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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INTRODUCTION

The primary step in aromatic amino acid biosynthesis involves the enzyme 3-deoxy-D-arabinohexitulosonate-7-phosphate (DAHP) synthase. This enzyme catalyses the condensation of erythrose-4-phosphate and phosphoenolpyruvate to form 3-deoxy-D-arabinohexitulosonate-7-phosphate. This step is followed by a series of

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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 isoforms 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 (10⁶ cells ml⁻¹) in SC liquid medium supplemented with 1 µg ml⁻¹ of both thiamin and biotin. Cultures were split into two: 3-aminoimidazole (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 (ODₕ₀, 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 ODₕ₀, 10, washing with sterilized 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 ODₕ₀, 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 ODₕ₀, 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 ∆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 PSIBLAST (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

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<td>ARO4</td>
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*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 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 SacI–XhoI fragment; Gillum et al., 1984; Losberger & Erst, 1989) flanked by 1150 bp direct repeat sequences of the Salmonella typhimurium bisG gene (Alani et al., 1987; Fonzi & Irwin, 1993). The C. albicans ARO4 disruption plasmid pMB7Aaro4 was made by first amplifying a portion of ARO4 from the isolated YEp13-ARO4 library plasmid using primers 5'TCCTTCGATCTCATCACCAGATGGTGTTTTCTTG-3' and 5'ATTATGAGGTCGATCTTCTAGGTAATAGAAAATGTA-3' (restriction sites italicized), digesting with BgIII and SacI 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'TTACAGATCTGGTATATGCTATGAAATGGG-3' and 5'TCCTTTCGATCTTCTAGGTAATAGAAAATGTA-3' (restriction sites italicized), digested with SacI and BgIII and subcloned into the corresponding sites of pMB7ARO43, resulting in pMB7Aaro4. This plasmid was digested with SacI and Spel 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 bisG::URA3::bisG. Plasmid pCaDis-aro4 was constructed by PCR-amplifying a portion of the coding region of C. albicans ARO4 (nucleotides 367 to 667 of the GenBank sequence) as above using primers 5'AAAGGATCTCCTAGTATTCTTGTCAAATAT-3' and 5'AAACTCTCGAGTAAATGATGACATTAC-3' (restriction sites italicized), digesting with BamHI and PstI, 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 BpII, 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 22 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 CYPI 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'ATGCGCAAATGTCGGATCAGAGCAGGAGT-3' and 5'CAAGAAACCATACCATCGGATA-3', which are designed to amplify the 205 bp deletion/insertion site (see above) in ARO4. PCR analysis of Met3::ARO4 strains was performed as above using primers 5'TCCTAAGTTGTGATCCTTTCTTTT-3' (MET3 promoter; nucleotides 5221 to 5242 of pCaDis) and 5'CATGATAAATTCACAGTACTAC-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'TCCTAAAGTTGTGATCCTTTCTTTT-3' (MET3 promoter; nucleotides
RESULTS AND DISCUSSION

Cloning and characterization of the C. albicans ARO4 gene

We previously generated a 222 bp genomic PCR fragment from a homoygous ar03-deletion strain of C. albicans, using a degenerate primer based on a conserved N-terminal region of Ar03p (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 the yeast 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 was sequenced and 370 bp genomic DNA with BstXI or BstXI and Hpal 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).

The 370 aa C. albicans protein, herein designated Ar04p, is 57.3–62.3% identical (67.1–70.9% similar) to the Ar03p 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 Ar04p proteins of S. cerevisiae and Sch. pombe, suggesting a functional relationship (Fig. 1).

Molecular evolution of the deduced Ar04p protein

When compared to prokaryotic orthologues, C. albicans Ar04p 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 Ar03p (P14843) is called Ar0F, but is inhibited by phenylalanine like E. coli Ar0G (P00886). Meanwhile, S. cerevisiae Ar04p, called Ar0G (P32449), is inhibited by tyrosine as is E. coli Ar0F (P00888). There is no evidence in any yeast genome for a biochemical orthologue to E. coli tryptophan-regulated Ar0H (P00887).

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, Ar0G and Ar0H) are shown in bold. The six proteins (GenPept accession numbers from top to bottom U53216, X61107, AL17210, L12217, X13514, Z54112) were aligned using the PIELOOP 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
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
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 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 YEpl3 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). Complementation 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
alleles of ARO4 in strain CAI4, which is wild-type for ARO3 (∆ara3::imm434/∆ara3::imm434 ARO3/ARO3). This involved the use of an ∆aro4::hisG-URA3-bisG 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 ∆aro4::hisG-URA3-bisG fragment from plasmid pMB7Δaro4 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 ∆aro4::hisG-URA3-bisG/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 (Δaro4::hisG/ARO4) (Fig. 5, lane 3) was transformed with the same ∆aro4::hisG-URA3-bisG fragment as before, and Ura⁺ transformants were again selected. Genomic PCR was used to screen the genotypes of

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

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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 Δaro4::hisG allele. A few Δaro4::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 Δaro4::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 Δaro4::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 Δaro4::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 aro3 Δaro4 double disruption strain.

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

The same Δaro4::hisG-URA3-hisG deletion/insertion cassette used successfully to disrupt both copies of ARO4 in C.a14 was introduced into an Aro3p-deficient (Δaro3::hisG/Δaro3::hisG) strain, SPC101 (derived from strain SPC64; Pereira & Livi, 1996). This resulted in strain SPC208 (Δaro3::hisG/Δaro3::hisG).

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**Fig. 5.** Southern blot analysis of C. albicans aro4 mutants. Genomic DNAs were digested with BstXI 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 (Δaro4::hisG-URA3-hisG/ARO4); lane 3, strain SSC2 (Δaro4::hisG/ARO4); lane 4, strain SSC2U-23 (Δaro4::hisG/Δaro4::hisG-URA3-hisG); duplication of disruption cassette verified by PCR, labelled as Δaro4::hisG-URA3-hisG (x 2) for brevity); lane 5, SSC3 (Δaro4::hisG/Δaro4::hisG).

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**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 (Δaro3::hisG/Δaro3::hisG ARO4::hisG/ARO4), and SSC2U-23 (Δaro4::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.

Δaro4::hisG-URA3-hisG/ARO4), and the subsequent URA3 popout strain (selected on 5-FOA) SPC311 (Δaro3::hisG/Δaro3::hisG Aro4::hisG/ARO4); again, genotypes were confirmed by genomic PCR (data not shown) and Southern blotting (Fig. 6b, 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 (Δaro4::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 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 pMB7Aro4, 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
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Fig. 7. (a) Schematic showing construction of *C. albicans* cells lacking Aro3p and Aro4p. Starting with strain SPC101 (ARO4/ARO4 Δaro3::hisG/Δaro3::hisG) (line 2), one wild-type ARO4 allele was disrupted via the Ura-blastere technique (Fonzi & Irwin, 1993) using plasmid pMB7Δaro4 linearized by digestion with SacI and SphI. A resulting strain, SPC208, contains one wild-type ARO4 allele (as in line 2) and an Δaro4::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 Δaro4::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 BplI and introduced into cells of SPC311, selecting for Ura⁺ transformants. Strain SSC12 contains the Δaro4::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, BamHI; Bp, BplI; Bs, BstXI; Hp, HpaI; Ps, PstI. 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 of MET3 promoter. Genomic DNA from each strain was digested with BstXI and HpaI. 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 Δaro3::hisG/Δaro3::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 Δaro3::hisG-URA3-hisG deletion/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
genes in this organism (Care et al., 1999; Warit et al., 2000). The product of the \textit{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 \textit{C. albicans} MET3 promoter. Transformation with this plasmid after restriction with BplI 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 (\textDelta{}aro3::hisG/\textDelta{}aro3::hisG \textDelta{}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 (\textDelta{}aro3::hisG/\textDelta{}aro3::hisG \textDelta{}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 \textit{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 methionine.
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 ar3/aro3 and ar4/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 isoforms 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 ar3Δ/aro3Δ ar4Δ/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 ar3Δ/aro3Δ ar4Δ/aro4Δ mutant under appropriately supplemented growth conditions for use in pathogenicity models for further target validation.

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