The AR04 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 AR03 and AR04, whose activities are feedback-inhibited by phenylalanine and tyrosine, respectively. AR03/4 gene transcription is controlled by GCN4. The authors previously cloned an AR03 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 AroM, 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 AR03 and AR04 arose from a single gene via a gene duplication event early in fungal evolution. C. albicans AR04 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 AR04 complements an aro3 aro4 double mutation in S. cerevisiae, an effect inhibited by excess tyrosine. The authors engineered ∆aro3/∆aro3 ∆aro4/MET3p::AR04 cells of C. albicans (with one wild-type copy of AR04 placed under control of the repressible MET3 promoter) and found that they fail to grow in the absence of aromatic amino acids when AR04 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 reactions leading to the formation of phenylalanine and tyrosine.
isozyme has also been reported in the fungus. Activity is inhibited by tryptophan. Existence of a third isozyme 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 anti-fungal 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 medium 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 (OD₅₆₂, 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 OD₅₆₂ 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 OD₅₆₂ 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 µM cysteine and methionine. Cell densities were adjusted to OD₅₆₂ 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 Δaro3 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 databases 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
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<table>
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<tr>
<td>S. cerevisiae</td>
<td>ARO3</td>
<td>-10\text{GTGACTAAT} -88</td>
<td>Paravincini et al. (1988)</td>
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<tr>
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<td>ARO4</td>
<td>-31\text{ATGACTCAAA} -34</td>
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</tr>
<tr>
<td>C. albicans</td>
<td>ARO3</td>
<td>-19\text{TTGACTAAT} -173</td>
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</tr>
<tr>
<td></td>
<td>ARO4</td>
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<td>This study†</td>
</tr>
</tbody>
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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.

Aromatic amino acid pathway in C. albicans

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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 (Perea & Livi, 1996). The nucleotide sequence of this PCR fragment predicts a 74 aa DAHP-synthase-related peptide with strong homology to known Aro3p 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 to position 57 (reverse complement of nucleotides 1330 to 1350 (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, AroH (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).
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 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). 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 (Δura3::imm434/Δura3::imm434 ARO3/ ARO3). This involved the use of an Δaro4::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 UraΔ− popout mutants.

Briefly, strain CAI4 was transformed with the 5.2 kb SacI–SphI Δaro4::hisG-URA3-bisG fragment from plasmid pMB7Δaro4 and UraΔ− 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 UraΔ− 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 Δaro4::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 UraΔ− (URA3 popout) mutants. Several UraΔ− 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 Δaro4::hisG-URA3-bisG fragment as before, and UraΔ− transformants were again selected. Genomic PCR was used to screen the genotypes of

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

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<td>Fonzi &amp; Irwin (1993)</td>
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<tr>
<td>SPC64</td>
<td>SPC10</td>
<td>Δaro4</td>
<td>Pereira &amp; Livi (1996)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S. cerevisiae strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH1368</td>
<td>MATα aro3-2 aro4-1 gcn1-1 trp1-1 leu2-3</td>
<td>Kuznler et al. (1992)</td>
</tr>
<tr>
<td>DC17</td>
<td>MATα bis1</td>
<td>Klar et al. (1985)</td>
</tr>
</tbody>
</table>
Attempts to create a homozygous \( \Delta \text{aro}3/\Delta \text{aro}3 \) \( \Delta \text{aro}4/\Delta \text{aro}4 \) mutant

The same \( \Delta \text{aro}4::\text{hisG-URA3-hisG} \) deletion/insertion cassette used successfully to disrupt both copies of \( \text{ARO}4 \) in CaI4 was introduced into an Aro3p-deficient \( \Delta \text{aro}3::\text{hisG}/\Delta \text{aro}3::\text{hisG} \) strain, SPC101 (derived from strain SPC64; Pereira & Livi, 1996). This resulted in strain SPC208 \( \Delta \text{aro}3::\text{hisG}/\Delta \text{aro}3::\text{hisG} \). To address this possibility, three alternative strategies were tried. First, strain SPC311 was transformed with plasmid DNA in which the orientation of the \( \text{hisG-URA3-hisG} \) cassette was reversed relative to the flanking \( \text{ARO4} \) sequences in plasmid pMB7\( \text{aro}4 \), with the intent to reduce the occurrence of double crossover events between the \( \text{hisG} \) repeats of the introduced DNA and the \( \text{hisG} \) of the previously disrupted allele. The second was a nested PCR-based disruption strategy (Wilson et
Aromatic amino acid pathway in \textit{C. albicans}\(^{a}\)

\(\text{Daro4::hisG-URA3-hisG}\)

\(\text{ARO4}\)

\(\text{MET3p::ARO4}\)

\(\text{Bh}\)

\(\text{Bs}\)

\(\text{Bp}\)

\(\text{Hp}\)

\(\text{Bp}\)

\(\text{probe}\)

Fig. 7. (a) Schematic showing construction of \textit{C. albicans} cells lacking Aro3p and Aro4p. Starting with strain SPC101 (\textit{ARO4/ARO4} \textit{Δaro3::hisG/Δaro3::hisG}) (line 2), one wild-type \textit{ARO4} allele was disrupted via the Ura-blaster technique (Fonzi \& Irwin, 1993) using plasmid pMB7\textit{Δaro4} linearized by digestion with Sac\textit{I} and Sph\textit{I}. A resulting strain, SPC208, contains one wild-type \textit{ARO4} allele (as in line 2) and an \textit{Δaro4::hisG-URA3-hisG} allele (as in line 3). Selection on 5-FOA resulted in loss of the \textit{URA3} marker via recombination between the bacterial \textit{hisG} repeats. One strain, SPC311, was obtained which contains one wild-type \textit{ARO4} allele (as in line 2) and an \textit{Δaro4::hisG} allele (as in line 4). The remaining wild-type \textit{ARO4} allele of strain SPC311 was subsequently placed under the control of the \textit{C. albicans MET3} promoter.

Plasmid pCaDis-aro4, containing the \(5\) \textit{ARO4} coding region of \textit{ARO4} (see Methods) was linearized with Bpl\textit{I} and introduced into cells of SPC311, selecting for Ura\textsuperscript{+} transformants. Strain SSC12 contains the \textit{Δaro4::hisG} allele (as in line 4) as well as the \textit{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, \textit{BamHI}; Bp, Bpl\textit{I}; Bs, Bst\textit{XI}; Hp, Hpal; Ps, Pst\textit{I}. \textit{MET3p} is the \textit{C. albicans MET3} promoter (Care \textit{et al.}, 1999). (b) Genomic Southern blot analysis demonstrating sequential disruption of \textit{ARO4} and knock-in of the \textit{MET3} promoter. Genomic DNA from each strain was digested with Bst\textit{XI} and Hpal. The probe was an \textit{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).

\textit{al.}, 1999) in which the \textit{URA3} marker was amplified using primers containing 90 nucleotides of flanking \textit{ARO4} homology. The third strategy was to use the \textit{AUR1} dominant selectable marker (Hashida-Okado \textit{et al.}, 1998) to disrupt the final copy of \textit{ARO4}. None of these strategies produced a strain in which the second \textit{ARO4} allele was disrupted. In addition, the medium used to select for double \textit{aro4} disruptants in a \textit{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 \textit{ARO4} in strain SPC311. Similarly, we were unable to disrupt the remaining wild-type \textit{ARO3} allele in strain SSC9 (Table 2) using the \textit{Δaro3::hisG-URA3-hisG} deletion/insertion cassette previously described by Pereira \& Livi (1996) (data not shown).

**Construction of an inducible double \textit{aro3} \textit{aro4} knockout strain**

Recently, a system has been developed that utilizes the promoter of the tightly regulated \textit{MET3} gene of \textit{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 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 BpiII 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 (Δaro3::hisG/Δaro3::hisG Δaro4::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 (Δaro3::hisG/Δaro3::hisG Δaro4::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 (YPD) medium, presumably due to the presence of some inhibitory levels of cysteine and

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.

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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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