Cloning, analysis and one-step disruption of the ARG5,6 gene of Candida albicans

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The ARG5,6 gene from the dimorphic fungus Candida albicans was cloned by functional complementation of the arginine auxotrophy present in strain EL2 (Arg') using a gene library constructed in the double autonomously replicating sequence vector pRM1. Sequence analysis revealed a putative 857 amino acid polypeptide (95 kDa) which showed high homology (63% protein identity) to the Saccharomyces cerevisiae ARG5,6 gene. Similarly to the S. cerevisiae gene, the C. albicans ARG5,6 gene is responsible for both the acetylglutamate kinase and acetylglutamyl-phosphate reductase activities, the second and third steps of arginine biosynthesis at the mitochondria. The C. albicans ARG5,6 gene complemented the arg6 mutation present in S. cerevisiae (strain D160-4D) on a yeast episomal plasmid using its own regulatory signals. A set of non-integrative high-efficiency plasmid vectors based on this gene marker was constructed and a null C. albicans arg5,6A strain was obtained using the common URA3-blaster strategy. In addition, we generated an arg5,6A null mutant in a single transformation event, thus improving the basic strategy for generating gene deletions in C. albicans.

Keywords: ARG5,6, Candida albicans, molecular biology, gene disruption, arginine

INTRODUCTION

Despite the importance of Candida albicans as a model pathogenic yeast, obtaining information about its genetics has been difficult due to its diploidy and lack of sexual cycle (Kurtz et al., 1990; Scherer & Magee, 1990). Although C. albicans genetics have largely relied on Saccharomyces cerevisiae as an intermediate genetic host, some of the most interesting and peculiar processes in C. albicans, such as its ability to switch between a yeast and a mycelial form of growth and its pathogenicity to humans (Odds, 1988), cannot readily be studied vicariously through a knowledge of S. cerevisiae. The development of an efficient transformation system, with the construction of both autoreplicative plasmids and suitable host strains, is then one of the major goals to facilitate genetic analysis of the biology and pathogenicity of C. albicans.

Although Kurtz and colleagues described both the integrative transformation (Kurtz et al., 1986) and the development of autoreplicative plasmids (Kurtz et al., 1987) in Candida, plasmids often multimerize in C. albicans leading to difficulties in their isolation (Goshorn et al., 1992). We recently developed plasmids carrying two independent autonomously replicating sequences (Cannon et al., 1990; Herreros et al., 1992) that allow the introduction and recovery of DNA in C. albicans (Pla et al., 1995). A second important tool in C. albicans molecular genetics is the availability of isogenic auxotrophic host strains. Although both UV-enhanced mitotic recombination (Kelly et al., 1987; Sadhu et al., 1992) and toxic metabolites (Gorman et al., 1991) have been used for gene disruption in C. albicans, the most commonly employed system now used makes use of the strategy adapted by Fonzi & Irwin (1993), which requires two sequential steps of gene disruption using the C. albicans URA3 gene flanked by a heterologous hisG sequence from Salmonella typhimurium. This process could, however, be shortened if different genetic markers and appropriate strains were available for sequential disruptions. Although some nutritional genes have been isolated (Rosenbluh et al., 1985; Goshorn et al., 1992; Hoyer et al., 1994; Pla et al., 1995), there are no reports of strains with more than one auxotrophic marker obtained entirely by gene disruption.

The EMBL accession number for the nucleotide sequence reported in this paper is X98880.
In this work, we describe the isolation of the ARG5,6 gene from *C. albicans* by complementation of a mutagenized *C. albicans* arginine auxotrophic strain and its use in developing a genetic transformation system. In addition, we have constructed a triple *ura3Δ his1Δ arg5,6Δ* strain and demonstrate the generation of a homozygous *arg5,6Δ* null strain in a single-transformation event.

**METHODS**

**Strains and growth conditions.** *C. albicans* and *Escherichia coli* strains are listed in Table 1. *C. albicans* strain 1001 (ATCC 64385), a wild-type strain from the Spanish Type Culture Collection, was used as the source of genomic DNA for the construction of a genomic library in plasmid pRM1 (Pla et al., 1995). The following *S. cerevisiae* strains, obtained from the Yeast Genetic Stock Center (Berkeley, CA, USA), were used to check the function of the cloned *C. albicans* ARG5,6 gene by complementation: X3163-4C (MATα arg1Δ met1 trp3 ade5 ura3 leu1 mal gal2); STX63-8B (MATα arg2 lys4 lys1 trp4 ade2 try1 rad2 gal2); STX9-1A (MATα arg3 ade2 gal2); AB18-20A (MATα arg4-8 ade2 ade5 cUP11 leu2-3, 112 ura3-52 met13 cyb6); D160-4D (MATα arg6 ura3 bom3 his1 trp2 ade1 met1 gal2); X1049-9C (MATα arg8 trp1 ura3 his3 asp5); XJ17-2 (MATα arg9 thr4 leu2 ade6 gal2); and STX14-1C (MATα arg10 ade2 gal2). Yeast strains were grown in either YED medium (1%, w/v, yeast extract; 2%, w/v, glucose) or SD minimal medium (0.67% Yeast Nitrogen Base without amino acids; 2%, w/v, glucose), supplemented with uridine, histidine or arginine at 25 μg ml⁻¹, depending on the nutritional requirements, with continuous shaking at 30°C. *C. albicans* Ura+ revertants were selected on 5-fluoroorotic acid plates as described previously by Navarro-García et al. (1995). *E. coli* strains were grown in Luria-Bertani (LB) or Terrific Broth (TB) at 37°C supplemented with 100 μg ampicillin ml⁻¹ for plasmid selection.

**DNA manipulations.** All DNA manipulations were carried out following standard procedures (Sambrook et al., 1989). Southern hybridization analysis was carried out using the Nonradioactive Labelling and Detection kit (Boehringer Mannheim) under high-stringency conditions on positively charged nylon membranes. For the determination of the ARG5,6 sequence, a 3.8 kb *BamHI-BamHI* insert from plasmid pAN1 was purified from agarose gels and sonicated to generate random fragments (300–600 bp in size) which were subcloned into the *SmaI* site of pUC19. Plasmid DNA was purified from *E. coli* transformants with Qiagen (Diagen) and both strands were sequenced with an Automated Sequencer (ALF; Pharmacia) according to Sanger et al. (1977) using the Universal and Reversal fluoresceinated primers. Sequence comparisons and homologies were carried out using the FASTA algorithm (Pearson & Lipman, 1988). DNA sequence analysis was performed with the PCGENE software.

**Plasmid constructions and gene disruption.** Plasmid pRM1, an efficient non-integrative plasmid, as well as its derived gene library have been described before (Pla et al., 1995). The *E. coli*/yeast shuttle vector YEp352 has also been described previously by Hill et al. (1986). YEpARG was obtained by subcloning a 3.8 kb *BamHI-BamHI* fragment from pAN1 into the *BamHI* site of YEp352. Plasmid pAN8, an autoreplicative plasmid which transforms *C. albicans* with high efficiency (>5 × 10⁶ transformants (μg DNA)⁻¹) and which is representative of a set of autoreplicative plasmids bearing this marker, was obtained by replacing the *NaeI–SmaI* fragment of pRM1 (Pla et al., 1995) with a blunt-ended *BamHI–BamHI* fragment from pAN1. For the disruption of the *C. albicans* ARG5,6 gene, the same 4 kb *BamHI–BamHI* fragment was subcloned into pUC19 yielding pUC-ARG. An internal *SnaBI–BglII* fragment (which comprised most of the ARG6 domain) was then replaced with either the *BamHI–BglII* fragment of pCUB-6 (Fonzi & Irwin, 1993) (carrying the *hisG–URA3–hisG* cassette) or the *KpnI–SspI* fragment from YEp-HISX (Pla et al., 1995) (carrying the *C. albicans* HIS1 gene).

**Table 1.** Strains used in this work

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Genotype/phenotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>1001*</td>
<td>Wild-type</td>
<td>Gil et al. (1988)</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>SC314</td>
<td>Wild-type</td>
<td>Gillium et al. (1984)</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>EL2†</td>
<td>Arg− Ade−</td>
<td>Gil et al. (1988)</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>CA14</td>
<td><em>ura3Δ:imm434/ura3Δ:imm434</em></td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>1006</td>
<td><em>MPA1 ser57 ura3 lys1 arg4</em></td>
<td>Unpublished</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>RM1000</td>
<td><em>ura3Δ:imm434/ura3Δ:imm434 his1Δ:hisG his1Δ:hisG</em></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>CNC40</td>
<td>(RM1000) <em>arg5,6Δ:hisG–URA3–hisG/ARG5,6</em></td>
<td>This work</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>CNC41</td>
<td>(RM1000) <em>arg5,6Δ:hisG/ARG5,6</em></td>
<td>This work</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>CNC42</td>
<td>(RM1000) <em>arg5,6Δ:hisG/arg5,6Δ:hisG–URA3–hisG</em></td>
<td>This work</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>CNC43</td>
<td>(RM1000) <em>arg5,6Δ:hisG/ARG5,6</em></td>
<td>This work</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>CNC44</td>
<td>(RM1000) <em>arg5,6Δ:hisG–URA3–arg5,6Δ:hisG</em></td>
<td>This work</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>MC1061</td>
<td><em>araD139 araL (ara-139) leu7776 (lacZΔM15 lacX74 galU galK strA</em></td>
<td>Hanahah (1988)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>DH5αF−</td>
<td><em>K12 Δ(lacZYA-argF)U169 supE44 thi1 recA1 endA1 hsdR17 gyrA relA1 (Δ80lacZAM15) F−</em></td>
<td></td>
</tr>
</tbody>
</table>

* A wild-type strain from the Colección Española de Cultivos Tipo (Spanish Type Culture Collection), ATCC 64385.
† An Arg− Ade− auxotroph derived from strain 1001.
‡ Designated arg57 (Goshorn & Scherer, 1989) and later shown to be arg4 mutant (Hoyer et al., 1994).
§ RM1000 background.
RESULTS AND DISCUSSION

Isolation of the C. albicans ARG5,6 gene

Strain EL2 is an arginine and adenine auxotroph obtained through ethylmethane sulfonate mutagenesis of strain 1001 as described previously (Gil et al., 1990). This auxotrophic mutant was used for the generation of a set of C. albicans mutants altered in their ability to undergo the dimorphic transition (Gil et al., 1988). We first verified that the arginine biosynthetic defect present in strains EL2 and 1006 (arg4; Hoyer et al., 1994) was different since it was possible to isolate prototrophic hybrids when parasexual crosses between both strains were carried out. A gene library constructed in the double autonomously replicating sequence vector pRM1 (Pla et al., 1995) was used to isolate the complementing gene. Screening of approximately 50,000 transformants yielded 14 prototrophs after 7 d growth in selective medium. Only one of them was characterized further. Plasmid DNA, designated pAN1, was extracted from this transformant and was shown to be capable of complementing the auxotrophic phenotype upon retransformation. Standard restriction analysis revealed an insert of approximately 7 kb, with a minimal 3-8 kb BamHI–BamHI complementing region (Fig. 1). When present in the episomal plasmid YEP332 (plasmid YEP-ARG), this DNA was shown to complement the arg6 defect present in strain D160-4D (an arg6 mutant). These results demonstrate the usefulness of these replicative plasmids (Cannon et al., 1992; Pla et al., 1995) in C. albicans genetic manipulation.

Sequence analysis of the C. albicans ARG5,6 gene

Sequence analysis of the insert revealed a putative ORF of 857 amino acids (95 kDa). This protein was 63% identical to the predicted translation product of the ARG5,6 gene of S. cerevisiae (54% identical to Schizosaccharomyces pombe Arg5p), thus indicating that we had cloned the C. albicans ARG5,6 gene. In S. cerevisiae, this gene is responsible for the generation of a polypeptide which is post-translationally processed yielding two mature polypeptides which encode the acetylglutamate kinase (ARG6) and acetylglutamate-phosphate reductase (ARG5) activities in the arginine biosynthetic pathway (Boonchird et al., 1991). Thus, in C. albicans, the genetic organization of the ARG5,6 locus is apparently similar to that in S. cerevisiae (Boonchird et al., 1991) and Schiz. pombe (van Huffel et al., 1992) and contrasts with the situation in prokaryotes where each activity is encoded by a separate gene. A putative TATA box was found around position 253 (Fig. 2), while a putative transcription termination signal was at 2943–2967 (based on their homology to S. cerevisiae consensus signals). No obvious S. cerevisiae GCN boxes (consensus sequence TGACTC) could be found in the 5′-upstream region, as has been observed for the C. albicans ARG3 (Pereira & Livi, 1995) and ARG4 (Hoyer et al., 1994) genes. In addition to the arg4 mutant strains 1006 and TMSU221, another complementation group designated arg100 has been described for strains A642, hOG318, hOG357, FC18-6 and WC-5-4 (Hoyer et al., 1994) located on the R chromosome. pAN8, an auto-replicative plasmid bearing the ARG5,6 gene, did not complement hOG318 arginine auxotrophy.

Construction of an arg5,6A null mutant

We used two different strategies to obtain an arg5,6A mutant in the ura3Δ his1A strain RM1000 background. We first used the URA3-blaster protocol to generate an arg5,6A mutant. Using the URA3 marker (construction
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Fig. 2. Nucleotide sequence of a Scal-BamHI fragment encoding C. albicans ARG5,6 with its deduced amino acid translation. The presumed TATA box region (underlined) and the putative transcription termination signal (double underlined) are shown. Scal and BamHI sites are highlighted in bold.

pUC-ARG-U), a clone was selected which was shown by Southern blotting to integrate the URA3-blaster construction at the ARG5,6 locus, yielding the heterozygous strain CNC40. Excision of URA3 was achieved on 5-fluoroorotic acid plates, yielding strain CNC43. Strain CNC40 was transformed with its deduced amino acid sequence of 15%.

Finally, excision of URA3 on 5-fluoroorotic acid plates yielded strain CNC43 (ura3Δ his1Δ arg5,6Δ). In an alternative approach, the C. albicans HIS1 gene (Pla et al., 1995) was used to replace an internal region of C. albicans ARG5,6 (ura3A his2A arg5,6A). Strain CNC40 was transformed with pUC-ARG-H making use of HIS1 (see Methods) as a selectable marker (Fig. 1). Fifteen of 21 Ura+ His+ transformants (70% were shown to be arginine auxotrophs. This strain was efficiently transformed into arginine protrophy with plasmid pAN8 (see Methods).
The availability of an appropriate genetic marker, the C. albicans ARG5,6 gene, allowed us to check the feasibility of a single-step gene disruption in C. albicans. We cotransformed strain RM1000 (his1A ura3Δ) with both URA- and HIS-blaster constructions. A cotransformation frequency of approximately 10% was found, similar to that quoted by other authors (Kurtz et al., 1986, 1990). Ten per cent of the prototrophic clones were shown to be arginine auxotrophs (strain CNC44) carrying the correct (i.e. homologous) recombination event at the ARG5,6 locus. This result is in general agreement with estimated frequencies for single recombination events (70% and 15%).

We conclude that it is possible to generate a deletion in a specific C. albicans gene in a single transformation step. This should expedite the generation of homoygous null mutants by integrative transformation by reducing substantially the number of steps required to achieve the disruption of the two alleles at each genetic locus. Strains with different gene markers (like the ura3Δ his1A arg5,6A strain described in this work) and suitable for genetic transformation should allow the deletion of both alleles of a specific gene while maintaining the wild-type gene on an episomal plasmid.

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