The *ARO4* gene of *Candida albicans* encodes a tyrosine-sensitive DAHP synthase: evolution, functional conservation and phenotype of Aro3p-, Aro4p-deficient mutants

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The enzyme 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthase catalyses the first step in aromatic amino acid biosynthesis in prokaryotes, plants and fungi. Cells of *Saccharomyces cerevisiae* contain two catalytically redundant DAHP synthases, encoded by the genes *ARO3* and *ARO4*, whose activities are feedback-inhibited by phenylalanine and tyrosine, respectively. *ARO3/4* gene transcription is controlled by *GCN4*. The authors previously cloned an *ARO3* gene orthologue from *Candida albicans* and found that: (1) it can complement an *aro3 aro4* double mutation in *S. cerevisiae*, an effect inhibited by excess phenylalanine, and (2) a homozygous *aro3*-deletion mutant of *C. albicans* is phenotypically Aro⁺, suggesting the existence of another isozyme(s). They now report the identification and functional characterization of the *C. albicans* orthologue of *S. cerevisiae* Aro4p. The two Aro4p enzymes share 68% amino acid identity. Phylogenetic analysis places the fungal DAHP synthases in a cluster separate from prokaryotic orthologues and suggests that *ARO3* and *ARO4* arose from a single gene via a gene duplication event early in fungal evolution. *C. albicans* *ARO4* mRNA is elevated upon amino acid starvation, consistent with the presence of three putative Gcn4p-responsive elements (GCRES) in the gene promoter sequence. *C. albicans* *ARO4* complements an *aro3 aro4* double mutation in *S. cerevisiae*, an effect inhibited by excess tyrosine. The authors engineered $\Delta aro3/\Delta aro3 \Delta aro4/MET3p::ARO4$ cells of *C. albicans* (with one wild-type copy of *ARO4* placed under control of the repressible *MET3* promoter) and found that they fail to grow in the absence of aromatic amino acids when *ARO4* expression is repressed, and that this growth defect can be partially rescued by aromatic amino acids and certain aromatic amino acid pathway intermediates. It is concluded that, like *S. cerevisiae*, *C. albicans* contains two DAHP synthases required for the first step in the aromatic amino acid biosynthetic pathway.

Keywords: aromatic amino acids, *GCN4*, *MET3* promoter, pathogenic fungi

INTRODUCTION

The primary step in aromatic amino acid biosynthesis involves the enzyme 3-deoxy-D-arabinoheptulosonate-

7-phosphate (DAHP) synthase. This enzyme catalyses the condensation of erythrose 4-phosphate and phosphoenolpyruvate to form 3-deoxy-D-arabinoheptulosonate-7-phosphate. This step is followed by a series of

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Abbreviations: 3AT, 3-aminotriazole; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; 5-FOA, 5-fluoroorotic acid; GCRES, Gcn4p-responsive element; SC, synthetic complete; UTR, untranslated region.

The GenBank accession number for the sequence reported in this paper is U53216.

reactions leading to the production of chorismic acid, which serves as a branchpoint compound for two separate pathways resulting in the production of phenylalanine/tyrosine and tryptophan (reviewed by Hinnebusch, 1990; Bently, 1990; Braus, 1991). Thus, DAHP synthases play a key role in catalysing the first committed step of the aromatic amino acid pathway, and as a consequence, cellular enzymes are tightly regulated.

Cells of the yeast *Saccharomyces cerevisiae* contain two isozymes of DAHP synthase encoded by the genes *ARO3* and *ARO4* (Teshiba *et al.*, 1986; Paravicini *et al.*, 1988, 1989; Kunzler *et al.*, 1992). Transcription of both genes is coordinately controlled by Gcn4p, which binds to Gcn4p-responsive elements (GCREs) in the promoters. Enzyme activity is also regulated at the post-translational level, i.e. the activity of Aro3p is feedback-inhibited by phenylalanine, whereas Aro4p is inhibited by tyrosine (Hinnebusch, 1990; Braus, 1991). Many prokaryotes possess a third DAHP synthase whose activity is inhibited by tryptophan. Existence of a third isozyme has also been reported in the fungus *Neurospora crassa*, although the corresponding gene has not been identified (Nimmo & Coggins, 1981).

We are studying the nature and complexity of DAHP synthases in the pathogenic fungi as potential antifungal drug targets, since: (1) humans lack a comparable biosynthetic pathway – instead they rely on dietary sources for phenylalanine and tryptophan, and can only synthesize tyrosine via hydroxylation of phenylalanine; (2) based on pathogenicity studies of other auxotrophic mutants of *Candida albicans* and *Cryptococcus neoformans* (Manning *et al.*, 1984; Shepherd, 1985; Kirsch & Whitney, 1991; Perfect *et al.*, 1993), aromatic amino acid auxotrophs are predicted to display decreased virulence *in vivo* as a result of poor growth due to suboptimal amino acid bioavailability; and (3) certain amino acid biosynthesis inhibitors have been used safely and effectively as herbicides (reviewed by Kishore & Shah, 1988).

We previously cloned an *ARO3* gene orthologue from the diploid pathogenic fungus *C. albicans* and found that it can complement an *aro3 aro4* double mutation in *S. cerevisiae*, and that complementation is inhibited by excess phenylalanine (Pereira & Livi, 1993). Expression of *C. albicans* *ARO3* mRNA is induced in response to amino acid starvation, consistent with the presence of two putative GCREs in the promoter sequence (Pereira & Livi, 1995). A homozygous *aro3*-deletion mutant strain was constructed and found to be prototrophic (Aro⁺) on synthetic complete media lacking aromatic amino acids (Pereira & Livi, 1996), suggesting the existence of at least one additional isozyme. A small genomic DNA fragment was PCR-amplified from the mutant strain using degenerate primers, and its nucleotide sequence was found to predict a DAHP-synthase-related peptide with a strong homology to *S. cerevisiae* Aro4p (Pereira & Livi, 1996). In this study, we have cloned the complete gene defined by this DNA fragment and determined its evolutionary relationship to known DAHP synthases. We have evaluated its expression in

response to nutrient deprivation and the effect of feedback inhibition on its gene product. In addition, we have created strains of *C. albicans* deficient in Aro3p and Aro4p, and show that they display a conditional growth phenotype *in vitro*, indicating the presence of only two DAHP synthases.

METHODS

Strains and growth conditions. Synthetic complete (SC) liquid and agar media and dropout derivatives thereof were prepared according to Hicks & Herskowitz (1976), with the addition of 1 µg ml⁻¹ each of thiamin and biotin. Cells of *C. albicans* strain B311-A were prepared for Northern blot analysis by shaking at 30 °C to mid-exponential phase (10⁷ cells ml⁻¹) in SC liquid medium supplemented with 1 µg ml⁻¹ of both thiamin and biotin. Cultures were split into two: 3-aminotriazole (3AT) was added to one culture to a final concentration of 10 mM, and cultures were incubated for 6 h to allow for induction of the starvation response. Cells of *S. cerevisiae* were prepared for functional expression studies by growing to mid-exponential phase (OD₅₄₀ 1) in SC–Leu liquid medium. These were then washed twice in sterile distilled water, and 15 µl of each culture was spotted onto each appropriate plate. Plates were incubated at 30 °C for 24 h. The same *S. cerevisiae* strains used for feedback regulation studies were prepared as above except that the cells were washed twice in either SC–Leu, SC–Leu–Tyr–Trp + 5 mM Phe or SC–Leu–Phe–Trp + 5 mM Tyr before being resuspended in an equal volume of each of these media and spotted onto the appropriate plates. Feedback-inhibition studies in *C. albicans* were performed by growing the indicated strains in SC medium to OD₆₀₀ 1.0, washing with sterile water and then resuspending in an equal volume of either SC, SC lacking aromatic amino acids or SC lacking aromatic amino acids to which excess (5 mM) tyrosine or phenylalanine had been added. Cultures were grown at 30 °C with shaking for 2 h then diluted to OD₆₀₀ 0.1, 0.01 and 0.001 in the same media and spotted (15 µl) onto plates containing the corresponding media. Plates were incubated for 16 h at 30 °C. Strains used in the *MET3* promoter repression assay were pregrown for 4 h in SC liquid medium with or without aromatic amino acids and with or without 2.5 mM cysteine and methionine. Cell densities were adjusted to OD₆₀₀ 0.1 in the appropriate media, 10-fold serial dilutions were spotted (15 µl) onto agar plates containing each medium, and plates were incubated for 16 h at 30 °C.

Cloning the *ARO4* gene. Pereira & Livi (1996) previously described using degenerate PCR primers to amplify a 222 bp DNA fragment encoding a DAHP-synthase-related peptide from *C. albicans* strain SPC64, a homozygous Δ *aro3* mutant. This DNA fragment was labelled and used as a probe to screen a *C. albicans* strain B792-derived YEp13-based genomic library carried in *Escherichia coli* (Rosenbluh *et al.*, 1985). Hybridizations and stringency washes were carried out using the Rapid Hyb system (Amersham Life Sciences) according to the manufacturer's protocol.

Phylogenetic analyses. Publicly available databases, including partial genomic sequences, were searched for homologous ORFs to class I DAHP synthases from *S. cerevisiae* (Aro3p and Aro4p) and *E. coli* (AroF, AroG, and AroH) using the programs BLASTP, TBLASTN (Altschul *et al.*, 1990) and PSI-BLAST (Altschul *et al.*, 1997). Individual protein datasets were initially aligned using the program CLUSTAL W v1.7 (Thompson *et al.*, 1994) with default settings. Multiple sequence alignments were further refined manually using the program

Table 1. Putative GCREs in the *C. albicans* *ARO3* and *ARO4* promoters compared to functional GCREs in *S. cerevisiae* gene orthologues

Species	Gene	Position and sequence*	Reference
<i>S. cerevisiae</i>	<i>ARO3</i>	-180GTGACTAAT ⁻¹⁸⁸	Paravicini <i>et al.</i> (1988)
	<i>ARO4</i>	-312ATGACTCAA ⁻³⁰⁴	Kunzler <i>et al.</i> (1992)
<i>C. albicans</i>	<i>ARO3</i>	-181TTGACTAAT ⁻¹⁷³	Pereira & Livi (1993)
		-332CTGACTAAT ⁻³²⁴	Pereira & Livi (1993)
	<i>ARO4</i>	-271ATGACTAAA ⁻²⁶³	This study†
		-301ATGACTGAA ⁻²⁹³	This study†
		-336CGGACTATT ⁻²⁹⁸	This study†

* Functionality of the *S. cerevisiae* *ARO3* and *ARO4* GCREs has been confirmed by mutagenesis and *in vitro* binding studies (Hinnebusch, 1990; Braus, 1991). The *S. cerevisiae* *ARO3* GCRE exists in an inverse orientation.

† GenBank accession number U53216.

SEQLAB of the GCG v10.0 software package (Genetics Computer Group) with reference to the three-dimensional structure of *E. coli* phenylalanine-regulated AroG (Shumilin *et al.*, 1999). Regions with residues that could not be unambiguously aligned or that contained insertions or deletions were removed from the alignments. Maximum-parsimony analysis was done using the software package PAUP* (Swofford, 1999). The number of minimal trees and their lengths were estimated from 100 replicate random heuristic searches. Confidence limits of branch points were estimated by 1000 bootstrap replications. Neighbour-joining analysis was performed using the programs NEIGHBOR and PROTDIST of the PHYLIP 3.57c package (Felsenstein, 1993). In PROTDIST, the 'Dayhoff' option was invoked, which estimates the expected amino acid replacements per position between all pairs of sequences based on the Dayhoff 120 substitution matrix (Dayhoff *et al.*, 1972). The programs SEQBOOT and CONSENSE were used to estimate the confidence limits of branching points from 1000 bootstrap replications. The program TREEVIEW (v1.6.1) was used to visualize trees and prepare figures (Page, 1996).

Plasmid constructions. The plasmid pMB-7 (constructed by Dr N. Gow and a kind gift from Dr W. Fonzi, Georgetown University, Washington, DC, USA) contains the *C. albicans* *URA3* gene (1365 bp *ScaI*-*XbaI* fragment; Gillum *et al.*, 1984; Losberger & Erst, 1989) flanked by 1150 bp direct repeat sequences of the *Salmonella typhimurium* *hisG* gene (Alani *et al.*, 1987; Fonzi & Irwin, 1993). The *C. albicans* *ARO4* disruption plasmid pMB7Δaro4 was made by first amplifying a portion of *ARO4* from the isolated YEp13-*ARO4* library plasmid using primers 5'-TCTTCAGATCTA-TACCGATGGATGTGTTTCTTG-3' and 5'-ATTATGAGCTCGATCTTGATGAATTAGAAATTGA-3' (restriction sites italicized), digesting with *Bgl*II and *Sac*I and subcloning into the corresponding sites of pMB-7, resulting in plasmid pMB7ARO43'. The subcloned 573 bp PCR fragment of pMB7ARO43' corresponds to nucleotides 1466 to 2038 of the *ARO4* gene (see GenBank U53216). Following sequence verification, a second 639 bp PCR product (corresponding to nucleotides 621 to 1259 of the GenBank sequence) was amplified as above using primers 5'-TTACAGCATGCG-TAATGATGACAGATGTATGT-3' and 5'-TCATAGT-CGACTTGGATCGGTACATTTGGCA-3' (restriction sites italicized), digested with *Sph*I and *Sal*I and subcloned into the corresponding sites of pMB7ARO43', resulting in pMB7Δaro4. This plasmid was digested with *Sac*I and *Sph*I to

liberate a 5.2 kb *ARO4* disruption cassette in which a 205 bp portion of the *ARO4* coding region (nucleotides 1260 to 1465) was replaced by *hisG::URA3::hisG*. Plasmid pCaDis-aro4 was constructed by PCR-amplifying a portion of the 5' coding region of *C. albicans* *ARO4* (nucleotides 367 to 667 of the GenBank sequence) as above using primers 5'-AAAGGAT-CCTAGTTATTCTTTGTCAAATAA-3' and 5'-AAACTG-CAGGTGAATTGAACATGGACCAAC-3' (restriction sites italicized), digesting with *Bam*HI and *Pst*II, and subcloning the resulting DNA fragment into the corresponding sites in pCaDis [Plasmid pCaDis (Care *et al.*, 1999) was kindly provided by P. E. Sudbery, University of Sheffield, UK]. pCaDis-*ARO4* was linearized with *Bp*II, which cuts the plasmid uniquely within the *ARO4* sequence, prior to transformation of *C. albicans*.

Transformation of *S. cerevisiae* and *C. albicans*. Lithium acetate transformation of *S. cerevisiae* and *C. albicans* was performed according to the methods of Gietz *et al.* (1992) and Sanglard *et al.* (1996), respectively.

Northern-blot analysis. Total RNA was extracted from *C. albicans* strain B311-A with and without histidine starvation (described above). Poly(A)⁺ RNA was prepared by oligo-dT affinity chromatography (mRNA purification kit; Boehringer Mannheim). mRNA was fractionated on a 1.5% denaturing agarose gel in the presence of 2.2 M formaldehyde, transferred to nitrocellulose and probed simultaneously with the ³²P-labelled (random primed) 222 bp PCR-generated fragment used to clone the *ARO4* gene (see above) and with an approximately 0.8 kb cDNA corresponding to the *CYP1* gene encoding cytoplasmic cyclophilin (Koser *et al.*, 1990).

Genomic analysis of *C. albicans* strains. Strains of *C. albicans* were analysed using whole-yeast cell PCR (Sathe *et al.*, 1991) and by Southern blotting (Southern *et al.*, 1975). Primers used in *ARO4* disruption analysis were 5'-ATTGCCAAATGTA-CCGATCCAAGT-3' and 5'-CAAGAAACACATCCATCG-GTGATA-3', which are designed to amplify the 205 bp deletion/insertion site (see above) in *ARO4*. PCR analysis of Met3p::*ARO4* strains was performed as above using primers 5'-TCCAAGTGTGTCACCTTTCTT-3' (*MET3* promoter; nucleotides 5221 to 5242 of pCaDis) and 5'-CATGATAA-TACCAAGTCATC-3' (reverse complement of *ARO4* nucleotides 728 to 749). PCR screening for the loss of the wild-type *ARO4* allele was performed using primers 5'-TCCAA-GTGTGTCACCTTTCTT-3' (*MET3* promoter; nucleotides

5221 to 5242 of pCaDis) and 5'-ATTTGTTTCAGCAACAC-TGTCT-3' (reverse complement of nucleotides 1330 to 1350 of ARO4, deleted in the *aro4Δ::his3* allele). Southern blotting was performed by digesting *C. albicans* genomic DNA with *Bst*XI or *Bst*XI and *Hpa*I overnight and separating on a 0.8% agarose gel prior to transfer to nitrocellulose and probing. Probes were generated by PCR and correspond to nucleotides 121 to 1611 and nucleotides 1456 to 1912 of ARO4; they were labelled with ³²P using the High Prime DNA Labelling Kit (Boehringer Mannheim Biochemicals).

RESULTS AND DISCUSSION

Cloning and characterization of the *C. albicans* ARO4 gene

We previously generated a 222 bp genomic PCR fragment from a homozygous *aro3*-deletion strain of *C. albicans*, using a degenerate primer based on a conserved N-terminal region of Aro3p (Pereira & Livi, 1993), plus a degenerate comeback primer encoding a conserved region of the protein that lies within the deleted portion of the gene (Pereira & Livi, 1996). The nucleotide sequence of this PCR fragment predicts a 74 aa DAHP-synthase-related peptide with strong homology to known Aro3p/Aro4p proteins. A *C. albicans* genomic YEp13-based plasmid library (Rosenbluh *et al.*, 1985) was screened using the PCR fragment as a probe. One plasmid isolated was found to contain an approximately 8 kb insert with a 1110 bp ORF predicting a 370 aa protein corresponding to the probe sequence (GenBank accession no. U53216). The 5'-UTR contains three putative GCRES, at positions -271 (5'-ATGACTA-AA-3'), -301 (5'-ATGACTGAA-3') and -336 (5'-CGGACTATT-3') (Table 1; see Braus, 1991). The 5'-UTR also contains a few potential TATAA elements downstream of these sites (Hinnebusch, 1990), whereas the 3'-UTR contains putative transcription termination signals that resemble the consensus TAGT... TAGT... TTT (Zaret & Sherman, 1982).

The 370 aa *C. albicans* protein, herein designated Aro4p, is 57.3–62.3% identical (67.1–70.9% similar) to the Aro3p proteins of *S. cerevisiae*, *C. albicans* and *Schizosaccharomyces pombe*. It shows greater sequence conservation (60.0–68.0% identity and 69.9–72.8% similarity) to the Aro4p proteins of *S. cerevisiae* and *Sch. pombe*, suggesting a functional relationship (Fig. 1).

Molecular evolution of the deduced Aro4p protein

When compared to prokaryotic orthologues, *C. albicans* Aro4p is more highly diverged, although several residues are conserved between *E. coli* and yeast DAHP synthases. It should be noted that naming conventions are not consistent with function among bacterial and yeast DAHP synthases. In *S. cerevisiae*, DAHP Aro3p (P14843) is called AroF, but is inhibited by phenylalanine like *E. coli* AroG (P00886). Meanwhile, *S. cerevisiae* Aro4p, called AroG (P32449), is inhibited by tyrosine as is *E. coli* AroF (P00888). There is no evidence in any yeast genome for a biochemical orthologue to *E. coli* tryptophan-regulated AroH (P00887).

Yeast ARO3 and ARO4 Alignment					
1					50
Ca_ARO4	~~~~~	~MSKTPVPTE	YDDTRILGYD	PLVPPALLQH	EIKASAESLD
Sc_ARO4	MSESPMFAAN	G-P-VNQA-	E-V-----	--AS-----V	Q-P-TPT--E
Sp_ARO4	~~~~MSPVFL	PSGE-YDQEH	L-N-V---N	---A---V-Q	--PV-ET-RK
Ca_ARO3	~~~~MFITN	..EHVGDERS	ME-W--R---	--T--D----	-YPLTP--QK
Sc_ARO3	~~~~MFIKN	..DHAGDRKR	LE-W--K---	--T--D----	-FPI-AKGEE
Sp_ARO3	~~MDKHTPLL	PGDSVFSRCK	TE-S--K---	-VIS---I-S	-LA--D-T-A
51					100
Ca_ARO4	VVIKGRYDSA	QILKGNDDRC	IVIVGPCSIH	DPPQALEYK	RLKKLADELK
Sc_ARO4	TAKR--REAI	D-IT-K---V	L-----	-LEA-Q--AL	-----S---
Sp_ARO4	-ITDS-KEIQ	A--NKQ---I	--V-----	--KL-MD-A-	L--PK----Q
Ca_ARO3	IIVE--NAAC	D--N-K---L	-IVI-----	--QA--D-CE	--YQASEKH-
Sc_ARO3	NI-KA-DSVC	D--N-K---L	VIVI-----L	--KA-YD-AD	--A-ISEK-S
Sp_ARO3	F-SDQ-RQA-	D-IA-R---L	LL-----L-	--VA-K-AI	--Q-E-IKH-
101					150
Ca_ARO4	DDLVIIMRAY	LEKPRTTVGW	KGLINDPDVD	NSFDINRGLK	ISRQLYSDLT
Sc_ARO4	G-S-----	-----	-----N	-T-N--K--Q	SA--FVN--
Sp_ARO4	-A-CVV--C-	-----I--	---V---NL-	G--A--K-IR	MA--M-C-V-
Ca_ARO3	GE-L-V----	-----	-----I-	GT-H--K--R	-A-K-FVQ--
Sc_ARO3	K--L-----	-----	-----MN	---Q--K--R	---EMFIK-V
Sp_ARO3	K--H-----	-----	-----L-	G-YN--K-IR	VA-RIFLE-L
151					200
Ca_ARO4	SVVGLPIGSE	MLDTISPOYF	SDFLSFGAIG	ARTTESQLHR	ELASGLSFIPI
Sc_ARO4	NI-----	-----L	A-LV-----	-----	-----V
Sp_ARO4	NF--I-LA--	--N-----	A-L-----	-----	-----A----
Ca_ARO3	..K--AG--	-----FL	--LF-V-----	-----	-----V
Sc_ARO3	E..K--AG--	-----FL	--CF-L-----	-----	-----
Sp_ARO3	E.T-VG-A--	-----L	A-LICW----	-----	-----
201					250
Ca_ARO4	GFKNGTDGGL	AVALDAVQAS	SKGHHFMGVT	KNGMAAITTT	KGNDCCFIIL
Sc_ARO4	-----T-	N--V--C--A	AHS-----	-H-V-----	---EH--V--
Sp_ARO4	-----TV	G--I--IG-T	AHP-TML--	-Q-L--M--	R--KDT----
Ca_ARO3	-----T-	G--I--LR-A	-HP--LS--	-P-VV--VG-	D--QD--V--
Sc_ARO3	-----Q-	I--MR-A	AHE-Y-LS--	-P-VT--VG-	E--KDT-L--
Sp_ARO3	----A--NI	GI-I--MNS-	ANP--LS--	-Q-VV--V--	T--PDTH--
251					300
Ca_ARO4	RGKKITNYD	VESVKAAKEA	IAKC....T	DPSIKLMVDC	SHDNSRKDYR
Sc_ARO4	-----G--	AK--AE--..	..QL....P	AG-NG--I-Y	--G--N--F-
Sp_ARO4	-----GP--	A-H-A-VRKD	LE-ANL...	..PPRI-I--	--G--S-NHL
Ca_ARO3	-----G--	AK--QETQ-E	LI-SKVVTES	K-GPRI----	--G--N--H-
Sc_ARO3	-----NG--F-	K--QNT-KQ	LE-AGLTDD	QK..RI-I--	--G--N--FK
Sp_ARO3	-----SG--F-	AD--AG--AK	LEE-NKL...	..PSI-I--	--G--S-NHK
301					350
Ca_ARO4	NQPQVLDSVA	EQISNGEDSI	IGVMIESNIH	EGKQPMPPAG	SG.KEALKYG
Sc_ARO4	---K-N-V-C	---A--NA-	T-----N	--N-GI--A	E--AG----
Sp_ARO4	---K-SK-I-	---R--DS-	V-----H-N	--R-DA-IRP	-V-DT----
Ca_ARO3	---K-AQVV-	--VAG-DK-	C-L-----	D-R-DV--KE	Q-G-D-----
Sc_ARO3	---K-AKCIY	D-LTE--N-L	C-----N	--R-DI--K	E-GR-G-----
Sp_ARO3	---K-AACI-	--VA--QKA-	T-----HLN	--K-AI--..	EDDLSSM--
351					387
Ca_ARO4	VSITDGCVSW	ETTVEMLTKL	SQAVQTRRS	LKKQKVS	N
Sc_ARO4	-----A-IG-	---EDV-R-	AA--RQ--EV	N-K-----	
Sp_ARO4	-----A----	-Q-AP--DD	AE--RA--QN	Q-SN----	
Ca_ARO3	-----A-IG-	---E-V-EV-	AN--K-----		
Sc_ARO3	C-V--A-IG-	-S-EQV-EL-	AEG-RN--KA	L-K-----	
Sp_ARO3	--V--A-IG-	DD-TAVFEQ-	AA--RS--H	-----	

Fig. 1. Deduced amino acid sequence alignment of yeast DAHP synthases. Species are *C. albicans* (Ca), *S. cerevisiae* (Sc) and *Schizosaccharomyces pombe* (Sp). Residues perfectly conserved between the yeast enzymes and also the DAHP synthases in *Escherichia coli* (AroF, AroG and AroH) are shown in bold. The six proteins (GenPept accession numbers from top to bottom U53216, X61107, AL117210, L12217, X13514, Z54142) were aligned using the PILEUP and PRETTY programs of the Genetics Computer Group sequence analysis software package. Dashes indicate identical amino acid sequences; periods and tildes indicate sequence gaps included to maximize alignments.

Database searches confirmed the existence of three DAHP synthases in the proteobacteria *E. coli* and *Salmonella typhimurium*. However, most complete genome sequences of bacteria as well as fungi reveal

Fig. 2. Neighbour-joining phylogenetic tree showing convergent evolution of fungal and bacterial DAHP synthases. Phylogenetic tree construction is described in Methods. Numbers along the branches show the percentage occurrence of nodes in 1000 bootstrap replicates of maximum-parsimony and neighbour-joining analyses. Nodes occurring in more than 70 % of resamplings by both methods are marked with an asterisk (*); values less than 50 % are either not shown or indicated as a dash (-). The scale bar represents 0.1 amino acid substitutions per site. In this diagram, we have included the prokaryotic nomenclature after each fungal protein name in order to indicate its functional equivalence (e.g. Aro4p is inhibited by tyrosine as is *E. coli* AroF).

fewer than three types of DAHP synthase. For example, the proteobacterium *Haemophilus influenzae* has only AroG. The sequenced genomes of three fungal species reveal AroG and AroF but not AroH. Thus, the evolution of the different feedback-inhibited types of DAHP synthases appears to be highly species specific.

Generally consistent phylogenetic trees were generated by the maximum-parsimony (MP) and neighbour-joining (NJ) methods. In 100 heuristic searches, MP detected only one shortest tree, which was 2158 steps in length. Phylogenetic analyses show four distinct clusters of DAHP synthases (Fig. 2). The yeast DAHP synthases form one cluster, which is highly divergent from bacterial enzymes. Within the fungal clade, *S. cerevisiae* and *C. albicans* Aro3p (AroG-type) and Aro4p (AroF-type) DAHP synthases occurred in clusters specific to each amino acid inhibitor. Although *Sch. pombe* Aro3p and Aro4p appear to be ancestral to other fungal DAHP synthases, this might be an artifact of their more rapid sequence evolution. Among the bacteria, the three different feedback-inhibited enzymes, AroF, AroG and AroH, formed separate clades. AroG was split into two groups: one consisted of proteobacteria and the other of high-G+C Gram-positive bacteria (*Mycobacterium*

avium) and actinobacteria (*Corynebacterium glutamicum* and *Amycolatopsis methanolica*). While bootstrap and minimal tree search support for five separate clades of DAHP synthases was generally high, the resolution of branching order among the groups was not resolved. NJ provided the best support for clustering the yeast enzymes with bacterial AroG although the bootstrap value (47 %) was less than 50 %. Regardless, phylogenetic analysis suggests that the two different fungal DAHP synthases arose from an early gene duplication in the fungi.

ARO4 mRNA levels increase during amino acid starvation

To determine whether the *C. albicans* ARO4 gene is subject to GCN-like transcriptional derepression, we measured the steady-state level of ARO4-specific mRNA in starved and unstarved cells. Starvation was induced with 3-aminotriazole (3AT), an inhibitor of the *HIS3* gene product (Hinnebusch & Fink, 1983). 3AT has been used to induce the GCN response in both *S. cerevisiae* and *N. crassa* (Ebbole *et al.*, 1991). Northern blot analysis of poly(A)⁺ RNA, probed with *C. albicans* ARO4, revealed an increase in abundance of a 1.5 kb

Fig. 3. Northern blot analysis of *ARO4* mRNA in unstarved versus histidine-starved cells. Lane 1, cells grown under unstarved, GCN-repressed (R) conditions; lane 2, cells grown under histidine-starved, GCN-derepressed (DR) conditions (addition of 10 mM 3AT). Each lane contains 2 µg poly(A)⁺ RNA (quantitated spectrophotometrically). The blot was simultaneously hybridized with ³²P-labelled *ARO4* and *CYP1* DNA probes (see Methods). *CYP1* encodes cytoplasmic cyclophilin (Koser *et al.*, 1990).

gene-specific transcript in starved (derepressed) cells (Fig. 3). The apparent basal level of *ARO4* mRNA in unstarved (repressed) cells is consistent with basal control of *ARO4* transcription by *GCN4* in *S. cerevisiae* (Kunzler *et al.*, 1992).

As an internal control for RNA abundance, the blot shown in Fig. 3 was simultaneously probed with a cDNA from the *C. albicans* *CYP1* gene, encoding cytoplasmic cyclophilin, which recognizes an approximately 800 bp mRNA (Koser *et al.*, 1990). The observed reduction in *CYP1* mRNA in 3AT-treated cells is consistent with earlier findings, and is probably due to a drop in the overall rate of protein synthesis (Pereira & Livi, 1995). This result serves to accentuate the observed increase in *ARO4*-specific mRNA, and our estimates of the derepression ratio compare favourably with those of many starvation-induced genes in *S. cerevisiae* (data not shown; Hinnebusch, 1990).

Functional expression in *S. cerevisiae*

The *C. albicans* *ARO4* gene carried on YEp13 was introduced into *S. cerevisiae* strain RH1368 (*aro3-2 aro4-1 gcn1-1 trp1-1 leu2-2*), selecting for Leu⁺ transformants. The *gcn1-1* mutation renders the cells Gcn4p-deficient, so that functional complementation should correspond to basal gene expression (Hinnebusch, 1990). As shown in Fig. 4(a, b), the *C. albicans* *ARO4* gene, like *ARO3* (Pereira & Livi, 1993), complements the *aro3 aro4* mutations in *S. cerevisiae*. The *ARO3* gene from *C. albicans* was previously defined by its inability to complement when cells are grown on excess (2 mM) phenylalanine, presumably due to isozyme-specific feedback inhibition (Pereira & Livi, 1993). Complemen-

Fig. 4. (a, b) Functional expression of the *C. albicans* *ARO4* gene in *S. cerevisiae*. The high-copy-number yeast episomal plasmid YEp13 (carrying the *LEU2* selectable marker) or derivatives thereof containing either the *C. albicans* *ARO3* gene (Pereira & Livi, 1993) or the *C. albicans* *ARO4* gene were introduced into cells of *S. cerevisiae* strain RH1368 (*aro3-2 aro4-1 gcn1-1 trp1-1 leu2-3*), selecting for Leu⁺ transformants. Numbered spots correspond to the following strains in each panel: spot 1, RH1368 plus YEp13-*ARO3*; spot 2, RH1368 plus YEp13-*ARO4*; spot 3, RH1368 plus YEp13 alone; spot 4, untransformed RH1368; spot 5, untransformed strain DC17 (*MATα his1*; Klar *et al.*, 1985). Growth media were as follows: (a) SC (synthetic complete) supplemented with 1 µg ml⁻¹ of both thiamin and biotin; (b) SC dropout medium lacking leucine, phenylalanine and tyrosine (SC-Leu-Phe-Tyr). (c) Regulation of *C. albicans* Aro4p activity in *S. cerevisiae* by aromatic amino acids. Cells of *S. cerevisiae* RH1368 containing either *C. albicans* *ARO3* or *C. albicans* *ARO4* carried on YEp13 were grown in SC dropout media in the presence (+) or absence (-) of the indicated amino acids (supplemented at 0.2 mM) or in the presence of excess (++) amino acids (supplemented at 5 mM).

tation by *ARO4* was similarly impaired (albeit to a lesser degree) by excess (5 mM) tyrosine (Fig. 4c), but not excess (5 mM) phenylalanine or tryptophan (Fig. 4c and data not shown). Taken together, these data suggest that *C. albicans* Aro4p is a structural as well as functional orthologue of *S. cerevisiae* Aro4p.

Engineering and phenotyping *aro4*-deficient mutants

A homozygous *aro3*-disruption (deletion/insertion) mutant strain of *C. albicans* (SPC64) (see Table 2 for complete genotypes of all strains) was previously constructed and found to be phenotypically Aro⁺, suggesting the existence of an additional DAHP synthase isozyme(s) (Pereira & Livi, 1996). To further investigate the genetic complexity of DAHP synthases in *C. albicans*, we started by sequentially disrupting both

Table 2. Strains of *C. albicans* and *S. cerevisiae*

<i>C. albicans</i> strain	Parent	Relevant genotype	Reference
CAF2-1	SC5314	$\Delta ura3::imm434/URA3$	Fonzi & Irwin (1993)
CAI4	CAF2-1	$\Delta ura3::imm434/\Delta ura3::imm434$	Fonzi & Irwin (1993)
SPC64	SPC10	$\Delta ura3::imm434/\Delta ura3::imm434$	Pereira & Livi (1996)
SSC1	CAI4	$\Delta aro3::hisG/\Delta aro3::hisG-URA3-hisG$	This study
SSC2	SSC1	$\Delta ura3::imm434/\Delta ura3::imm434$	This study
SSC2U-23	SSC2	$\Delta aro4::hisG/\Delta aro4::hisG-URA3-hisG-URA3-hisG$	This study
SSC3	SSC2U-23	$\Delta ura3::imm434/\Delta ura3::imm434$	This study
SSC6	SSC3	$\Delta aro4::hisG/\Delta aro4::hisG$	This study
SSC9	SSC6	$\Delta ura3::imm434/\Delta ura3::imm434$	This study
SPC101	SPC64	$\Delta aro3::hisG/\Delta aro3::hisG$	This study
SPC208	SPC101	$\Delta aro4::hisG-URA3-hisG/ARO4$	This study
SPC311	SPC208	$\Delta ura3::imm434/\Delta ura3::imm434$	This study
SPC311	SPC208	$\Delta aro4::hisG/ARO4$	This study
SSC12	SPC311	$\Delta ura3::imm434/\Delta ura3::imm434$	This study
SSC13	SPC311	$\Delta aro4::hisG/MET3p::ARO4$	This study
SSC13	SPC311	Same as SSC12	This study
<i>S. cerevisiae</i> strain	Genotype	Reference	
RH1368	$MAT\alpha aro3-2 aro4-1 gcn1-1 trp1-1 leu2-3$	Kunzler <i>et al.</i> (1992)	
DC17	$MAT\alpha his1$	Klar <i>et al.</i> (1985)	

alleles of *ARO4* in strain CAI4, which is wild-type for *ARO3* ($\Delta ura3::imm434/\Delta ura3::imm434$ *ARO3/ARO3*). This involved the use of an $\Delta aro4::hisG-URA3-hisG$ deletion/insertion cassette (see Methods) and the Ura-blaster technique (Alani *et al.*, 1987; Fonzi & Irwin, 1993), which allows repeated use of the *URA3* selectable marker due to homologous intrachromosomal recombination between flanking *hisG* repeats and the generation of Ura⁻ popout mutants.

Briefly, strain CAI4 was transformed with the 5.2 kb *SacI-SphI* $\Delta aro4::hisG-URA3-hisG$ fragment from plasmid pMB7 $\Delta aro4$ and Ura⁺ transformants were selected. Southern blot analysis of genomic DNA digested with *BstXI* (which cuts outside of *ARO4*) and probed with an *ARO4* PCR product consisting of

nucleotides 121 to 1611 (GenBank accession no. U53216), revealed the presence of a second ~ 9 kb band in Ura⁺ transformants in addition to the single 5 kb wild-type *ARO4*-specific band observed in CAI4 (Fig. 5, lanes 1 and 2). This pattern is indicative of a heterozygous $\Delta aro4::hisG-URA3-hisG/ARO4$ genotype. One heterozygote (strain SSC1, Fig. 5, lane 2) was chosen for subsequent experiments. Cells of SSC1 were plated on SC medium plus 5-FOA to select for Ura⁻ (*URA3* popout) mutants. Several Ura⁻ mutants were analysed by genomic Southern as before, and a few appeared to have lost the *URA3* gene. One strain, SSC2 ($\Delta aro4::hisG/ARO4$) (Fig. 5, lane 3) was transformed with the same $\Delta aro4::hisG-URA3-hisG$ fragment as before, and Ura⁺ transformants were again selected. Genomic PCR was used to screen the genotypes of

Fig. 5. Southern blot analysis of *C. albicans* *aro4* mutants. Genomic DNAs were digested with *Bst*XI and probed with an *ARO4*-specific PCR product (nucleotides 121–1611). Lane 1, strain CA14 (*ARO4/ARO4*), which contains a single 5 kb *ARO4*-specific band; lane 2, strain SSC1 (Δ *aro4::hisG-URA3-hisG/ARO4*); lane 3, strain SSC2 (Δ *aro4::hisG/ARO4*); lane 4, strain SSC2U-23 (Δ *aro4::hisG/Δaro4::hisG-URA3-hisG-URA3-hisG*; duplication of disruption cassette verified by PCR, labelled as Δ *aro4::hisG-URA3-hisG* ($\times 2$) for brevity); lane 5, SSC3 (Δ *aro4::hisG/Δaro4::hisG*).

multiple transformants using primers flanking the sequence targeted for deletion. In each individual transformant, the deletion/insertion event occurred at either the wild-type allele or the Δ *aro4::hisG* allele. A few Δ *aro4::hisG/Δaro4::hisG-URA3-hisG* transformants were identified and analysed by genomic Southern blotting, and one strain, SSC2U-23, was chosen for further study (Fig. 5, lane 4). It was determined that the larger size of the band corresponding to the Δ *aro4::hisG-URA3-hisG* allele in this strain is the result of a duplicative integration event at this allele (verified by PCR; data not shown). Thus, the correct genotype is Δ *aro4::hisG/Δaro4::hisG-URA3-hisG-URA3-hisG* (Table 2). Cells of SSC2U-23 were plated on 5-FOA to select for Ura[−] popouts, resulting in strain SSC3, which contains two Δ *aro4::hisG* alleles (Fig. 5, lane 5).

Both strains containing homozygous null alleles, SSC2U-23 and SSC3, retain aromatic amino acid prototrophy (data not shown), presumably due to the presence of functional DAHP synthase activity provided by Aro3p. Growth of *C. albicans* strains carrying homozygous disruptions of either *ARO3* or *ARO4* are inhibited by excess tyrosine or phenylalanine, respectively (Fig. 6), indicating specific feedback inhibition of the remaining isozyme. These results suggest that as in *S. cerevisiae*, only two DAHP synthase isozymes (encoded by *ARO3* and *ARO4*) exist in *C. albicans*. To demonstrate this more directly, we sought to engineer a homozygous *aro4* double disruption strain.

Attempts to create a homozygous Δ *aro3/Δaro3 Δaro4/Δaro4* mutant

The same Δ *aro4::hisG-URA3-hisG* deletion/insertion cassette used successfully to disrupt both copies of *ARO4* in CA14 was introduced into an Aro3p-deficient (Δ *aro3::hisG/Δaro3::hisG*) strain, SPC101 (derived from strain SPC64; Pereira & Livi, 1996). This resulted in strain SPC208 (Δ *aro3::hisG/Δaro3::hisG*

Fig. 6. Effect of feedback inhibition on the growth of *C. albicans* wild-type and *aro* mutant strains. The panels show growth of *C. albicans* strains CAF2-1 (*ARO3/ARO3 ARO4/ARO4*), SPC64 (Δ *aro3::hisG/Δaro3::hisG-URA3-hisG*) and SSC2U-23 (Δ *aro4::hisG/Δaro4::hisG-URA3-hisG-URA3-hisG*) on SC medium, SC lacking aromatic amino acids, and SC lacking aromatic amino acids to which excess (5 mM) tyrosine or phenylalanine was added.

Δ *aro4::hisG-URA3-hisG/ARO4*), and the subsequent *URA3* popout strain (selected on 5-FOA) SPC311 (Δ *aro3::hisG/Δaro3::hisG Δaro4::hisG/ARO4*); again, genotypes were confirmed by genomic PCR (data not shown) and Southern blotting (Fig. 7b, lanes 2 and 3).

The same deletion/insertion cassette was then used to transform strain SPC311 in an attempt to disrupt the remaining wild-type allele of *ARO4*. Nearly 1000 Ura⁺ transformants were screened by PCR, and in all cases the disruption cassette was found to have integrated either at the previously disrupted (Δ *aro4::hisG*) allele, or at a much lower frequency, at some other site in the genome (data not shown). This type of event may have been favoured over gene replacements at the wild-type allele because of more extensive homology between the *hisG* sequences in the transforming DNA fragment and the Δ *aro4::hisG* allele in the chromosome, resulting in more relaxed recombination intermediates, or may be due to recently revealed allele preferences during targeting integration as described by Yesland & Fonzi (2000).

To address this possibility, three alternative strategies were tried. First, strain SPC311 was transformed with plasmid DNA in which the orientation of the *hisG-URA3-hisG* cassette was reversed relative to the flanking *ARO4* sequences in plasmid pMB7 Δ *aro4*, with the intent to reduce the occurrence of double crossover events between the *hisG* repeats of the introduced DNA and the *hisG* of the previously disrupted allele. The second was a nested PCR-based disruption strategy (Wilson *et*

Fig. 7. (a) Schematic showing construction of *C. albicans* cells lacking Aro3p and Aro4p. Starting with strain SPC101 (*ARO4/ARO4* $\Delta aro3::hisG/\Delta aro3::hisG$) (line 2), one wild-type *ARO4* allele was disrupted via the Ura-blaster technique (Fonzi & Irwin, 1993) using plasmid pMB7 $\Delta aro4$ linearized by digestion with *SacI* and *SphI*. A resulting strain, SPC208, contains one wild-type *ARO4* allele (as in line 2) and an $\Delta aro4::hisG-URA3-hisG$ allele (as in line 3). Selection on 5-FOA resulted in loss of the *URA3* marker via recombination between the bacterial *hisG* repeats. One strain, SPC311, was obtained which contains one wild-type *ARO4* allele (as in line 2) and an $\Delta aro4::hisG$ allele (as in line 4). The remaining wild-type *ARO4* allele of strain SPC311 was subsequently placed under the control of the *C. albicans* *MET3* promoter. Plasmid pCaDis-aro4, containing the 5' coding region of *ARO4* (see Methods) was linearized with *BpI* and introduced into cells of SPC311, selecting for Ura⁺ transformants. Strain SSC12 contains the $\Delta aro4::hisG$ allele (as in line 4) as well as the *MET3p::ARO4* allele (as in line 1). The region of probes used for genomic Southern blotting is indicated by a heavy line, and the expected size of bands hybridizing to the probes is indicated for each allele. Bh, *Bam*HI; Bp, *Bp*II; Bs, *Bst*XI; Hp, *Hpa*I; Ps, *Pst*I. *MET3p* is the *C. albicans* *MET3* promoter (Care *et al.*, 1999). (b) Genomic Southern blot analysis demonstrating sequential disruption of *ARO4* and knock-in of the *MET3* promoter. Genomic DNA from each strain was digested with *Bst*XI and *Hpa*I. The probe was an *ARO4* PCR product corresponding to nucleotides 1456 to 1912. Individual alleles represented by each band are indicated on the right, with positions of size markers on the left. Lane 1, strain CAI4; lane 2, SPC208; lane 3, SPC311; lane 4, SSC12; lane 5, SSC13 (see Table 2 for relevant genotypes).

al., 1999) in which the *URA3* marker was amplified using primers containing 90 nucleotides of flanking *ARO4* homology. The third strategy was to use the *AUR1* dominant selectable marker (Hashida-Okado *et al.*, 1998) to disrupt the final copy of *ARO4*. None of these strategies produced a strain in which the second *ARO4* allele was disrupted. In addition, the medium used to select for double *aro4* disruptants in a $\Delta aro3::hisG/\Delta aro3::hisG$ background was supplemented with increased concentrations of each of the three aromatic amino acids in an attempt to rescue the potential auxotrophy of the double mutant. Despite these efforts we were unable to generate a disruption of

the remaining wild-type copy of *ARO4* in strain SPC311. Similarly, we were unable to disrupt the remaining wild-type *ARO3* allele in strain SSC9 (Table 2) using the $\Delta aro3::hisG-URA3-hisG$ deletion/insertion cassette previously described by Pereira & Livi (1996) (data not shown).

Construction of an inducible double *aro3 aro4* knockout strain

Recently, a system has been developed that utilizes the promoter of the tightly regulated *MET3* gene of *C. albicans* to assess essentiality and null phenotype of

Fig. 8. Effect of switching off *ARO4* expression on growth of *C. albicans*. Strains were pregrown for 4 h in SC liquid medium with or without 0.2 mM aromatic amino acids (SC+Aro or SC-Aro, respectively) and with or without 2.5 mM cysteine (C) and methionine (M). Cell densities were adjusted to OD₆₀₀ 0.1 in the appropriate medium, and 10-fold serial dilutions were spotted (15 µl) onto agar plates containing each medium.

genes in this organism (Care *et al.*, 1999; Warit *et al.*, 2000). The product of the *C. albicans* *MET3* gene is required for the biosynthesis of cysteine and methionine and its expression is strongly repressed in the presence of these amino acids (Care *et al.*, 1999). We constructed an integrating plasmid (pCaDis-Aro4) in which an extreme 5' fragment of the *ARO4* coding region was placed immediately 3' of the *C. albicans* *MET3* promoter. Transformation with this plasmid after restriction with *Bpl*I within the *ARO4* sequence results in a duplicative integration in the genome resulting in a 3' truncated version of the *ARO4* gene as well as a full-length copy under the control of the *MET3* promoter (see schematic in Fig. 7a).

We started with strain SPC311 ($\Delta aro3::hisG/\Delta aro3::hisG \Delta aro4::hisG/ARO4$), created using the Ura-blaster technique (as described above). Linearized plasmid pCaDis-Aro4 was introduced into cells of SPC311 and Ura⁺ transformants were selected. Transformants were screened by genomic PCR to identify those containing plasmid integrations at the remaining *ARO4* locus. A strain with the correct genotype was identified and called SSC12 ($\Delta aro3::hisG/\Delta aro3::hisG \Delta aro4::hisG/Met3p::ARO4$). Proper integration was verified by Southern blotting (Fig. 7b, lane 4). A second

isolate with identical genotype was also identified (strain SSC13, Fig. 7b, lane 5).

This strain was tested for growth in the presence or absence of aromatic amino acids under conditions of *ARO4* expression or repression (Fig. 8). In the absence of cysteine and methionine and the aromatic amino acids, strain SSC12 grows equally as well as its isogenic parent SPC208, and its progenitor strain CAI4, presumably as a consequence of *ARO4* expression. In contrast, strain SSC12 fails to grow in media supplemented with 2.5 mM cysteine and methionine in the absence of the aromatic amino acids tryptophan, tyrosine and phenylalanine. Thus, switching off expression of the last remaining copy of *ARO4* results in a conditional growth defect, and both Aro3p and Aro4p are apparently necessary for the growth of *C. albicans* on synthetic complete medium in the absence of exogenously supplied aromatic amino acids (Fig. 8). Inclusion of the three aromatic amino acids at standard concentrations (0.2 mM each) did not restore growth to wild-type *ARO4* levels (e.g. CAI4 and SPC208), but under these conditions some slow growth of SSC12 was observed (data not shown). Slow growth was also observed on rich (YEPD) medium, presumably due to the presence of some inhibitory levels of cysteine and

Fig. 9. Filter disc zone-of-growth assays to determine utilization of aromatic amino acids and pathway intermediates. Overnight cultures of *C. albicans* strain SSC12 were grown in SC-Aro-Cys-Met. Cysteine and methionine were added to 2.5 mM each while the culture was at mid-exponential phase, and the culture was allowed to grow for an additional 4 h. The culture was diluted with SC-Aro+2.5 mM Cys and Met to OD₆₀₀ 0.01 in 100 ml and poured onto agar plates containing SC-Aro+2.5 mM Cys and Met medium. Filter discs were soaked in water (control), 45 mM chorismic acid, or a mixture of tryptophan, phenylalanine and tyrosine at 5 mM each. The plates were allowed to grow at 30 °C for 24–36 h. In contrast to the spot assays shown in Figs 4, 6 and 8, photographs were taken with backlighting, so that the filter disc appears as dark a circle, and zones of growth are indicated by a dark halo of small colonies.

methionine, and was inhibited further with the addition of 2.5 mM each of cysteine and methionine (data not shown).

These data support the idea that, like *S. cerevisiae*, *C. albicans* contains two DAHP synthases, Aro3p and Aro4p. However, our results stand in contrast to studies in *S. cerevisiae*, where disruption of both *ARO3* and *ARO4* results in aromatic amino acid auxotrophy that can be fully relieved upon supplementation with the appropriate amino acids. To further investigate the nature of the observed phenotype we attempted to rescue the growth defect with higher concentrations of aromatic amino acids as well as several pathway intermediates. We found that the growth defect in strain SSC12 (under conditions where the *MET13* promoter is repressed) can be partially rescued in the presence of relatively high concentrations (5 mM each) of the three aromatic amino acids, or with chorismic acid (45 mM), as illustrated in filter disc zone-of-growth assays (Fig. 9). The zone-of-inhibition format was used because the response was not as easily observed in a spot assay (e.g. as in Fig. 8). Similar results were found with shikimic acid (100 mM) (data not shown). Chorismic acid is the branchpoint intermediate precursor for the synthesis of tryptophan, phenylalanine and tyrosine, as well as *p*-aminobenzoic acid, which is a necessary precursor for folate biosynthesis, whereas shikimic acid occurs upstream of chorismic acid in the pathway.

Concluding remarks

In contrast to the situation in *S. cerevisiae*, growth of *C. albicans* engineered to lack DAHP synthase activity is severely inhibited even upon provision of aromatic amino acids. Only a partial restoration of this defect is observed when these amino acids or certain metabolic pathway intermediates are supplied at high concentrations. We conclude that DAHP synthase activity is important for the normal growth of cells under standard laboratory conditions, even in the presence of 0.2 mM aromatic amino acids. A deficiency in amino acid uptake

appears unlikely since exogenous tyrosine and phenylalanine were found to cause feedback inhibition in *aro3/aro3* and *aro4/aro4* strains, respectively (Fig. 6).

In the present study we have demonstrated that cells of *C. albicans* contain a second, Aro4p-related, DAHP synthase whose expression is coordinately controlled by a GCN-like mechanism and whose activity is sensitive to feedback inhibition by tyrosine. In addition we have shown that in *C. albicans*, as in *S. cerevisiae*, Aro3p and Aro4p represent the only functional isozymes of DAHP synthase present in the cell. In contrast to studies in *S. cerevisiae*, however, we have found that when these isozymes are absent, cells growth is severely inhibited even when supplemented with phenylalanine, tyrosine and tryptophan. These results may partially explain our inability to generate a true *aro3Δ/aro3Δ aro4Δ/aro4Δ C. albicans* mutant. Cells devoid of DAHP synthase activity are highly compromised in terms of growth *in vitro*, but the observed growth defect can be restored to some extent by excess aromatic amino acids as well as certain metabolic intermediates of the aromatic amino acid pathway. The question remains, however, whether this pathway offers tractable targets for development of antifungal drugs. Although it has proven difficult thus far, we are continuing to try to create a true *aro3Δ/aro3Δ aro4Δ/aro4Δ* mutant under appropriately supplemented growth conditions for use in pathogenicity models for further target validation.

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