VADLLKTMPV	MTIYTASRTL	QGAEVRKGFD	AGFADLYHDL	DQGFSPVNFV	FPWLPLPRNR	RRDRAHKIMQ	KTYLKIIKDR	R
0.	maydis	KTRKT-I	TVDIFQAMSE	LIILTASRTL	QGKEVRQGLD	KSFAQLYHDL	DSGFTPINFV	IPNLPLPSNF	KRDRAQKKMS	QFYQDIVAKR	RAA
м.	globosa	QKGATSV	TVDIENVESE	ITILTASRTL	QGKEVRESLD	KTFAKLYHDL	DSGFTPINEV	I PNLPLPNNF.	REDRAQELMS	DFYLGIIKKR	REG
Ρ.	chrysosporium	QM-NDINEWG	AFDVLKVMSE	ITILTASRTL	QGREVRANIT	KDYAQVYNDL	DGGFTPLHFM	FPNLPLESYR	KRDAAHKKIS	DFYISIIRKR	REN
A.	cinnamomea	QM-KDINEWG	TEDAIKILAE	MILLIAARIL	LGREVRESLD	KGFADLISDL	DHGMTPLHWM	FSDLPLPSIR	KRDAAHQKIS	GFIQSILQKR	KRQ
<i>C</i> .	cinereus	QQRNPANEWG	RENAVKIMUE	TTILTASETL	QGREVRSSLT	KEFAQTINDL	DGGLTPLNFL	FPNLPLESIK	REDRAQKEMS	DFTIDIIKKR	KLA
С.	cinerea	QQ-NDVNEWG	TENAVKVMQE	ITILTASETL	QGREIRNGLT	KEFAQIINDL	DGGFTPLNFL	FPNLPLESIK	KRDRAHKKMS	DFIVDIIKRR	RAG
C.	neorormans	RrSA	1 LDLLKAMSE	LILLTASRTL	QGKEVRESLN	GUFARTIEDL	DGGFTPLNFM	FFNLFLFSYK	REDEAUKAMS	DETLKIMENR	RKG
£.	graminis		DURKVASE	. ** *	QGPEVREGLN	NOF ANLIHOL	DGGFTPLHFA	**	REDEAQVAME	NEIMNIIQKR	RED
CI	ustar consens		•	•			M22	20			

		310	320	330	340	350	360	370	380	390	400
с. a	lbicans	RGDID	PNRDLIDSLL	IHSTYKDGVK	MTDQEIANLL	IGILMGGQHT	SASTSAWFLL	HLGEKPHLQ-	DVIYQEVV	ELLKEKG	-GDLNDLTYE
7. t	ropicalis	SGDID	PKRDLIDSLL	VNSTYKDGVK	MTDQEIANLL	IGVLMGGQHT	SASTSAWFLL	HLAEQPQLQ-	DDLYEELT	NLLKEKG	-GDLNDLTYE
в. с	erevisiae	NNDIQ	-DRDLIDSLM	KNSTYKDGVK	MTDQEIANLL	IGVLMGGQHT	SAATSAWILL	HLAERPDVQ-	QELYEEQM	RVLD	-GGKKELTYD
. g	labrata	KNDIQ	-NRDLIDELM	KNSTYKDGTK	MTDQEIANLL	IGVLMGGQHT	SAATSAWCLL	HLAERPDVQ-	EELYQEQM	RVLN	-NDTKELTYD
l. g	ossypii	NNDVQ	-DRDLIDALM	TSAQYKDGVK	MTDQQIANLL	IGVLMGGQHT	SAATSAWVLL	HLAERPDIQ-	EELYEEQM	RVLD	-GGAKELTYE
1. n	ecator	KRSGE	NKHDIMWELM	R-STYKDGTP	VPDREIAHMM	IALLMAGQHS	SSSTSSWIML	WLAARPDIM-	EELYEEQL	RIFGSEK	PFPPLQYE
3. a	raminis	KETDD	SNLDIMWOLM	R-SSYKDGTP	VPDKEIAHMM	IALLMAGOHS	SSSSSTWIML	WLAARPDIT-	EELYQEQL	ELLGSE	LPPLKYE
1. f	ructicola	ATEAE	FKSDIMWQLM	R-SSYKDGTP	VPDKEIANMM	IALLMAGQHS	SSSSISWIML	RLAARPDIM-	EELYQEQI	EVLGAD	LPDLKYE
B. f	fuckeliana	ATEAE	FKSDIMWOLM	R-SSYKDGTP	VPDKEIANMM	IALLMAGOHS	SSSSISWIML	RLASRPDIM-	EELYOEOI	OVLGAD	LPALKYE
0. 1	allundae	KKSLD	N-MDIMSOLM	R-STYKNGVP	VPDMETAHMM	TALLMAGOHS	SSSSSSWIML	RLASEPDIM-	EELYOEOL	EVLGSD	LPPLOYE
0. 1	artornie	KKSLD	N-MDIMSOLM	R-STYKNOVP	VDDMETAHMM	TALIMAGOHS	SSSSSSWIMI.	RLASEPDIM-	FELYOFOL	EVLGSD	LPPLOYD
D. a	iaanii	KKSIF	N-I DIMODIM	R-STYKNOVE	VDDMETAUMM	TALIMACOUS	CCCCCCWITT	PLAAPPOTM-	==FELYOFOV	FOLGAD	LPPLOYE
ы. ј м. 4	iaapii Fiaabami B	CCVKD	C-EDMUMINI M	C-CUVENCED	VEDEETAUMM	TALLMACOUS	CCCTACWIUI	DI ATRODIM-	FEI VOEOT	DVI CS	DI PDI TED
N. 1	ischeri B	GSKKD	C EDMUMNIM	S-CVIRNGIP	VEDEEIAHMM	TALLMACOUC	COCTRONIVI	DIATEDDAM-	EELIQEQI	DULCS	DIRDITYD
A. I	umigatus B	GSKKD	S-EDMVWNLM	S-CVIRNGTP	VPDEEIAHMM	TALLMAGQHS	SSSTASWIVL	RLATRPDIM-	EELIQEQI	RVLGS	DLPPLIID
A. C	clavatus B	GNEKD	S-EDMVWNLM	S-CVIKNGVP	VPDEEIAHMM	TALLMAGQHS	SSSTAAWIVL	RLATRPDIM-	EELIQEQL	RVLGS	DLPPLIID
A. 1	lavus B	GSKKD	S-EDMVWNLM	S-CMYRDGTP	VPDEEIAHMM	TALLMAGQHS	SSSTAAWIVL	HLAASPEIT-	EELIQEQL	RILGH	DMPPLIYE
A. n	nidulans B	GEKKD	S-EDMVWNLM	S-CVYKNGTP	LSDEEIAHMM	IALLMAGQHS	SSSTLSWILL	HLARHPEIV-	EELYQEQL	KVLGS	DMH-MTYD
P. #	narneffei B	GGEKD	S-EDMVWNLM	S-CVYKDGTP	VPDMEVAHMM	IALLMAGQHS	SSSTISWIIL	RLASQPKIT-	EQLYQEQI	RVLGP	DLPPLTHE
T. s	stipitatus B	GGQKD	S-EDMVWNLM	S-CAYKDGTP	VPDIEVAHMM	IALLMAGQHS	SSSTISWIVL	RLASQPKIT-	EQLYQEQI	RVLGA	DLPPLTHE
V. 1	inaequalis	GVKKD	S-EDMIWNLM	Q-CVYKNGTP	IPDKEIAHMM	IALLMAGQHS	SSSTSSWILL	RLATRPDIQ-	EELYQEQI	RVCGA	DLPPLQYE
М. с	raminicola	KTGEH	E-EDMIHNLM	Q-CKYKDGNA	IPDKEIAHMM	IALLMAGQHS	SSATESWITL	RLASRPDIQ-	DELLQEQK	DMLGVNAD	-GSIKELTYA
P. 6	ligitatum	GRDKT	KGTDMISNLM	R-CVYRDGTP	IPDKEIAHLM	ITLLMAGOHS	SASISCWILL	RLASOPEMT-	EKLFAEQV	NNLG	-ADLPPLQYK
P. i	italicum	RODKS	KGTDMISNLM	R-CVYRDGTP	TPDKETAHMM	ITLLMAGOHS.	SSAISCWILL	RLASOPEMA-	EKLHAEOI	KNLG	-ADLPPLOYK
N. 4	fischeri A	GEKDS	OKSDMIWNT.M	N-STYKNGOO	VPDKEIAHMM	ITLLMAGOHS	SSSISAWIMI.	RLASOPKVI	EELYOFOL	ANLGPVGPD-	-GSLPPLOYK
A. 4	fumicature A	GEKD9	OKSDMTWNTM	N-CTYKNGOO	VPDKETAHMM	TTLLMAGONG	SSSISAWIMI	RLASOPKVI -	EELYOEOT	ANLGPAGPD-	-GSLPPLOVK
A .	alavatue A	GG====FDT	OKSDMTWNTM	N=CSYKNCOO	VDDKETAUMM	TTLIMAGOUG	SSSTCSWIMI	RLASOPEVI -	EKI YOFOT	DKLAOGGPG-	-SNLEPLOVE
A. 0	flame A	SSEDI	ETCONTWINDING	H_CTYKNCOD	I DOKETAUMM	TTLIMACOUC	CCCTCCWIML	DIDGEDNU-	FELVOPOT	TKLSPDG	-RTI.PPI.OVP
л. I х	idulan-	PDR	ODUDMININUM	U_CTINNGQP	UDDVETAUM	THITMACONS	CCCTCNMIT!	DIACEDOTT	FEI YOROT	TUDOL DO-	-CAFEDIOVY
A. I	niculans A	PDA	<b>ODHDWIMNTW</b>	n-STIKNGNP	VEDKEIAHIM	I I LLMAGQHS	SSSISAWILL	RLASEPUIL-	RELIGEOL	ANLEKEDPRT-	-GALEPLQIK
S. F	pombe	SSTEN	PGTDMIWTLM	5-CKYRDGRP	LNEHEIAGMM	IALLMAGQHT	SAATIVWVLA	LLGSKPEII-	EMLWEEQK	RVVGEN	DEPKED
υ. п	naydis	GASTSADDAS	GENDMIAALI	E-QKYKNGRA	LSGVEIAHMM	IALLMAGQHT	SSATSSWAFL	KLASKPEII-	EELYEEQL	NVISDG	NGGLKELDYE
М. с	globosa	NTEG	TEHDMISALM	E-QSYKNGRN	INDREIAHMM	IALLMAGQHT	SSATGSWAML	RLASRPEII-	EELYEEQK	RVYSDG	TGGFAPLDYD
P. 0	chrysosporium	PGQE	EEHDMIAALM	N-QKYRVGRP	LKDHEIAHIM	IALLMAGQHT	SSATGSWALL	HIADRPDVA-	EALYEEQV	KHFRQS	DGSWRTPEYE
A. (	cinnamomea	ESE	YEDDVMGSLM	K-QQYRDGRA	LQDHEIAHIL	IALLMAGQHT	SSSSTSWALL	HLADRPDVA-	DALYQEQV	EHFGTP	DGGLRDMTYE
C. 0	cinereus	QVEE	VEHDMIASLS	E-QRYRSGAP	LPDHEIAHIM	IALLMAGQHT	SSATSSWALL	HLAHNVEVAY	VSEALYNEQV	EHFSNP	DGSFRPLTYD
C. (	cinerea	HSDE	TDHDMISALL	E-QRYRNGTA	LKDHEIAHIM	IALLMAGQHT	SSATGAWTLL	HLANNPDVA-	EALYKEQL	EHFTNP	DGSFREMTYD
C. 1	neoformans	ESD	HEHDMIENLO	S-CKYRNGVP	LSDRDIAHIM	IALLMAGOHT	SSATSSWTLL	HLADRPDVV-	EALYQEQK	QKLGNP	DGTFRDYRYE
-			-					UTROPODIU	DELDOROT	BURGKRGOMP	DKELDPLDLE
Clus	stal Consens	NREG	QLGDMIDSLQ *:: *	G-QTYKDGRP *: *	: ::* ::	* :**.***	*:: *	:. :	AELKQEQI	PALOVEGÄID	0100010000
P. g Clus	stal Consens	NREG	QLGDMIDSLQ *:: *	G-QTYKDGRP *: * 	LTDKEIAHIM : ::* ::	IALLMAGQHT * :**.***:	*:: *	:. :	WERKÖRÖI : *	EVEGNEGUID	
P. g Clus	stal Consens	410	QLGDMIDSLQ *:: * 420	G-QTYKDGRP *: * 	LTDREIAHIM : ::* :: ) 440	1ALLMAGQHT * :**.***: 450	SAATGSWLLL *:: *	+LASRPDIV- :. :	AELRQEQI : *	2010 490	500
Clus	lbicans	410 DLQ-KLPSVN	QLGDMIDSLQ *:: * 420 NTIKETLRMH	G-QTYKDGRP *: * SRS-5 430 MPLHSIFRKV	LTDKEIAHIM : ::* :: ) 440 TNPLRIP	450	SAATGSWLLL *:: * 0 460 ETNYIVPKGH	+LASRPDIV- :. : ) 470 YVLVSPGYAH	AELKQEQI : * ) 480 TSERYFDNPE	DFDPTRWDTA	) 500 AAK
2. g Clus C. a C. t	stal Consens blbicans cropicalis	NREG 410 DLQ-KLPSVN DLQ-KLPLVN	QLGDMIDSLQ *:: * 420 NTIKETLRMH NTIKETLRMH	G-QTYKDGRP *: * SRS-5 430 MPLHSIFRKV MPLHSIFRKV	LTDKEIAHIM : ::* :: ) 440 TNPLRIP MNPLRVP	450	SAATGSWLLL *:: * 0 460 ETNYIVPKGH NTKYVIPKGH	HLASRPDIV- :. : 470 YVLVSPGYAH YVLVSAGYAH	AELKQEQI : * ) 480 TSERYFDNPE TSDRWFEHPE	0 490 DFDPTRWDTA HFNPRRWESD	0 500 AAK DTK
F. 9 Clus C. a C. t S. c	stal Consens lbicans ropicalis erevisiae	410 DLQ-KLPSVN DLQ-KLPLVN LLQ-EMPLLN	QLGDMIDSLQ *:: * 420 NTIKETLRMH NTIKETLRMH QTIKETLRMH	G-QTYKDGRP *: * SRS-5 430 MPLHSIFRKV MPLHSIFRKV HPLHSLFRKV	LTDKEIAHIM : ::* :: 0 440 TNPLRIP MNPLRVP MKDMHVP	450	SAATGSWLLL *:: * 0 460 ETNYIVPKGH NTKYVIPKGH NTSYVIPAGY	HLASRPDIV- :. : 470 YVLVSPGYAH YVLVSAGYAH HVLVSPGYTH	AELKQEQI : * ) 480 TSERYFDNPE TSDRWFEHPE LRDEYFPNAH	0 490 DFDPTRWDTA HFNPRRWESD QFNIHRWNKD	0 500 AAK DTK SAS
F. 9 Clus C. a C. a C. t S. c C. g	stal Consens lbicans cropicalis erevisiae labrata	410 DLQ-KLPSVN DLQ-KLPJVN LLQ-EMPLLN DLQ-NMPLLN	QLGDMIDSLQ *:: * 420 NTIKETLRMH NTIKETLRMH QTIKETLRMH QMIKETLRLH	G-QTYKDGRP *: * MPLHSIFRKV MPLHSIFRKV HPLHSLFRKV HPLHSLFRKV	LTDREIAHIM : ::* :: D 440 TNPLRIP MNPLRVP MKDMHVP MRDVAIP	450	SAATGSWLLL     *:: *     GETNYIVPKGH     NTKYVIPKGH     NTSYVIPAGY     NTSYVVPRDY	HLASRPDIV- :. : yulvspgyah yulvsagyah hvlvspgyth hvlvspgyth	AELROEQI : * TSERYFDNPE TSDRWFEHPE LRDEYFPNAH LQEEFFPKPN	0 490 DFDPTRWDTA HFNPRRWESD QFNIHRWNKD EFNIHRWDGD	) 500 AAK DTK SAS AAS
C. a C. t C. t C. c C. g A. g	stal Consens lbicans ropicalis erevisiae Habrata Tossypii	410 DLQ-KLPSVN DLQ-KLPLVN LLQ-EMPLLN DLQ-NMPLLN LLQ-EMPLLN	QLGDMIDSLQ *:: * 0 420 NTIKETLRMH NTIKETLRMH QTIKETLRMH QMIKETLRLH QVIKETLRMH	G-QTYKDGRP *: * MPLHSIFRKV MPLHSIFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV	LTDREIAHIM : ::* :: D 440 TNPLRIP MNPLRVP MRDVAIP TRDMPVP	450	*:: * 0 460 ETNYIVPKGH NTKYVIPKGH NTSYVIPAGY NTSYVVPRDY NTSYVIPKDH	HLASSPDIV- : YVLVSPGYAH YVLVSPGYTH HVLVSPGYTH YVLASPGFCH	AELROEQI : * TSERYFDNPE TSDRWFEHPE LRDEYFPNAH LQEEFFPKPN LSEEYFPNAK	0 490 DFDPTRWDTA HFNPRRWESD QFNIHRWNKD EFNIHRWDGD EFNPHRWDND	) 500 AAK DTK SAS AAS
P. 9 Clus C. a C. t S. c C. g A. g U. n	stal Consens blbicans rropicalis erevisiae habrata rossypii ecator	410 DLQ-KLPSVN DLQ-KLPLVN LLQ-EMPLLN DLQ-NMPLLN LLQ-EMPLLN DLS-KLQLHQ	QLGDMIDSLQ *:: * 0 420 NTIKETLRMH NTIKETLRMH QMIKETLRMH QVIKETLRMH QVIKETLRMH NVLKEVLRLH	G-QTYKDGRP *: * SRS-5 0 430 MPLHSIFRKV MPLHSIFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV APIHSIMRKV	LTDREIAHIM : ::* :: MYPLRIP MNPLRVP MRDVAIP TRDMPVP KNPMIVP	450	*:: * 0 460 ETNYIVPKGH NTKYVIPKGH NTSYVIPAGY NTSYVIPRDY NTSYVIPKDH GTKYVIPTSH	470 YVLVSPGYAH YVLVSPGYAH HVLVSPGYTH YVLASPGFCH VLISSPGCTS	AELROEQI : * TSERYFDNPE TSDRWFEHPE LRDEYFPNAK LSEEYFPNAK QDATFFPDPL	0 490 DFDPTRWDTA HFNPRRWESD QFNIHRWDKD EFNIHRWDGD EFNPHRWDDG KWDPHRWDIG	) 500 AAK DAK SAS AAS SGS
F. 9 Clus C. a C. t S. c C. g A. g U. n B. g	stal Consens lbicans ropicalis erevisiae Habrata rossypii ecator rraminis	410 DLQ-KLPSVN DLQ-KLPLVN LLQ-EMPLLN DLQ-NMPLLN LLQ-EMPLLN DLS-KLQLHQ DLS-KLSLHQ	QLGDMIDSLQ *:: * 420 NTIKETLRMH NTIKETLRMH QMIKETLRHH QWIKETLRHH NVLKEVLRHH NVLKEVLRLH	G-QTYKDGRP *: * SRS-5 MPLHSIFRKV MPLHSIFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV APIHSILRKV	LTDREIAHIM : ::* :: MNPLRIP MKDMHVP MKDMPVP KNPMIVP KNPMIVP	450	*:: * ETNYIVPKGH NTSYVIPAGY NTSYVIPAGY NTSYVIPAGY NTSYVIPKDH GTKYVIPTSH GTSYVIPKTH	112 122 123 123 123 123 123 123	AELROEQI : * TSERYFDNPE TSDRWFEHPE LREYFPNAH LQEEFFPKPN LSEEYFPNAK QDATFFPDPL RDASYFPNPL	D 490 DFDPTRWDTA HFNPRRWESD QFNIHRWNKD EFNIHRWDGD EFNPHRWDDIG KWDPHRWDIG	) 500 AAK DTK SAS AAS AAS SG SG
F. 9 Clus C. a C. t S. c C. g U. n B. g M. f	stal Consens stal Consens ropicalis erevisiae dabrata tossypii tecator rraminis fructicola	410 DLQ-KLPSVN DLQ-KLPIVN DLQ-KLPIVN LLQ-EMPLLN DLS-KLQLHQ DLS-KLSLHQ DLS-KLTLHO	QLGDMIDSLQ *:: * 0 420 NTIKETLRMH 0 VIKETLRMH QVIKETLRMH 0 VIKETLRMH NVLKEVLRLH NVLKEVLRLH NILKETLRLH	G-QTYKDGRP *: * SRS-5 0 430 MPLHSIFRKV MPLHSIFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV APIHSIMRKV APIHSIMRKV	LTDREIAHIM : ::* :: TNPLRIP MKDURVP MRDVAIP TRDMPVP KNPMIVP KNPMIVP TTPMPVS	450	*:: * ETNYIVPKGH NTKYVIPKGH NTSYVIPAGY NTSYVIPAGY NTSYVIPRDH GTKYVIPTSH GTKYVIPTSH	1112 1112	AELROEQI : * TSERYFDNPE TSDRWFEHPE LRDEYFPNAK QDATFFPDPL RDASYFPNPL RDASYFPPEPL	D 490 DFDPTRWDTA HFNPRRWESD QFNIHRWNKD EFNIHRWDGD EFNPHRWDDG KWDPHRWDIG KWDPHRWDIG EWDPHRWDIG	) 500 AAK DTK SAS AAS SG SG SG
E. 4 Clus C. a C. c C. c C. c G. g U. n B. g M. f B. f	lbicans lbicans ropicalis verevisiae labrata tossypii vecator traminis ructicola "uckeliana	410 DLQ-KLPSVN DLQ-KLPSVN LLQ-EMPLLN DLQ-NMPLLN LLQ-EMPLLN DLS-KLQLHQ DLS-KLQLHQ DLS-KLTLHQ DLS-KLTLHQ	QLGDMIDSLQ *:: * 0 420 NTIKETLRMH QTIKETLRMH QTIKETLRHH QVIKETLRHH NVLKEVLRLH NVLKEVLRLH NULKETLRLH NVLKETLRLH	G-QTYKDGRP *: * SRS-5 0 430 MPLHSIFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV APIHSILRKV TPIHSIMRKV TPIHSIMRKV	17DKE1AHIM : ::* :: D 440 TNPLRIP MKDMHVP MKDMHVP TRDMPVP KNPMIVP KNPMPVP TTPMPIS	450	SAATGSWILL *:: * 0 460 ETNYIVPKGH NTSYVIPKGH NTSYVIPKGH NTSYVIPKGH GTKYVIPTSH GTKYVIPTSH GTKYVIPTSH	HLASRPDIV- :. : yVLVSPGYAH YVLVSPGYTH HVLVSPGYTH YVLSSPGCTS SLLAAPGWTS TLMASPGCTS	ALLKODUI : * TSERYFDNE LRDEYFPNAH LQEEFFFKPN LSEEYFPNAH LSEEYFPNAH RDASYFPNPL RDASYFPNPL RDASYFPPAL	0 490 DFDPTRWDTA HFNPRRWESD QFNIHRWNKD EFNIHRWDG EKWDPHRWDIG EWDPHRWDIG EWDPHRWDIG	) 500 AAK DTK SAS AAS AG SG SG SG
F. 9 Clus C. a C. c C. c C. c G. c G. c G. c G. c G. c G. c G. c G	stal Consens libicans ropicalis rerevisiae labrata rossypii recator rraminis fructicola fuckeliana rallundae	4100 DLQ-KLPSVN DLQ-KLPLVN LLQ-EMPLLN DLQ-MPPLLN DLQ-MPPLLN DLS-KLSLHQ DLS-KLSLHQ DLS-KLSLHQ DLS-KLFLHQ DLS-KLFLHQ	QLGDMIDSLQ *:: * 0 4200 NTIKETLRMH NTIKETLRMH QTIKETLRHH QVIKETLRHH NVLKEVLRLHH NVLKEVLRLHH NVLKETLRLH NVLKETLRLH	G-QTYKDGRP *:* SRS-5 ) 43( MPLHSIFRKV MPLHSIFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV APIHSIMRKV TPIHSIMRKV TPIHSIMRKV	17081AHIM : ::* :: ) 4400 TNPLRIP MNDVALPVP MRDVAIP TRDMPVP TRDMPVP TTPMPVS TTPMPIS TTPMPIS	450	SAATGSWILL *:: * 0) 4600 ETNYIVPKGH NTSYVIPKGH NTSYVIPKDH GTKYVIPTSH GTKYVIPTSH GTKYVIPTSH GTKYVIPTSH	HLASRPDIV- :. : ) 47(0 YVLVSPGYAH YVLVSAGYAH HVLVSPGYTH YVLASPGFTH YVLASPGFTS TLMASPGCTS TLMASPGCTS TLMASPGCTS	) 480 TSERYFONPE TSDRWFEHPE LRDEYFPNAK QDATFFPDPL RDASYFPNPL RDASYFPNPL RDAEYFPPLR RDDFFFPAL	D) 490 DFDPTRMDTA HFNPRRWESD QFNIHRWNKD EFNPHRWDGD EFNPHRWDIG EWDPHRWDIG EWDPHRWDIG EWDPHRWDIG	) 500 AAK DAK SAS AAS SG SG SG SG
E. 4 Clus C. at c S. c g g J. n g g f f S. f g g f f S. f g g f f S. f g	Jramin's stal Consens lbicans rcopicalis erzevisiae tlabrata toosypii ecator rraminis Tructicola tuckeliana rallundae uufeorm's	410 DLQ-KLPSVN DLQ-KLPEVN DLQ-EMPLLN LLQ-EMPLLN LLQ-EMPLLN DLS-KLQLHQ DLS-KLSLHQ DLS-KLSTHQ DLS-KLFLHQ DLS-KLFLHQ	QLGDMIDSLQ *:: * 0 420 NTIKETLRMH NTIKETLRMH QTIKETLRMH QWIKETLRHH NULKEVLRLH NULKEVLRLH NULKEVLRLH NULKEVLRLH NULKEVLRLH NULKEVLRLH	G-QTYKDGRP *:* sRS-5 MPLHSIFRKV MPLHSIFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV APIHSILRKV TPIHSIMRKV TPIHSIMRKV TPIHSIMRKV	LTDREIAHIM           :         ::*:*::           O         440           TNPLRIP           MNDLRVP           MRDVAIP           TRDMPVP           KNPMVP           TTPMPS           TTPMPS           TTPMPS           KTPMPVP           KTPMPVS	45(	SAATGSWILL *:: * 0 460 ETNYIVPKGH NTSYVIPRGY NTSYVIPRGY NTSYVIPRGY NTSYVIPRGY GTKYVIPTSH GTKYVIPTSH GTKYVIPTSH GTKFYVPTSH	1	ALLRODY 3 480 TSERYFDNPE TSDRWFEHPE LQEEFFPKPN LSEEYFPNAH LQEEFFPKPN RDASYFPNPL RDASYFPNPL RDDEYFPEAL RDDFYFPDAM	0 490 DFDPTRWDTA HFNPRRWESD GFNIHRWNKD EFNIHRWDGD EFNPHRWDGG EWDPHRWDIG EWDPHRWDIG EWDPHRWDIG KWEPHPWDPE	) 500 AAK DAS AAS AAS SG SG SG S
2. 4 Clus C. 4 C. 5 C. 5 C. 7 C. 7 C. 7 C. 7 C. 7 C. 7 C. 7 C. 7	stal Consens libicans ropicalis erevisiae labrata iossypii ecator rraminis fructicola fructicola fuckeliana allundae ecuformis ioarii	4100 DLQ-KLPSVN DLQ-KLPLVN LLQ-EMPLLN DLQ-MPPLN DLQ-MPPLN DLS-KLSLHQ DLS-KLSLHQ DLS-KLSLHQ DLS-KLFLHQ DLA-KLTKHQ DLA-KLTKHQ	QLGDMIDSLQ *:: * 0 420 NTIKETLRMH NTIKETLRMH QTIKETLRLH QVIKETLRLH NVLKEVLRLH NVLKEVLRLH NVLKEVLRLH NVLKEVLRLH NVLKEVLRLH	G-QTYKDGRP *:* SRS-5 3 430 MPLHSIFRKV MPLHSIFRKV MPLHSIFRKV HPLHSLFRKV HPLHSLFRKV APIHSIMRKV TPIHSIMRKV TPIHSIMRKV TPIHSIMRKV TPIHSIMRKV	17081AHIM : ::* :: 0) 4400 TNPLRIP MNDVAIP TROMPVP TROMPVP TTPMPVS TTPMPVS TTPMPS KTPMPVA KTPMPVA KTPMPVA	450	SAATGSWILL *:: * 0) 4600 ETNYIVPKGH NTSYVIPKGH NTSYVIPKDH GTKYVIPKDH GTKYVIPKSH GTKYVIPTSH GTKYVIPTSH GTKFVVPTSH GTKFVVPTSH GTKFVVPTSH	LLASPETU- ) 47( YVLVSPGYAH YVLVSAGYAH HVLVSPGYTH VVLSSPGTS SLLASPEGTS TLMASPEGTS TLMASPEGTS VLLASPEFSS VLLASPEFSS	ALLKOUI : * TSERYFDNPE TSDRWFEHPE LRDEYFPNAK QDATFFPDPL RDASYFPPL RDDEYFPPAL RDDTYFPDAM RDDTYFPDAM	D) 490 DFDPTRWDTA HFNPRRWESD QFNIHRWNKD EFNPHRWDDG EWDPHRWDIG EWDPHRWDIG EWDPHRWDIG EWDPHRWDIG EWDPHRWDE KWEPHRWDPE	) 500 AAK SAS AAS SG SG SG SG SG
2. § Clus c. a t c g g n g f f B. f y a j	Jainis stal Consens hipotalis erevisiae (labrata tossypii tecator tructicola tuckeliana rallundae cuformis iaapii jisabari P	410 DLQ-KLPSVN DLQ-KLPSVN DLQ-KLPLVN DLQ-MPFLN LLQ-EMPLLN DLS-KLQLHQ DLS-KLSLHQ DLS-KLFLHQ DLS-KLTHQ DLA-KLTKHQ DLA-KLTKHQ DLA-KLTKHQ	QLGDMIDSLQ *:: * 0 420 NTIKETLRMH NTIKETLRMH QTIKETLRHH QWIKETLRLH NULKEULRLH NULKEULRLH NULKEULRLH NULKEULRLH NULKEULRLH NULKEULRLH NULKEULRLH	G-QTYKDGRP *:* SRS-5 J MPLHSIFRKV MPLHSIFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV TPIHSIMKV TPIHSIMKV TPIHSIMKV TPIHSIMKV TPIHSIMKV TPIHSIMKV TPIHSIMKV TPIHSIMKV TPIHSIMKV TPIHSIMKV	LTDREIAHIM           :         ::*:*::           D         440           TNPLRIP           MNDLRVP           MRDWAIP           TRDMPVP           TRDMPVP           TTPMPVS           TTPMPIS           KTTPMPVA           KTPMPVA           KTPMPVA           VENDNUD	450	SAATGSWILL *:: * 0 460 ETNYIVPKGH NTSYVIPRGY NTSYVIPRGY NTSYVIPRDY NTSYVIPRDY GTKYVIPTSH GTKYVIPTSH GTKYVIPTSH GTKFVVPTSH GTKFVVPTSH GTKFVVPTSH GTKFVVPTSH GTKFVVPTSH GTKFVVPTSH	ILASPEDIV- i. i yvLvSpGYAH yvLvSpGYTH HVLVSPGYTH HVLVSPGYTH VVLSSPGCTS SLLAAPGWTS TLMASPGCTS VLLASPGFSS VLLASPGFSS VLLASPGSS	ALLROUT : * TSERYFDNPE TSDRWFEHPE LDEYFPNAH LQEEFFPRNN LSEEYFPNAH LQEFFFPDPL RDASYFPNPL RDDFYFPDAM RDDFYFPDAM RDDFYFPDAM RDEAHFPEPL RDDTYFPDAM	D 490 DFDPTRWDTA HFNPRRWESD GFNTHRWNKD EFNTHRWDGD EFNPHRWDND KWDPHRWDIG EWDPHRWDIG EWDPHRWDIG EWDPHRWDES SWDPHRWDPE SWDPHRWDPE	) 500 AAK DAS AAS AG SG SG SG SG SG S
Clus C. at c g g n g f f y B. f f	Jraminis stal Consens hibicans roppicalis recevisiae labrata cossppii eccator rraminis Tructicola tuckeliana atallundae conformis iaspii fischeri B	410 DLQ-KLPSVM DLQ-KLPSVM LLQ-EMPLLN DLQ-NMPLLN DLQ-KLQLM DLS-KLSLHQ DLS-KLSLHQ DLS-KLSLHQ DLS-KLFLHQ DLA-KLTKHQ DLA-KLTKHQ DLA-KLTKHQ NLQ-KLDLHA	QLGDMIDSLQ *:: * 0 420 NTIKETLRMH NTIKETLRMH QUIKETLRMH NVLKEVLRH NVLKEVLRH NVLKEVLRH NVLKEVLRH NVLKEVLRH KVIKETLRH VUKETLRH	G-OTKHORP *:* SRS-5 433 MPLHSIFRKV MPLHSIFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV APIHSINRKV TPIHSIMRKV TPIHSIMRKV TPIHSIMRKV APIHSINRKV DPIHSINRKV DPIHSITRAV DDINOT	LTDREIAHIM           :         ::*:*::           MNDLRUP           MNDLRUP           MRDVAIP           TRUMPUP           KNEMUVP           TTPMPVS           TTPMPVS           TTPMPVS           TTPMPVS           KTEMPVA           KTEMPVA           KNEMAVD           WUMMUE	450	SAATGSWILL *:: * 0 460 ETNYIVPKGH NTSYVIPKGH NTSYVIPKGH GTKYVIPTSH GTKYVIPTSH GTKYVIPTSH GTKYVIPTSH GTKVVIPTSH GTKVVIPTSH GTKVVIPTSH GTKVVIPTSH GTKVVIPTSH GTKVVIPTSH GTKVVIPTSH	HLASPEDIU- :. : yvLvsGyah HVLVSGYTH HVLVSGYTH HVLVSPGTH VULSSPGTS SLLAPCGTS TLMASPGTS VLLASPGFSS VLLASPGFSS VLLASPGSS NVLSSPGTT	480 TSERVFONDE LRDEYFPNAH LQEEFFPRDAH LSEEVFPNAK UDSEVFPNAL RDASYFPNAL RDASYFPNAL RDASYFPPAL RDDTYFPDAM RDDTYFPDAM RDDTYFPDAM RDEAHFPEPL SSEEHFPNPL	0 490 DFDPTRWDTA HFNPRRWESD QFNIHRWNKD EFNIHRWDND EWDPHRWDIG EWDPHRWDIG EWDPHRWDIG EWDPHRWDE KWEFHRMDPE KWEFHRMDPE EWDPHRWDEN	) 50( AAK DAS AAS SG SG SG SG SG SG SG
Clus C. at c g g n g f f B. f f y B. f f N. f f A. f	Jraminis stal Consens http://www.consens iropicalis erevisiae (labrata toosppii wecator raminis fructicola fructicola fructicola ruckeliana rallundae cuformis isoheri B fruigatus B	410 DLQ-KLPLVN DLQ-KLPLVN DLQ-EMPLLN DLQ-EMPLLN DLQ-EMPLLN DLS-KLGLHQ DLS-KLTHQ DLS-KLTHQ DLA-KLTKHQ DLA-KLTHQ DLA-	QLGDMIDSLQ *:: * 0 420 NTIKETLRMH NTIKETLRMH QTIKETLRMH QVIKETLRMH NVLKEVLRLH NVLKEVLRLH NVLKEVLRLH NVLKEVLRLH KVIKETLRLH KVIKETLRLH KVIKETLRLH	G-QTYKDGRP s:* sRS-5 433 MPLHSIFRKV MPLHSIFRKV MPLHSIFRKV HPLHSLFRKV HPLHSLFRKV APIHSINRKV TPIHSIMRKV TPIHSIMRKV TPIHSIMRKV TPIHSIMRKV PHSIIRAV APIHSIIRAV APIHSIIRAV PHSIIRAV	LTDREIAHIM           :         ::*:*::           O         440           TNPLRIP         MKDMHVP           MRDVAIP         TRDMVP           TRDMVP         TTMPNVS           TTPMPIS         KTPMPVS           KTPMPVS         TMPMVS           KTPMPVS         KTPMPVS           KTPMPVT         KSPMPVP           KNPMAVD         KNPMAVD	450	SARTGSWILL *:: * 0 460 ETNYIVPKGH NTSYVIPKGH NTSYVIPKGH GTSYVIPKTH GTSYVIPKTH GTSYVIPKTH GTKYVIPTSH GTKYVIPTSH GTKVVIPTSH GTKVVIPTSH GTSYVIPTSH GTSYVIPTSH GTSYVIPTSH GTSYVIPTSH	LLASPEDIU- :. : YVLVSBCYAH HVLVSBCYTH HVLVSPCGYTH VVLSSPCGYT VVLSSPCGYT TLMASPCCTS VLLASPCFSS VVLSSPCGYTA NVLSSPCGYTA	ALLROUT 3 480 TSERVFDNPE TSDRWFEHPE LQEDFFPRNAK LQESFFPRAK QDATFFPDPL RDAEYFPPAL RDDEYFPPAL RDDTYFPDAM RDENFFPPAL RSEEHFPNPL RSEEHFPNPL	0 490 DFDPTRWDTA HFNPRRWESD QFNIHRWNKD EFNIHRWNDG EFNIHRWDG EWDPHRWDIG KWEPHRWDIG KWEPHRWDE SWDPHRWDE SWDPHRWDE EWDPHRWDED EWDPHRWDED	) 500 AAK DAS AAS SG SG SG SG SG SG SG SG SG SG
Clus at c g g n g f f f y a j f f f y a j f y a j f f y a j f y a j f f y a j f y a j f y a j f y a j f f y a j f y a	Jraminis stal Consens hibicans roppicalis recevisiae labrata cossppii eccator rraminis Tructicola tuckeliana rallundae cuformis iaspii fischeri B fumigatus B lavatus B	410 DLQ-KLPSVN LLQ-EMPLLN DLQ-KLPIVN LLQ-EMPLLN DLQ-KLQLM DLS-KLSLHQ DLS-KLSLHQ DLS-KLSLHQ DLS-KLSLHQ DLA-KLTKHQ DLA-KLTKHQ DLA-KLTKHQ NLQ-KLDLHA SLQ-KLDLHA SLQ-KLDLHA	QLGDMIDELQ *:: * 0 420 NTIKETLRMH NTIKETLRMH QUIKETLRMH NULKEVLRLH NULKEVLRLH NULKEVLRLH NULKEVLRLH NULKEVLRLH KVIKETLRLH KVIKETLRLH KVIKETLRLH	G-OTKHORP *:* SRS-5 	LTDREIAHIM           :         ::*:*:           O         440           TNPLRIP           MKDMVP           MKDMVP           MRDVAIP           TRDMPVP           KNPMVP           TTPMPVS           TTPMPVS           TTPMPVS           KTPMPVA           KNPMAVD           KNPMAVD           KNPMAVD           KNPMAVD           KNPMAVD	450	SAATGSWILL *:: * 0 460 ETNYIVPKGH NTSYVIPKGH NTSYVIPKDH GTKYVIPTSH GTSYVIPKTH GTKYVIPTSH GTKYVIPTSH GTKFVVPTSH GTKVVIPTSH GTKVVIPTSH GTSVVIPTSH GTSVVIPTSH GTSVVIPTSH GTSVVIPTSH GTSVVIPTSH	HLASPEDIU- :. : yvLvSeyAH HVLVSPGYH HVLVSPGYH HVLVSPGYTH HVLVSPGYTH VLISSPGTS SLLASPGWTS TLMASPGTS VLLASPGFSS VLLASPGSS NVLSSPGYTA NVLSSPGYTA	0 480 TSERVFONPE LRDEYFPNAH LQEEFFFRPN LSEEVFFNAK QDATFFPDPL RDDEVFFPAL RDDEVFFPAL RDDTYFFDAM RDDTYFFDAM RDDTYFFPAM RDEAHFPPL RSEEHFNPL SSEEHFNPL SSEEHFNPL	0 490 DFDPTRMDTA HFNPRRMESD QFNIHRWNKD EFNIHRWDKD EFNIHRWDKD EWDPHRWDIG EWDPHRWDIG EWDPHRWDE KWEPHRWDPE EWNPHRWDEN EWNPHRWDEN EWNPHRWDEN	) 50( AAK DAK SAS AAS SG SG SG SG SG SG SG SG SG SG
2. S Clus c. at c g g n g f f b N. a j f f S. a f f f S. a t c g g n g f f f S. a f f f f f f f f f f f f f f f f f f f	Justilis stal Consens libicans ropicalis exervisis labrata iossypii ecator rmatioala fuckelians allunds cuformis aapii lischeri B umigatus B lavatus B lavatus B lavatus B	410 DLQ-KLPSVN LLQ-EMPSLN LLQ-EMPSLN DLQ-WFPLM DLS-KLSLHQ DLS-KLSLHQ DLS-KLSLHQ DLS-KLSLHQ DLS-KLSLHQ DLA-KLTKHQ DLA-KLTKHQ DLA-KLTKHQ NLQ-KLDLHA SLQ-KLDLHA SLQ-KLDLHA	QLGONIDELQ *::: * 0 420 NTIKETLRMH QTIKETLRMH QVIKETLRLH QVIKETLRLH NVLKEVLRLH NVLKEVLRLH NVLKEVLRLH NVLKEVLRLH KVIKETLRLH KVIKETLRLH KVIKETLRLH	G-OTYKDGRP *:* SRS-5 430 MPLHSIFRKV HPLHSIFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV APIHSILRKV TPIHSIMRKV TPIHSIMRKV TPIHSIMRKV PHSIIRAV APIHSIIRAV APIHSIIRAV APIHSIIRAV APIHSIIRAV	LTDREIAHIM           :         ::*:*:           D         440           TNPLRIP         MKDMHVP           MRDVAIP         KNPMVP           KNPMPVS         TTPMPIS           KTPMPVA         KNPMVP           KNPMVP         KNPMVP           KNPMVP         KNPMVP           KNPMPVE         KNPMVE	450	AARIGSWILL *:: * 0 460 ETNYIVPKGH NTSYVIPKGH NTSYVIPKGH GTKYVIPTSH GTKYVIPTSH GTKYVIPTSH GTKYVIPTSH GTKYVIPTSH GTSVIPTSH GTSVVIPTSH GTSVVIPTSH GTSVVIPTSH GTSVVIPTSH GTSVVIPTSH	LLASPEDIU- :. : YVLVSBCYAH HVLVSBCYTH HVLVSPCYTH VVLSSPCGTS SLLAPGWTS TLMASPCCTS VLLASPCFSS VLLASPCFSS VLLASPCFSS VLLASPCFSS NVLSSPCVTA NVLSSPCVTA	ALLROWI 	0 490 DFDPTRWDTA HFNPRRWESD QFNTHRWNKD EFNTHRWDGD EFNTHRWDGG EWDPHRWDIG KWDPHRWDIG KWDPHRWDIG KWEPHRWDE EWDPHRWDE EWDPHRWDEN EWNPHRWDEN EWNPHRWDEH EWNPHRWDEH	) 500 AAK DTK SAS AAS SG SG SG SG SG SG SG SG SG SG
C. at c g g n g f f g g g g g g g g g g g g g g	Justial Consens lbicans tropicalis erevisiae labrata tossypii tecator tructicola tuckeliana rallundae cuformis isscheri B tunigatus B lavatus B lavatus B lavatus B	410 DLQ-KLPSVN LLQ-EMPLLN LLQ-EMPLLN LLQ-EMPLLN LLQ-EMPLLN LLQ-EMPLLN LLQ-EMPLLN DLS-KLQLHQ DLS-KLTLHQ DLS-KLTHQ DLA-KLTKHQ DLA-KLTKHQ DLA-KLTHN NLQ-KLDLHA NLQ-KLDLHA NLQ-KLDLHA	QLGDNIDSLQ *::: * NTIKETLRMH QTIKETLRMH QTIKETLRH QTIKETLRH QVIKETLRLH NVLKEVLRLH NVLKEVLRLH NVLKEVLRLH KVIKETLRLH KVIKETLRLH KVIKETLRLH KVIKETLRLH	G-OTYKDGRP *:* SRS-5 430 MPLHSIFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV HPLHSLFRKV APIHSIRKV APIHSIRKV APIHSIRKV APIHSIRAV APIHSIRAV APIHSIRAV APIHSIRAV APIHSIRAV	LTDREIAHIM           :         ::*:*::           O         440           TNPLRIP         MKDMHVP           MKDMHVP         KNEMVP           KNEMVPVP         TTPMPVS           TTPMPVS         KNPMAVD           KNPMAVD         KNPMAVD           KNPMPVF         KNPMPVF           KNPMPVF         KNPMPVF	45(	SARTOSWILL *:: * 0 460 ETNYIVPKGH NTSYVVPRDY NTSYVVPRDY NTSYVVPRDY NTSYVVPRDY GTKYVIPTSH GTKYVIPTSH GTKVVIPTSH GTSYVIPTSH GTSYVIPTSH GTSYVIPTSH GTSYVIPTSH	ILASPEDIU- :. : YULVSPGYAH YULVSPGYAH HULVSPGYTH HULVSPGYTH YULSSPGTS SULASPGTS VLLASPGFSS VLLASPGFSS VLLASPGFSS VLLASPGFSS NVLSSPGVTA NVLSSPGVTA NVLSSPGVTA	ALLKQUI TSERYFDPE TSDRWFEHPE LRDEYFPNAH LQEDFFPKPN LSEEVFPNAL RDDRYFPDPL RDDRYFPDAM RDDTYFPDAM RDDTYFPDAM RDDRYFPPPL RSEEHFPNPL RSEEHFPNPL RSEEHFPNPL RSEEHFPNPL	0 490 DEPDTRWDTA HFNPRRWESD QFNITHRWNKD EFNPHRWDGD EFNPHRWDDG EWDPHRWDIG EWDPHRWDIG EWDPHRWDE EWDPHRWDE EWDPHRWDE EWNPHRWDEN EWNPHRWDEN EWNPHRWDEN EWNPHRWDEN	) 500 AAK SAS AAS AAS SG SG SG SG SG SG SG I I PI PI
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с.	glabrata	SSAAGGD	EVDYGFGAIS	KGVSSPYLPF	GGGRHRCIGE	LFAYCQLGVL	MSIFIRTMKW	RYPTEGET	VPPSDFTS	MVTLPTAPAK	IYWEKRHPEQ	KY
A.	gossypii	SVSTGE	KVDYGFGAIS	KGVSSPYLPF	GGGRHRCIGE	GFAYMQLGTI	FSVVVRSMKW	HFPADMKG	VPNPDFTS	MVTLPSEPCR	TAWERRVPDQ	11
υ.	necator	KVLGNDAVDE	KYDYGYGLTS	TGASSPYLPF	GAGRHRCIGE	QFATLQLVTI	MATMVRFFRF	RNIDGKQG	VVKTDYSS	LFSMPLAPAL	IGWEKR	
в.	graminis	GVIGTDMEDE	KFDYGYGLIS	TGAASPYLPF	GAGRHRCIGE	QFATVQLVTI	MATMVRSFKF	HNLDGRNS	VAETDYSS	MFSRPMAPAT	TAWEKRDKKD	KTEC
м.	fructicola	RVIGNDQDEE	FQDYGYGMIS	KGASSPYLPF	GAGRHRCIGE	QFANVQL1T1	MATVVRLFKF.	KNPDGSKD	VIGTDYTS	LFTRPLEPAV	VAWERR	
в.	fuckeliana	RVVGNDQDEE	FQDYGYGMIS	KGASSPYLPF	GAGRHRCIGE	QFATVQLVTI	MATVVRLFKF	KNIDGSKD	VIGTDYAS	LFTRPLAPAV	VAWERR	
0.	yallundae	GVLGTDVEEE	SFDYGYGLIS	KGAKSPYLPF	GAGRHRCIGE	QFANVQLITI	TAVMVRYFKF	KNLDNSGK	VVETDYTS	LFSRPLAPAV	VEYERREKVK	V
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в.	Jaapii	KVLNNQVEEE	TYDYGYGLVS	KGVKSPYLPF	GAGRHRCIGE	QFANVQLVT1	TAIMVRYFRF	RNVDKSTN	VIDTDYTS	LFSRPQAPAV	VEWERRN	
N.	fischeri B	AASAEDDE	KVDYGYGLVN	KGTNSPYLPF	GAGRHRCIGE	QFAYLQLGTI	TAVLVRLFKF	RNLPGVDG	VPDTDYSS	LFSKPLGRSF	VEFERRESAT	KA
A.	fumigatus B	AASAEDDE	KVDYGYGLVS	KGTNSPYLPF	GAGRHRCIGE	QFAYLQLGT1	TAVLVRLFRF	RNLPGVDG	IPDTDYSS	LFSKPLGRSF	VEFERRESAT	KA
Α.	clavatus B	VENDEDEE	KIDYGYGLVN	KGTNSPYLPF	GAGRHRCIGE	QFAYVQLGTI	TAGLARLFKF	RNLPDIEG	IPDTDYSS	LFSKPLGKSV	VQFEKREPAL	KA
Α.	flavus B	AVSSEDEE	KVDYGYGLVT	KGTNSPYLPF	GAGRHRCIGE	QFAYVQLGAI	TAALVRLFKF	SNLPGVQT	LPDTDYSS	LFSKPLGNSK	IQPERREPVT	KA
A.	nidulans B	ANSTEDEE	KIDYGYGLVS	KGTNSPYLPF	GAGRHRCIGE	QFAYVQLITV	TAALVRLFKF	DTVSESDKSS	VPETDYSS	LFSRPAGKCF	VQYEKRNVTT	KA
Ρ.	marneffel B	-KAAEDDE	QVDYGYGLIS	KGAGSPYLPF	GAGRHRCIGE	QFAYVQLGAI	CAALVRQLKF	KNLPNVEG	VPATDYSS	LFSRPWANSV	VQYERRDATT	KA
T.	stipitatus B	-KATEDDE	QVDYGYGLIS	KGAGSPYLPF	GAGRHRCIGE	QFAYVQLGAI	CATLVRLLKF	KNLPGVEG	IPATDYSS	LFSRPWANSV	VEYERRDATT	KA
v.	inaequalis	ISGGENGGEE	KEDYGYGLIT	KGASSPYLPF	GAGRHRCIGE	QFAYMQLNTV	LATQVREFKF	S-LREGES	FPKTDFSS	LFSGPLRPAW	LNWERREKSS	
м.	graminicola	TTALGSIAEE	KEDYGYGLVS	KGAASPYLPF	GAGRHRCIGE	QFAYVQLQTI	TATMVRDFKF	YNVDGSDN	VVGTDYSS	LFSRPLSPAV	VKWERREEKE	EKN
Ρ.	digitatum	RVEAE-DSSD	TVDYGYGAVS	KGTRSPYLPF	GAGRHRCIGE	KFAYLNLGVI	IATLVREFRF	FNPEGMEG	VPDTDYSS	LFSRPMQPAT	VRWEVRS	
P.	italicum	RVEVE-DSSD	TVDYGYGAVS	KGTRSPYLPF	GAGRHRCIGE	KFAYLNLEVI	VATLVREFRF	FNPEGMEG	VPDTDYSS	LFSRPVQPAT	VRWEVRS	
N.	fischeri A	QAAKEQENDE	VVDYGYGAVS	KGTSSPYLPF	GAGRHRCIGE	KFAYVNIGVI	LATIVRHLRL	FNVDGKKG	VPETDYSS	LFSGPMKPSI	IGWEERSKDT	SK
Α.	fumigatus A	QATKEQENDK	VVDYGYGAVS	KGTSSPYLPF	GAGRHRCIGE	KFAYVNLGVI	LATIVRHLRL	FNVDGKKG	VPETDYSS	LFSGPMKPSI	IGWEKRSKNT	SK
А.	clavatus A	QTTKE-DSGE	MVDYGYGAVS	KGTASPYLPF	GAGRHRCIGE	KFAYVNIGVI	LATLVRHLRL	SNMDGKEG	VPATDYSS	LFSGPMKPSI	IQWGKRSNDL	SK
Α.	flavus A	RVEKE-DEED	IVDYGYGTVS	KGTSSPYLPF	GAGRHRCIGE	KFAYVNLGVI	VATMARHMKL	FNVDGKKG	VPATDYSS	MFSGPSKPAI	IGWERRFPEK	S
A.	nidulans A	RPDKEDEEGE	LIDYGYGAVS	KRMSSPYLPF	GGGRHRCIGE	KFAYVNLGVI	VATIVRNLKL	YNVDGKTG	VPATDYSS	MFMGPMKPAV	VGWERRFPAR	S
s.	pombe	VNEDENAE	QIDYGYGLVT	KGAASPYLPF	GAGRHRCIGE	QFAYMHLST1	ISKEVHDYTW	TLIGKVPN	VDYSS	MVALPLGPVK	TAWKRRN	
0.	maydis	MQDSGED	KQDFGFGMIS	TGANSPYLPF	GAGRHRCIGE	QFAYLQIGVI	LATEVRIEKW	HLDSK-	FPDPDYQS	MVVLPSKNGC	AIVLTPRAES	THTD
м.	globosa	MNQTDDA	QEDFGWGMVS	TGANSPYLPF	GAGRHRCIGE	QFAYLQLGT1	ISTEVRAEDW	RLETK-	LPAPDYTS	MAAPLG5-V	NLVFTPRKNK	A
Р.	chrysosporium	YKQYDDAEGA	KVDFGFGLVS	KGTDSPYQPF	GAGRHRCIGE	QFAYLQLGTI	ISTEVRHVEM	RLPETG	VPPPNYHT	MITLPKAPRN	ILYRRRNFD-	
A.	cinnamomea	HRQYDEDG	KVDF1FS	KGTGSPYLPF	GAGRHRCIGE	QFATLQIGVI	ISTEVRKIEM	RLDQP-	FPKPDYAS	MMVIPLRPCQ	ILYRRREPD-	
с.	cinereus	YSTYVDGNGE	KIDYGFGAVS	KGTESPYQPF	GAGKHRCIGE	QFAYLQLGTL	IATIIRKLEL	RVDKV-	-PEHNYHVGS	SVFCCHTPGN	AHMARY	
с.	cinerea	YKTYVDENGE	KIDYGFGAVS	KGTESPYQPF	GAGKHRCIGE	QFAYLQLGVL	ISTVIRKLEL	RVDII-	-PEHNYHT	MIMMPKTPHT	ISTRRRKAD-	KODU
с.	neoformans	MVQYTKAE	QVDYGFGSVS	KGTESPYQPF	GAGRHRCVGE	QFAYTQLSTI	FTYVVRNFTL	KLAVPK	FPETNYRT	WINGSNND-P	VTETLRNAEV	KQEV
P.	graminis	GGETQEE	MVDYGFGMIS	SGANSPFLPF	GAGRHRCIGE	QFAYLQLSTL	GATVIRNCEL	ELVSNQ	FFKPDYTT	MTACLIKLUD	VETRENTHS	
Cl	ustal Consens		*:: .	. **: **	· . * : * * * : * *	** :: .:	:		::			

FIG. 1. Sequence alignment of fungal CYP51 proteins. The ClustalX (version 1.8) program was used to construct the sequence alignment from selected fungal CYP51 proteins deposited in the ExPASy protein database (http://www.expasy.ch/), as detailed in the Materials and Methods section. SRSs were annotated on the basis of an alignment of the CYP51 proteins performed by Strushkevich et al. (38). The four main AF51A mutation sites associated with azole resistance (G54, L98, M220, and G448) in A. fumigatus are labeled along with the heme-thiolate-forming cytseine residue (C<sub>H</sub>454). Clustal consensus sequence indicates absolutely conserved residues (\*), conserved strong (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW) groups (:), and conserved weaker (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY) groups (.) (http://www.clustal.org/).



FIG. 2. Phylogenetic tree of fungal CYP51 proteins. The ClustalX (version 1.8) and Treeview (version 1.6.1) programs were used to construct the phylogenetic tree from selected fungal CYP51 protein sequences deposited in the ExPASy proteomics database (http://www.expasy.ch/), as detailed in the Materials and Methods section.

(8), with the modification that 0.1% (wt/vol) L-histidine in 0.1 M Tris-HCl (pH 8.1) and 25% (wt/vol) glycerol were used to elute nonspecifically bound *E. coli* proteins after the salt washes. The AF51A and AF51B proteins were eluted with 1% (wt/vol) L-histidine in 0.1 M Tris-HCl (pH 8.1) and 25% (wt/vol) glycerol. The Ni<sup>2+</sup>-NTA agarose-purified AF51A and AF51B proteins were used for all subsequent spectral determinations. The AF51A protein was also isolated as a membrane suspension for sterol binding experiments by omitting the sodium cholate from the sonication buffer and then resuspending the membrane pellet recovered after ultracentrifugation in 0.1 M Tris-HCl (pH 8.1) and 25% (wt/vol) glycerol. Protein purity was assessed by SDS-polyacrylamide gel electrophoresis using staining intensity analysis with the UTHSCSA ImageTool (version 3.0) program (http://ddsdx.uthscsa.edu/dig/itdesc.html).

**Determination of cytochrome P450 protein concentrations.** Reduced carbon monoxide difference spectra (12) were used to determine cytochrome P450 concentrations using an extinction coefficient of 91 mM<sup>-1</sup> · cm<sup>-1</sup> (34) for the absorbance difference between 448 and 490 nm. In this method, carbon monoxide is passed through the cytochrome P450 solution prior to the addition of sodium dithionite to the sample cuvette. Absolute spectra between 380 and 700 nm were determined using 1 µM native AF51A and AF51B in 0.1 M Tris-HCI (pH 8.1) and 25% (wt/vol) glycerol for the oxidized protein, the 10 mM sodium dithionite reduced protein, and the reduced carbon monoxide-P450 complex, as described previously (8). The spin state of P450 samples was estimated from the ratio  $\Delta A_{393.470} \Delta A_{417.470}$  as previously described by Lepesheva et al. (25). All spectral determinations were made using an Hitachi U-3310 UV-visible spectrophotometer (San Jose, CA), and quartz semimicrocuvettes with a light path of 10 mm was used for the CO difference spectra.

Sterol binding properties. Lanosterol and eburicol (0.05%, wt/vol) were solubilized in a 1:10 mixture of chloroform-acetone containing 0.5% (vol/vol) Tween 80, prior to evaporation to dryness under nitrogen. The Tween 80-sterol residue was then dissolved in water to produce a stock 0.05% (wt/vol) aqueous sterol solution used for substrate binding experiments. Lanosterol and eburicol were progressively titrated against 10  $\mu$ M AF51B and 2  $\mu$ M AF51A in 50 mM potassium phosphate (pH 7.5) and 10% (wt/vol) glycerol in the sample cuvette, and equivalent amounts of 5% (vol/vol) Tween 80 were added to the reference cuvette also containing 10  $\mu$ M AF51B or 2  $\mu$ M AF51A. The absorbance difference spectrum between 500 and 350 nm was determined after each incremental addition of sterol. The sterol saturation curves were constructed from the difference in the absorption between 385 and 419 nm ( $\Delta t_{385-419}$ ) including corrections for changes in sample volume. The substrate binding constants ( $K_s$ ) were determined by nonlinear regression (Levenberg-Marquardt algorithm) using the Hill equation:  $\Delta t = \Delta t_{max}/(1 + K_s/[sterol]^n)$ , where *n* is the apparent Hill number. Sterol binding experiments using *E. coli* membrane suspensions containing 1  $\mu$ M AF51A were also performed.

Azole binding properties of AF51A and AF51B proteins. Binding of azole antifungal agents to the AF51A and AF51B proteins was performed as described previously (23) using split cuvettes with a 4.5-mm path length and with dimethyl sulfoxide (DMSO) also added to the cytochrome P450-containing compartment of the reference cuvette. Stock 0.05-mg  $\cdot$  ml<sup>-1</sup> solutions of clotrimazole, flucon-azole, itraconazole, posaconazole, and voriconazole were prepared in DMSO. The azole was progressively titrated against 1  $\mu$ M either AF51A or AF51B protein in 0.1 M Tris-HCl (pH 8.1) and 25% (wt/vol) glycerol. Difference spectra between 500 and 350 nm were determined after each incremental addition of azole. Binding saturation curves were constructed from the change in the absorbance between the spectral peak and the trough against the azole concentration. A rearrangement of the Morrison equation [ $\Delta A = \Delta A_{max} \times \{(E_t + azole concentration + K_d)^2 - (4 \times E_t \times azole concentration)^{0.5}/(2 \times E_t)$  (26, 32), where  $\Delta A_{max}$  is the maximum change in

absorbance difference and  $E_t$  is the total concentration of CYP51 available to bind azole] was used to determine  $K_d$  (dissociation constant) values when ligand binding was tight. Tight binding is observed when the  $K_d$  for the ligand is similar to or less than the concentration of CYP51 present (10). The Michaelis-Menten equation  $[\Delta A = (\Delta A_{\text{max}} \times \text{azole concentration})/(K_d + \text{azole concentration})]$  was used where the ligand binding was no longer tight, and the Hill equation  $[\Delta A = \Delta A_{\text{max}}/(1 + K_d/[\text{azole}]^n)]$  was used when binding appeared to be allosteric.

Sequence alignment and phylogenetic analysis of fungal CYP51 proteins. An alignment of 37 selected fungal CYP51 protein sequences deposited in the ExPASy protein database (http://www.expasy.ch/) was constructed using the ClustalX (version 1.8) program (http://www.clustal.org/) and a phylogenetic tree from the Phylip-dnd file using the Treeview (version 1.6.1) program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The CYP51 sequences (ExPASy protein database accession number) used were those of Antrodia cinnamomea (A8DBU6), Ashbya gossypii (Q759W0), Aspergillus clavatus CYP51A (A1CGZ8), Aspergillus clavatus CYP51B (A1CD61), Aspergillus flavus CYP51A (B8N2C8), Aspergillus flavus CYP51B (B8NFL5), Aspergillus fumigatus CYP51A (Q4WNT5), Aspergillus fumigatus CYP51B (Q96W81), Aspergillus nidulans CYP51A (Q9P462), Aspergillus nidulans CYP51B (Q5ATU7), Blumeria graminis (Q7Z7R3), Blumeriella jaapii (Q1KX12), Botryotinia fuckeliana (Q9P428), Candida albicans (P10613), Candida glabrata (P50859), Candida tropicalis (P14263), Coprinopsis cinerea (A8NUS3), Coprinus cinereus (Q68HC4), Cryptococcus neoformans (AAF35366), Malassezia globosa (A8Q3I7), Monilinia fructicola (B3VMU9), Mycosphaerella graminicola (Q5XWE5), Neosartorya fischeri CYP51A (A1CXT9), Neosartorya fischeri CYP51B (A1DC38), Oculimacula acuformis (Q9UV04), Oculimacula yallundae (Q9HDH9), Penicillium digitatum (Q9P340), Penicillium italicum (Q122664), Penicillium marneffei CYP51B (B6Q8Q4), Phanerochaete chrysosporium (B6DX27), Puccinia graminis (PGTG07202; http://www.broad.mit.edu /annotation/genome/puccinia graminis), Saccharomyces cerevisiae (P10614), Schizosaccaromyces pombe (Q09736), Talaromyces stipitatus (B8M0V2), Uncinula necator (O14442), Ustilago maydis (P49602), and Venturia inaqualis (Q9P330).

Data analysis. Curve fitting of the numerical data was performed using the computer program ProFit (version 5.0.1; QuantumSoft, Zurich, Switzerland). Protein-targeting signal peptide prediction was performed using Predotar (http://urgi.versailles.inra.fr/predotar/predotar.html), SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/), and TargetP1.1 (http://www.cbs.dtu.dk/services/TargetP/) software.

**Chemicals.** All chemicals, unless otherwise stated, were obtained from Sigma Chemical Company (Poole, United Kingdom). Voriconazole was supplied by Discovery Fine Chemicals (Bournemouth, United Kingdom). Growth media, sodium ampicillin, IPTG, and 5-aminolevulenic acid were obtained from Foremedium Ltd. (Hunstanton, United Kingdom). Ni<sup>2+</sup>-NTA agarose affinity chromatography matrix was obtained from Qiagen (Crawley, United Kingdom). Eburicol was synthesized and purified by W. David Nes (Texas Tech University). Posaconazole was a gift from Schering-Plough Ltd. (Welwyn Garden City, United Kingdom).

# **RESULTS AND DISCUSSION**

Sequence alignment and phylogenetic analysis of fungal CYP51 proteins. Alignment of 37 fungal CYP51 protein sequences (Fig. 1) indicated that 47 residues were conserved, with the heme-binding domain (S442 to A460 in AF51A and S451 to A469 in AF51B) being the most highly conserved region. The level of sequence conservation among the putative fungal CYP51 substrate recognition sites (SRSs) was relatively low for all sites with the exception of SRS-4, suggesting that sequence alignment may not necessarily be a good means of predicting the residues associated with substrate binding. Mutation of the G54 residue in AF51A is associated with itraconazole and posaconazole cross-resistance (27), whereas mutation of the G448 residue confers voriconazole and ravuconazole cross-resistance (37). Mutation of the L98 and M220 residues in AF51A is associated with cross-resistance to all four azoles (37). G448 is conserved in all 37 fungal CYP51 proteins, with G54 being conserved in



FIG. 3. Absolute and reduced carbon monoxide spectra of AF51A and AF51B proteins. The absolute spectra of purified AF51A (A) and AF51B (B) were determined in the oxidized resting state (line 1), the one-electron dithionite reduced state (line 2), and the reduced state bound to carbon monoxide (line 3). Carbon monoxide difference spectra (12) are shown as insets to the main panels. Protein dilutions equivalent to 1  $\mu$ M CO-P450<sub>red</sub> were chosen for comparison. Spectral determinations were made using quartz semimicrocuvettes with path lengths of 4.5 mm for absolute spectra and 10 mm for CO difference spectra.

all fungal CYP51 proteins except A. flavus CYP51B. L98 is conserved in 26 of the 37 fungal CYP51 proteins, including AF51B and C. albicans CYP51, while M220 is conserved in only 24 of the fungal CYP51 proteins, including AF51B but not C. albicans CYP51. Further investigations are required to resolve the apparent contradiction that nearly all the residues outside the heme-binding domain associated with azole resistance in C. albicans CYP51 (28) are not widely conserved among other fungal CYP51 proteins, in contrast to the case for the residues associated with azole resistance in AF51A, which are more widely conserved. This observation suggests that in C. albicans CYP51, altering the internal three-dimensional structure of the substrate/inhibitor binding pocket and access channel is important in conferring azole resistance, whereas for AF51A, the two most frequent mutation sites (G54 and M220) are thought to interfere with the entry of azole drugs into the hydrophobic access tunnel (9, 27, 37). Such a hydrophobic access tunnel (9) immersed in the endoplasmic reticulum membrane would allow highly lipophilic sterol substrates and azoles to enter the CYP51 active site directly from the lipid bilayer and restrict access to foreign metabolites from the cytosol. The amino acids that interact with either bound azole drug or sterol substrate differ between CYP51 proteins (9, 36, 38), with most involving hydrophobic interactions between substrate/azole and the amino acid side chains lining the internal substrate binding pocket and access channel in conjunction with weaker van der Waals interactions; an exception is the direct coordination of the azole ring nitrogen to the heme ferric iron. The degree of conformational change induced by ligand



FIG. 4. Eburicol and lanosterol binding properties of AF51A and AF51B. Eburicol and lanosterol bound weakly to 1  $\mu$ M AF51A in an *E. coli* membrane suspension (A). Line 1, the baseline; line 2, difference spectrum obtained with 4.8  $\mu$ M eburicol; line 3, difference spectrum obtained with 6.2  $\mu$ M lanosterol. (B) The eburicol (filled circles) and lanosterol (hollow circles) saturation curves for AF51A were constructed from the difference in absorbance at 395 and 419 nm. (C) Eburicol and lanosterol were progressively bound to 10  $\mu$ M purified AF51B to produce type I difference in absorbance at 385 and 419 nm. The Hill equation was used to fit the binding data. No reproducible type I difference spectra were obtained using 2  $\mu$ M purified AF51A with either eburicol or lanosterol.

binding also differs between CYP51 proteins, with azole binding to human CYP51 causing a large structural change in the relative positions of the B', F, F', and G helices (38), whereas azole binding to the *Trypanosoma brucei* and *Trypanosoma cruzi* CYP51 proteins induces far fewer structural changes (10).

Phylogenetic analysis of the 37 fungal CYP51 proteins included (Fig. 2) indicated that AF51A had the greatest homology with the CYP51A proteins of other Aspergillus species, with which it had 74 to 81% sequence identities, and to the closely related species N. fischeri CYP51A, with which it shared 96% sequence identity. Similarly, AF51B had the greatest homology with the CYP51B proteins of other Aspergillus species (79 to 89% sequence identity) and shared the greatest sequence identity (98%) with N. fischeri CYP51B. AF51B had 78% and 79% identities with the CYP51B proteins of the closely related eurotiomycetes P. marneffei and T. stipitatus. AF51A had 66 and 68% identities with the CYP51 proteins of the closely related eurotiomycetes P. italicum and P. digitatum. However, the sequence identity between AF51A and AF51B was only 63%. Sequence identities with the S. cerevisiae and C. albicans CYP51 proteins were 49 and 47%, respectively, for AF51A and 50 and 47%, respectively, for AF51B, whereas the sequence identities with human CYP51 were only 38 and 39% for AF51A and AF51B, respectively. Before the discovery of CYP51 isoenzymes in A. fumiga*tus* (29) and among other ascomycete filamentous fungi (http: //www.expasy.ch/), the presence of more than one CYP51 gene was a characteristic of higher plant species, such as arabidopsis and poplar (33, 35; http://www.expasy.ch/). Recently, the genomes of three *Fusarium* species (*F. oxysporum*, *F. verticilloides*, and *F. graminearum*) have been published (http://www.broadinstitute.org/annotation/genome /fusarium\_graminearum/GenomesIndex.html). Preliminary annotation has indicated the presence of three CYP51 genes in each *Fusarium* species, with one gene being closely related to AF51A (65 to 68% identity), a second gene being closely related to AF51B (65% identity), and a third gene (*CYP51C*) having 49 to 51% identity to AF51A and 52 to 57% identity to AF51B.

Heterologous expression and purification of recombinant proteins. AF51A and AF51B were expressed in *E. coli* with the protein localized in the membrane fraction. This was in agreement with predictions made using the Predotar, SignalP3.0, and TargetP1.1 programs that AF51A and AF51B were membrane-bound proteins located in the endoplasmic reticulum with N-terminal membrane anchors of 1 to 26 and 1 to 33 amino acid residues, respectively. Protein isolation by cholate extraction using sonication (3) gave yields of 17 ( $\pm$ 6) and 134 ( $\pm$ 31) nmol per liter culture for AF51A and AF51B, respectively. The levels of expression of AF51B were comparable to



FIG. 5. Azole binding properties of AF51A and AF51B proteins. Azole antifungals were progressively titrated against AF51A (filled circles) and AF51B (hollow circles). The resultant type II difference spectra obtained with clotrimazole (A) and fluconazole (B) are shown. Saturation curves for clotrimazole (C) and fluconazole (D) were constructed. The Hill equation was used to fit the fluconazole data, the Michaelis-Menten equation was used to fit the AF51A clotrimazole data, and the Morrison equation was used to fit the tight binding observed for clotrimazole with AF51B. Native P450 concentrations of 1  $\mu$ M, as determined by CO-P450 difference spectroscopy (12), were used.

those achieved for C. albicans CYP51 in the pCWori<sup>+</sup> vector (7, 24); however, the 8-fold lower yield observed with AF51A was in part due to the instability of the solubilized AF51A protein during the measurement of the reduced CO difference spectrum. Purification by Ni<sup>2+</sup>-NTA agarose chromatography resulted in 36% and 54% recoveries of native AF51A and AF51B, respectively, with most of the lost CYP51 protein being recovered by inclusion of 2% (wt/vol) sodium cholate in the elution buffer, indicating a hydrophobic interaction with the column matrix. SDS-polyacrylamide gel electrophoresis confirmed that the purities of Ni2+-NTA agarose-purified AF51A and AF51B were greater than 90% when the purity was assessed by the staining intensity. Apparent molecular masses of 58.9 kDa were obtained for both proteins and were close to the predicted values of 58,888 and 59,751 Da for AF51A and AF51B, respectively, including the 6-histidine Cterminal extensions.

Spectral properties of recombinant CYP51 protein. The absolute spectra (Fig. 3) and reduced CO-P450 difference spectra (Fig. 3, insets) of AF51A and AF51B were characteristic of those for a native cytochrome P450 enzyme (6, 14). AF51A was predominantly in the ferric low-spin state with a heme iron Soret ( $\gamma$ ) band at 419 nm, in addition to  $\alpha$ ,  $\beta$ , and  $\delta$  bands at 575, 539, and 350 nm, respectively. AF51B was also predominantly in the ferric low-spin state with a heme Soret peak at 419 nm, in addition to  $\alpha$ ,  $\beta$ , and  $\delta$  bands at 568, 539,

and 349 nm, respectively. A dithionite one-electron reduction caused a red shift of the Soret peak to 421 nm for AF51A, whereas it caused a blue shift to 415 nm for AF51B. Binding carbon monoxide to the dithionite-reduced ferrous form of AF51A did not produce the characteristic red shift of the Soret band from 419 to 448 nm but produced a small red shift to 423 nm instead, indicating the formation of the inactive P420 complex with CO. Carbon monoxide binding to ferrous AF51B, in contrast, did result in the characteristic red shift of the Soret peak to 448 nm. The carbon monoxide difference spectra (12), where dithionite was added to the sample cuvette just prior to measurement, did give characteristic Soret spectral peaks at 450 and 448 nm for the AF51A and AF51B proteins, respectively (Fig. 3, insets), although the AF51A spectral peak at 450 nm quickly degraded to inactive P420 over 3 min, whereas the reduced AF51B-CO complex remained stable.

Substrate binding properties. Progressive titration of 10  $\mu$ M AF51B with eburicol gave a strong type I difference spectrum (Fig. 4C) with a peak at 385 nm and a trough at 419 nm, indicating a change in spin state from low to high spin. The absolute spectra of AF51B indicated that the presence of 35  $\mu$ M eburicol (data not shown) caused a 10% increase in the high-spin fraction of AF51B from 35 to 45%. The spin state change occurs due to substrate binding to the apoprotein displacing the water molecule coordinated as the sixth ligand to

	T	ype II differe	ence spectrun	n (nm)					
Azole		AF51A							
	$\lambda_{\text{peak}}$	$\lambda_{trough} 1$	$\lambda_{trough} 2^a$	$\overline{\lambda_{peak}}$	$\lambda_{trough}$				
Clotrimazole	429	386	None	428	407				
Fluconazole	$427 \rightarrow 418$	376	438	428	403				
Itraconazole	$421 \rightarrow 418$	375	437	426	397				
Posaconazole	$424 \rightarrow 419$	376	438	427	404				
Voriconazole	$419 \rightarrow 417$	373	436	427	406				

 TABLE 1. Type II difference spectra characteristics of AF51A and AF51B bound to azole antifungal agents

 $^{a} \lambda_{trough}^{2}$  is a second spectral trough obtained only when AF51A was bound to triazole antifungal compounds.

the low-spin hexacoordinated heme, resulting in the adoption of the high-spin pentacoordinated heme conformation (14). The substrate molecule does not coordinate with the heme prosthetic group. The Hill equation best fitted the eburicol saturation curve (Fig. 4D), yielding a  $K_s$  value of 22.6  $\pm$  0.9 µM. The relatively small change in spin state caused by the binding of sterol was previously observed for other CYP51 enzymes (2, 7, 16, 25), with spin state changes usually not exceeding 10%. Lanosterol binding to AF51B gave a much weaker type I binding spectrum, although with a higher affinity  $(K_{\rm s}, 8.6 \pm 1.02 \ \mu {\rm M})$ . The absolute spectra of AF51B in the presence and absence of 70 µM lanosterol indicated that a less than 1% change in spin state had occurred. The  $K_s$  value obtained for lanosterol was comparable to the  $K_m$  value of 7.4 µM for lanosterol reported for C. albicans CYP51 in metabolism studies (39) but 2-fold higher than the  $K_m$  value for S. cerevisiae CYP51 (20). Further investigations are required to fully characterize the substrate binding specificity of AF51B. No reproducible type I binding spectra could be obtained with either lanosterol or eburicol using 2 µM purified AF51A, in part probably due to the apparent instability, as judged by the CO difference spectrum, of AF51A in free solution. However, weak type I binding spectra were obtained with both eburicol  $(K_s, 4.1 \pm 1.2 \,\mu\text{M})$  and lanosterol  $(K_s, 3.1 \pm 1.3 \,\mu\text{M})$  using an E. coli membrane suspension containing 1 µM recombinant AF51A protein (Fig. 4A and B), suggesting that incorporation into a lipid membrane stabilizes the AF51A tertiary structure. This result for AF51A and AF51B is in agreement with the sterol pathway producing eburicol before sterol 14- $\alpha$  demethylation by CYP51.

Azole binding properties. Clotrimazole bound to AF51A and AF51B, producing type II difference spectra (Fig. 5A) caused by the imidazole ring N-3 nitrogen coordinating as the sixth ligand with the heme iron (15). However, spectral differences between AF51A and AF51B were evident, with the spectral trough of AF51A being at 386 nm and that for AF51B being at 407 nm (Table 1). The trough at 386 nm indicated that AF51A was predominantly in the high-spin conformation prior to binding to the azole ligand to form the low-spin CYP51azole complex (14). This suggests that clotrimazole preferentially binds to the high-spin conformation of the AF51A protein. We have previously shown that voriconazole preferentially binds to the high-spin form of Mycobacterium smegmatis CYP164A2 (40). The trough at 407 nm indicated that AF51B was predominantly in the low-spin conformation prior to binding to the clotrimazole ligand (14). Fluconazole bound to AF51A and AF51B, producing spectra that, while being type II in nature, were significantly different from each other (Fig. 5B). AF51B gave the characteristic type II spectrum of a predominantly low-spin P450 binding to an azole ligand with a peak at 428 nm and a trough at 403 nm. Similar spectra were observed with AF51B binding to itraconazole and posaconazole (data not shown). AF51A, however, had a spectral peak that became progressively blue shifted from 427 to 418 nm as the fluconazole concentration increased from 1 to 250  $\mu M$  and two troughs at 376 and 438 nm, respectively. The first trough at 376 nm indicated that prior to binding to fluconazole, AF51A was predominantly in the high-spin conformation, and the second trough at 438 nm in conjunction with the progressively blue-shifted spectral peak indicated the existence of two distinct perturbations of the heme environment within AF51A during fluconazole binding, suggesting the possibility of two binding orientations for fluconazole molecules with the AF51A heme prosthetic group. Chen et al. (9) have recently demonstrated two binding conformations of fluconazole and posaconazole in T. brucei and T. cruzi CYP51 proteins. Itraconazole and posaconazole gave spectra (data not shown) with AF51A similar to the spectrum of fluconazole. The azole binding saturation curves (Fig. 5C and D) indicated profound differences between AF51A and AF51B. AF51A had relatively low affinities for clotrimazole, itraconazole, and posaconazole, obeying the Michaelis-Menten binding model with  $K_d$  values of 4.8, 1.0, and 2.7 µM, respectively. In contrast, AF51B tightly bound to clotrimazole, itraconazole, and posaconazole, obeying the

TABLE 2. Azole binding properties of AF51A and AF51B proteins

		AF51A		AF51B						
Azole	$\Delta A_{\max}^{a}$	$K_d \; (\mu \mathrm{M})$	Apparent Hill no. <sup><i>a,b</i></sup>	$\Delta A_{\rm max}$	$K_d \; (\mu \mathrm{M})$	Apparent Hill no.	Tight-binding $K_d \ (\mu M)^c$			
Clotrimazole	0.05628	4.79 (±0.27)	1.00	0.01679	$NA^d$	NA	$0.103(\pm 0.007)$			
Fluconazole	0.02697	$11.93(\pm 0.99)$	0.53	0.01143	$4.03(\pm 0.25)$	0.51	ŇA			
Itraconazole	0.01880	$1.01(\pm 0.08)$	1.00	0.01222	NA	NA	$0.031(\pm 0.005)$			
Posaconazole	0.02494	$2.69(\pm 0.11)$	1.00	0.01263	NA	NA	$0.073(\pm 0.006)$			
Voriconazole	0.02379	3.63 (±0.12)	0.70	0.00990	$0.423(\pm 0.031)$	0.99	0.429 (±0.057)			

<sup>*a*</sup> The standard deviations of the  $\Delta A_{\text{max}}$  and apparent Hill number values were less than 5%.

<sup>b</sup> An apparent Hill number of 1.00 indicates that the Michaelis-Menten equation gave the best fit to the binding data.

<sup>c</sup> Tight-binding  $K_d$  values for AF51B were calculated using the Morrison equation.

<sup>d</sup> NA, not applicable.

MIC ( $\mu g \cdot ml^{-1}$ ) Mutant Wild type Azole CEA10 and ΔAF51B  $\Delta AF51A$ AF293 CM-237  $>100^{b} (>327)$ 6.2-12.5<sup>b</sup> (20-41)<sup>c</sup> 32-64<sup>d</sup> (105-210)  $>100^{b}(>327)$ Fluconazole Itraconazole  $0.037 - 0.075^{b}$  (0.053 - 0.107)  $0.6^{b}(0.85)$  $0.6^{b}(0.85)$  $0.5^{e}(0.71)$  $0.5^{g}(0.72)$ ND/ ND ND Posaconazole  $0.12 - 0.50^{h} (0.34 - 1.44)$  $0.52^{h}(1.49)$  $0.25^{e} - 0.50^{g} (0.72 - 1.44)$ Voriconazole ND

TABLE 3. MICs for azole antifungals with A. fumigatus<sup>a</sup>

<sup>a</sup> Previously published MICs for wild-type A. funigatus strains and CYP51-knockout mutants were correlated. The molecular weights of fluconazole, itraconazole, posaconazole, and voriconazole were 306, 706, 701, and 349, respectively.

<sup>b</sup> Data for *\Deltaerg11A* and *\Deltaerg11B* mutants constructed from the wild-type CEA10 A. *fumigatus* strain (13).

<sup>c</sup> Values in parentheses are the equivalent MICs in µM.

<sup>d</sup> MIC reported by Kim et al. (19).

<sup>e</sup> MIC reported by Balajee et al. (4).

<sup>f</sup> ND, not determined.

<sup>g</sup> MIC reported by Lamaris et al. (21).

<sup>h</sup> ΔAF51A mutants constructed from the wild-type CM-237 A. fumigatus strain (31).

Morrison quadratic tight-binding model (26, 32), with  $K_d$  values of 0.10, 0.03, and 0.07 µM, respectively (Table 2). Although itraconazole and posaconazole are structurally similar, both AF51A and AF51B had a 2.5-fold higher affinity for itraconazole than posaconazole. The difference in azole binding mechanisms between AF51A and AF51B is exemplified for clotrimazole in Fig. 5C. AF51A bound to fluconazole weakly  $(K_d, 11.9 \,\mu\text{M})$  and voriconazole relatively weakly  $(K_d, 3.6 \,\mu\text{M})$ , with the Hill equation best describing the resulting binding saturation curves. The apparent Hill numbers of 0.53 and 0.70 obtained for fluconazole and voriconazole, respectively, suggest that negative cooperativity was occurring between AF51A monomers during the binding of these two azole drugs. AF51B also bound to fluconazole weakly  $(K_d, 4 \mu M)$  with an apparent Hill number of 0.51, indicating negative cooperativity between AF51B monomers. However, AF51B bound to voriconazole tightly ( $K_d$ , 0.42  $\mu$ M) with an apparent Hill number close to 1, suggesting no cooperativity between AF51B monomers during voriconazole binding. Mechanistically, AF51B is more analogous to C. albicans CYP51 than to AF51A, as AF51B displayed tight binding toward clotrimazole, itraconazole, and voriconazole like C. albicans CYP51 (41), whereas AF51A did not exhibit tight binding with any of the azoles examined. Weak fluconazole binding ( $K_d$ , 19  $\mu$ M) was also observed with M. smegmatis CYP51 (40). The fluconazole saturation curves for AF51A and AF51B (Fig. 5D) account for the intrinsic fluconazole resistance of wild-type A. fumigatus (11, 30) in comparison with the resistance of wild-type C. albicans CYP51, which has a 250-fold higher affinity ( $K_d$  0.045  $\mu$ M) for fluconazole than AF51A (41).

MIC values for azole antifungal drugs with wild-type and CYP51 gene-knockout mutants of *A. fumigatus* (Table 3) indicate the importance of AF51A in conferring enhanced resistance to azole antifungal agents compared with that which would otherwise be the case if AF51B was the sole CYP51 present. MIC results are influenced by a variety of factors, including inoculum density and the medium used, besides cell permeability, cellular efflux rates, drug lipophilicity, and the availability and affinity of a drug for the target. This makes direct comparisons between observed MIC and  $K_d$  values for CYP51 difficult. However, the observed  $K_d$  values for itracon-

azole, posaconazole, and voriconazole with AF51A and AF51B were broadly comparable to the MIC values reported previously (Table 3), whereas fluconazole gave a MIC value for the wild-type organism far higher than the CYP51  $K_d$  value. The direct ligand binding studies described here had correctly identified fluconazole to be the weakest antifungal agent and itraconazole, posaconazole, and voriconazole to be effective inhibitors of A. fumigatus CYP51 proteins A and B. Previous MIC determinations using AF51A- and AF51B-knockout A. fumigatus mutants (13) have shown that AF51A was responsible for conferring resistance to fluconazole and itraconazole (Table 3), with the AF51A knockout causing a 16-fold increase in azole susceptibility. The finding that the wild-type A. fumigatus strains with AF51A-knockout mutations were 17 and 40 times more sensitive than the parental strain to fluconazole and ketoconazole, respectively (31), agrees with our finding that AF51B is more sensitive to azole antifungal agents than AF51A and supports a rationale for the appearance of mutations in CYP51A as the main cause of resistance.

We are presently investigating the azole binding properties of AF51A mutations at the three mutation hot spots Gly54, Leu98, and Met220 to gain further insight into the azole resistance mechanism of this CYP51 isoenzyme. Preliminary results have indicated that the point mutations so far examined yielded AF51A proteins that, while being relatively stable in the *E. coli* membrane fractions, were unstable when they were solubilized. We are currently optimizing a methodology to produce these mutant proteins.

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