Large-Scale Identification of Putative Exported Proteins in *Candida albicans* by Genetic Selection

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In all living organisms, secreted proteins play essential roles in different processes. Of special interest is the construction of the fungal cell wall, since this structure is absent from mammalian cells. The identification of the proteins involved in its biogenesis is therefore a primary goal in antifungal research. To perform a systematic identification of such proteins in *Candida albicans*, we carried out a genetic screening in which in-frame fusions with an intracellular allele of invertase gene *SUC2* of *Saccharomyces cerevisiae* can be used to select and identify putatively exported proteins in the heterologous host *S. cerevisiae*. Eighty-three clones were selected, including 11 previously identified genes from *C. albicans* as well as 41 *C. albicans* genes that encode proteins homologous to already described proteins from related organisms. They include enzymes involved in cell wall synthesis and protein secretion. We also found membrane receptors and transporters presumably related to the interaction of *C. albicans* with the environment as well as extracellular enzymes and proteins involved in different morphological transitions. In addition, 11 *C. albicans* open reading frames (ORFs) identified in this screening encode proteins homologous to unknown or putative proteins, while 5 ORFs encode novel secreted proteins without known homologues in other organisms. This screening procedure therefore not only identifies a set of targets of interest in antifungal research but also provides new clues for understanding the topological locations of many proteins involved in processes relevant to the pathogenicity of this microorganism.

Fungal infections are currently an important source of morbidity and mortality in several countries. Fungi cause a range of diseases, ranging from relatively moderate superficial infections to severe systemic diseases that are often life threatening. Their treatment is difficult because of the close similarity between mammalian and microbial cells and is mainly based on polyenes and azoles (6). Polyenes have several side effects which can be partially overcome with the introduction of new liposome-based formulations. In contrast, emerging resistance to azoles may seriously compromise their usefulness in the near future (67, 85). All the foregoing aspects have prompted the development of new strategies for the search for novel antifungal targets. Because of its absence in mammalian cells, the fungal cell wall, like that of bacteria, is the most attractive multienzymatic target, conferring potential selectivity to anti-microbial agents through its inhibition (21). The cell wall is a highly dynamic structure that is composed mainly of glucan (β-1,3 and β-1,6 type), chitin, and cell wall mannoproteins. These components are essential to fungal cells either as cross-linking enzymes that covalently join different proteins to the β-1,3, β-1,6, or chitin fractions of the wall or as structural components. They are also involved in morphogenesis and may regulate the exchange of compounds with the extracellular medium. In pathogenic fungi, cell wall proteins play a key role in the relationship between the fungal cell and the host, participating in pathogenesis through adhesion phenomena and modulation of the immune response (15).

The cell wall is not the only important source of potential targets, and other exported proteins can be also considered in this context. Membrane proteins, for example, play an essential role in fungal physiology because they are involved in nutrient transport, energy generation, and signal transduction pathways, ultimately leading to growth and host adaptation. Extracellular enzymes encode hydrolytic enzymes, such as lipases (47, 81), proteases (36, 37), and cell wall-hydrolyzing enzymes (32, 50), that are involved in pathogenicity and virulence, mainly through their contribution to invasion. The identification of these secreted proteins is thus of enormous interest because their inhibition may ultimately lead to cellular death, either as a direct consequence of cellular processes—such as the inhibition of cell wall assembly—or because they are novel virulence factors whose inhibition enables the host immune response to control and eradicate infection.

The identification of exported proteins has remained elusive for several reasons. Biochemical separation of cell wall proteins is complicated by their high level of glycosylation and cross-linking to other cell wall components (17, 43). Genetic methods are therefore an alternative approach to analyzing the localization of these proteins. Although there are no intrinsic specific domains characteristic of exported proteins, most of them have a signal peptide: a 10- to 30-amino-acid-long amino-terminal domain that has a characteristic hydrophobicity profile, that is cleaved off during secretion, and that is responsible for introducing the protein into the secretory pathway (18, 78). Although its existence is neither necessary nor sufficient to unambiguously determine whether a given protein will be present at the cell surface, it can be used as an attractive

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topological signal to devise genetic screenings with gene reporters and/or gene tags whose localizations can be efficiently traced inside the cell (66).

In this work, we undertook a genetic approach to the identification of exported proteins in *Candida albicans*; the most frequently isolated fungal pathogen in clinical samples. We made use of genetic selection based on the Saccharomyces cerevisiae invertase gene, SUC2, by means of a strategy in which in-frame fusions with an intracellular allele of this gene can identify putative export signals. This strategy has already proved successful in a mammalian system (45). We thus identified several exported proteins—with several putative new cell wall components—constituting an important source of potential antifungal targets.

**MATERIALS AND METHODS**

Strains and DNA manipulations. Due to the poor transformation efficiencies obtained with the original *S. cerevisiae* sac2 mutant strain, SEY2101 (MATa sac2-1 yEP3-1-112 ade2-1 sac2-29) (27), an Ade· spontaneous revertant, named SS10, was obtained from this strain. *C. albicans* 1001 is a wild-type strain from the Spanish Type Culture Collection (ATCC 64385) (30) and was used as the source of genomic DNA for the preparation of the genomic libraries. *C. albicans* SC5314 is a wild-type strain (31) frequently used in *C. albicans* genetic studies. All DNA manipulations were carried out by standard procedures (2, 72). Escherichia coli DH5α (K-12 lacZΔM15 tetR65 supE44 thr-1 relA1 endA1 hsdR17 recA1 araD139 proAB thi-1 gyrA96 Δ(lac-proA)15Tc proAB1) (Stratagene) was used as the source of DNA-modifying enzymes and was obtained from Boehringer Mannheim (Mannheim, Germany).

**Genetic constructions.** Three different suc2 alleles lacking the signal peptide and Met131 (suc2m3, suc2m5, and suc2m6) were constructed by PCR amplification of the SUC2 gene in order to achieve in-frame fusions with fragments obtained from the genomic DNA. The three alleles were amplified by using an Expand High-Fidelity PCR system from Boehringer Mannheim and pLC1 (a centromeric plasmid [obtained from P. Sanz] that comprises the wild-type *SUC2* gene) (23) as a template. Three pairs of oligonucleotides were used: SUC2mut (5'-AAAGATATCCTGGATATGCTT-3') and SUC2mut2 (5'-AAAGAATCCATCCTTCAAGACCTATCATTGG-3') for suc2m3 amplification, SUC2mut and SUC2mut2 (5'-AAAGAATCCATCCTTCAAGACCTATCATTGG-3') for suc2m5 amplification, and SUC2mut and SUC2mut3 (5'-ATAG ATCCCTCCGGGACACCGAATTACAGC-3') for suc2m6 amplification, and SUC2mut2 and SUC2mut3 (5'-ATAG ATCCCTCCGGGACACCGAATTACAGC-3') for suc2m6 plus amplification. A BglII restriction site was introduced within the oligonucleotides at the 5' end for allowing the construction of in-frame fusions in all three reading frames with Sac3A-derived fragments (Fig. 1A). After PCR amplification, suc2m3 was rendered blunt ended with the Klenow fragment of DNA polymerase and inserted into the Smal site of the EcoRI shuttle vector pSE1. The Sac-HindIII fragment of pSE1, which carries suc2m2, was subcloned into the Sac-HindIII sites of YCplac33B (a centromeric yeast vector obtained as a YCplac33 derivative from which the BglII site of the pSC2 and pSE2 to produce plasmids pSE2phex, pSE2phexxi, and pSE2phexxii, and the suc2m6 and suc2m7 alleles were first subcloned into vector pGEM-T (Promega), excised as a BamHI fragment, and finally inserted into pSac or EcoRI-PhII opened YEp352 in order to generate pSac and pSacE, respectively. The SacI-SacI fragment carrying suc2m6 was also subcloned into the SacI site of YCplac33B to obtain pSacC.

The primers used were (GGCGAGCTCATTTTATCATGTTTCGTTTGT) and lo-hex1 (GAGCATCAGATCAGATATTTTGGGC) were designed and used for the amplification of a DNA fragment carrying the *S. cerevisiae* SUC2 promoter and signal sequence from plasmid pLC1. The PCR fragment was cloned into vector pT7Blue-T (Novagen) to make pT7psuc. sucpl, has been designed in order to obtain a set of fragments encoding different amino acids at position 21 (Met in SUC2), although sequencing eventually revealed that the fragment in pT7psuc encoded Thr21. After the amplified DNA was excised from this plasmid with BamHI-BamHI, it was subcloned into the BglII sites of plasmids pSE1, pSE1C, and pSE3, resulting in pSE1psuc, pSE1Cpsuc, pSE1Cpsuc, pSE3psuc, and pSE3psuc. A DNA fragment containing the promoter, the signal sequence, and the coding sequence up to amino acid 37 of the HEX7 promoter and signal sequence fused in frame with the suc2m2 allele, while pSE3psuc contains the same DNA insert cloned in the opposite orientation.

For assessing the functionality of these constructs (Fig. 1B), 10· cells of *S. cerevisiae* SS10 transformants carrying various plasmids were spotted onto YEP-sucrose solid medium (1% yeast extract, 2% peptone, 2% sucrose, 2% agar, 1 μg of ampicillin A/ml) and grown at 24°C. pSC1psuc, pSC1psuc, and pSC2psuc bear the SUC2 promoter and signal sequence fused in frame with the corresponding suc2m2 allele, while in pSC1psuc and pSC3psuc the same DNA insert is cloned in the opposite orientation. pLC1 is a centromeric plasmid that contains the SUC2 gene.

**Library construction and screening.** *C. albicans* genomic DNA was extracted from strain 1001 grown at 30°C overnight in SD medium (2% glucose, 0.67% yeast nitrogen base without amino acids) supplemented with a mixture of amino acids. Genomic DNA was partially digested with Sac1A by use of appropriate dilutions to obtain a high proportion of 0.5- to 4-kb fragments. After preparation electrophoresis, fragments of 0.5 to 2 kb were eluted with dialysis bags; incorporated into episomal vectors pSE1, pSE2, and pSE3; digested with BglII; and treated with calf intestinal alkaline phosphatase to avoid recircularization. After standardization of the optimal vector DNA/gene-fragment ratio for ligations with each of the three vectors, a series of ligation mixtures were pooled. This procedure afforded three fusion libraries in the three different frames, each of which accounted for more than 95% of the recombinant clones. Totals of 231,000, 192,360, and 128,860 colonies were obtained from the pSE1, pSE2, and pSE3 libraries, respectively. The recombinant clones had an average insert size of 1 to 1.3 kb, as determined with several small-scale preparations of plasmid DNA. After the first screening experiments, SS10 was transformed by electroporation (ElectroCell Manipulator 600; BTX Laboratories) with published protocols (4) and with 186 and 1,400 V as optimal experimental conditions. *Ura°* transformants were selected on solid SD medium supplemented with amino acids and 1 M sorbitol for osmotic protection. They were then replica plated on YEP-sucrose solid medium, and growth was observed after 3 to 10 days at 28°C. Plasmids were recovered from yeast colonies (65) and amplified in E. coli by electroporation (24). All these transformations were carried out by the lithium acetate procedure (40).

**Sequencing and in silico analyses.** Inserts of the clones selected were sequenced at the Automatic DNA Sequencing Unit of the Universidad Complutense de Madrid by using oligonucleotide sucesq (5'-CATTCTACATGCACCGTTGGT-3'), which hybridizes to the 3'-terminal region of suc2m2 alleles. Homology searches were made by using the BLASTx and BLASTn programs (29) against nonredundant databases or by using the BLASTn program against the *C. albicans* sequence at the Stanford Genome Technology Center. Sequence data for *C. albicans* were obtained from the Stanford Genome Technology Center website at www.sequence.stanford.edu/group/candida. The putative *C. albicans* open reading frames (ORFs) and their homologues were detected within assembly 6 of the *C. albicans* sequence (www.sequence.stanford.edu/group/candida/download). DNA sequence analysis of signal sequences of translated fusion proteins was performed by using the SignalP World Wide Web server (SignalP V1.1 World Wide Web Prediction Server; www.cbs.dtu.dk/services/SignalP) (59); when the results were not very clear, the iPSORT WWW Service at the Human Genome Center, Institute of Medical Science, University of Tokyo (www.HypothesisCreator.net/iPSORT), was also used. Putative glycosylation and/or protein N- and O-linked sites were predicted by the PSORTII (Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences) program at the PSORT World Wide Web server (www.psort.nibb.ac.jp/).

**RESULTS**

Development of a genetic selection scheme for the identification of heterologous exported proteins. The final goal of the genetic strategy devised was the identification of *C. albicans* proteins that contain export signals. This was achieved by using the *S. cerevisiae* invertase encoded by *SUC2* as a reporter of protein localization. *SUC2* encodes an extracellular invertase able to hydrolyze sucrose into glucose and fructose. It thus enables the utilization by yeast cells of sucrose as a sole carbon source. Targeting of secreted proteins to the secretory pathway occurs via a short amino-terminal sequence known as the sig-
nal peptide. Deletion of the native invertase signal peptide (amino acids 1 to 19) prevents secretion and results in accumulation of the enzyme within the cell, impairing its ability to grow in a medium with sucrose or raffinose as the sole carbon source (42). This system is therefore convenient not only because the enzymatic activity of invertase is readily detectable but also because it allows a positive selection scheme. We screened the *C. albicans* fusion libraries with intracellular *suc2* alleles in order to isolate gene fragments able to restore secretion ability to the invertase; these would presumably correspond to normally secreted proteins.

Different intracellular alleles (*suc2*<sup>m<sub>ic1</sub></sup>, *suc2*<sup>m<sub>ic2</sub></sup>, and *suc2*<sup>m<sub>ic3</sub></sup>) were constructed, enabling us to obtain in-frame fusions in the three open reading frames. They start at amino acid 22 and therefore lack the native signal peptide and the two initiator methionines of the *SUC2* gene (14) (Fig. 1A). The intracellular alleles were introduced into YEp352 and YCplac33 to obtain the multicopy (pSE1, pSE2, and pSE3) and centromeric (pSC1/pSC2) vector series. Episomal vectors were used for the construction of the genomic libraries. The use of multicopy plasmids facilitates a priori the chance of isolating *C. albicans* genes poorly expressed in *S. cerevisiae* in this heterologous screening. The functionality of these constructions was checked by using the native *SUC2* promoter and signal peptide in both types of vectors in different frames (*suc2*<sup>m<sub>ic1</sub></sup>-pSE1psuc, *suc2*<sup>m<sub>ic1</sub></sup>-pSC1psuc, and *suc2*<sup>m<sub>ic3</sub></sup>-pSE3psuc). SS10 transformants carrying the *SUC2* promoter and signal peptide displayed growth on solid (Fig. 1B) as well as in liquid sucrose-antimycin A medium.

In order to validate this strategy for our heterologous screening, we used the *C. albicans* *HEX1* gene. This gene encodes the secreted hydrolytic enzyme β-N-acetylglucosaminidase (12). The *HEX1* gene was selected because of the presence of a clear signal peptide that includes the first 22

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**FIG. 1.** Genetic constructions and testing of the system. (A) Schematic representation of the *S. cerevisiae* *SUC2* gene and the *suc2* intracellular alleles constructed. The cleavage site of the signal sequence is indicated by a vertical arrow. Sequence details of the fusion zone are given in the box. The names of the centromeric (pSC series) or episomal (pSE series) versions of the gene library vectors carrying the three different alleles are given. aa, amino acids. (B) Complementation of the *Suc2<sup>−</sup>* phenotype with the library vectors and genetic constructions including the *SUC2* promoter and signal sequence. (C) Analysis of the functionality of the *C. albicans* *HEX1* promoter and signal sequence.
amino acids. Expression of the \textit{HEX1} gene in \textit{S. cerevisiae} cells has been shown to occur at moderate levels, although it is not inducible in response to \textit{N}-acetylglucosamine, as occurs in \textit{C. albicans} (12). A chimera comprising the sequence encoding the first 37 amino acids of \textit{\beta-N-acetylglucosaminidase} and the \textit{suc2m} allele was constructed with centromeric (pSC2) and episomal (pSE2) vectors (generating plasmids pSC2aphex and pSE2aphex, respectively). pSC2aphex showed slight complementation of the Suc" phenotype after 8 days of incubation on sucrose-antimycin A plates, while complementation with the episomal version was faster (Fig. 1C). We observed that insertion of the native \textit{SUC2} (plasmids pSC1psuci and pSE3psuci) or \textit{C. albicans} \textit{HEX1} (plasmid pSE2phexi) signal peptides in opposite orientations did not complement the Suc" phenotype. These results indicated that the vectors constructed enabled the functional characterization of signal sequences within \textit{C. albicans} DNA, even with its own regulatory signals.

**Screening.** A set of three libraries was constructed with \textit{C. albicans} strain 1001 as a source of genomic DNA. Genomic DNA was used because the expression of \textit{C. albicans} genes in \textit{S. cerevisiae} is not a major drawback (63). This DNA was partially digested with Sau3A, and fragments of 0.5 to 2 kb—presumably corresponding to promoters and partial open reading frames—were selected. After SS10 transformation, a total of about 107,900 transformants obtained with the three episomal libraries were screened. Transformants were first grown on SD plates to a cell density of about 2,000 transformants/plate. After replica plating on sucrose medium and 3 to 10 days of incubation, 571 positives clones were selected (\(\approx 0.5\%\)). Plasmids were rescued from the corresponding \textit{S. cerevisiae} transformants and, after amplification in \textit{E. coli}, were transformed back into the host \textit{suc2} strain. Some of these plasmids were eliminated because they failed in these recomplementation assays. First, we sequenced 189 clones and obtained 143 different inserts (after eliminating some \textit{S. cerevisiae} \textit{SUC2} genes, some very small inserts, and some repeated clones). Analysis of these inserts identified several proteins lacking a signal peptide. For this reason, new recomplementation assays were carried out, and only the 83 clones that showed the best complementation of the Suc" phenotype were selected (they were able to grow from 3 to 7 days of incubation and, more importantly, they showed homogeneous growth) (Fig. 2).

**Analysis of screening.** Plasmids from the 83 isolated clones (named Cep, for \textit{Candida} exported protein) included the 5' regions of 83 \textit{C. albicans} putative genes in frame with \textit{suc2m} alleles that encoded different types of proteins (Fig. 2). Eleven of these proteins are already present in public databases (Table 1), while 52 share homology with proteins present in public databases (Tables 2 and 3). These correspond to genes already described (Table 2) as well as to putative ORFs determined by sequencing programs (orphan genes) (Table 3). We also found 5 clones that correspond to novel \textit{C. albicans} ORFs without homologues in any of the public databases (Table 4) and 15 clones that do not match any \textit{C. albicans} ORF identified at the Stanford \textit{Candida} Genome Center (Assembly 6 Contig Index) (Table 5).

**(i) Previously identified \textit{C. albicans} proteins.** As shown in Table 1, many of the previously identified proteins are related to cell wall biogenesis and include enzymes involved either in the hydrolysis or biosynthesis of major cell wall components, mainly chitin (Cht1p) and glucan (Xog1p, Gsc1p, and Kre9p), or in the proper glycosylation of cell wall mannoproteins (Mnn9p). In addition, and as expected, most of the isolated clones correspond to exported proteins whose localization is the cell surface. Some of the proteins are localized at the plasma membrane, such as Gsc1p/Fks1p (involved in glucan biosynthesis), while other clones identify enzymes that are secreted into the medium. Examples are the major glucanase in \textit{C. albicans} cells, Xog1p, or members of families involved in the pathogenic process, such as the secretory lipase or the secretory aspartyl proteinase families (Lip4p, Sap10p, and Plb1p).

It is important to note that a signal peptide was detected in 10 of these 11 proteins. The remaining one (\(\beta-1,3\)-glucan synthase, Gsc1p) is a cytoplasmic membrane protein with no apparent signal peptide. Interestingly, although the insert isolated in clone Cep74 is short, in silico analysis of the peptide starting at Met648 revealed that the insert could encode a functional signal peptide.

**(ii) Proteins homologous to other proteins in databases.** The criterion for the inclusion of genes in this subset was a specific degree of homology determined by the \textit{E} parameter of the BLAST algorithm. DNA sequences were submitted to the Stanford \textit{Candida} Genome Center, and putative \textit{C. albicans} ORFs were identified in the Assembly 6 Contig Index. Among the ORFs identified, we selected those that displayed homology to sequences encoding already described or putative proteins with an \textit{E} value of less than e\(^{-10}\). These are listed in Table 2 (41 clones) or Table 3 (11 clones), respectively.

Twenty-six of the 41 proteins in Table 2 contain \textit{C. albicans} peptides reported to be located at the cell surface, either as secreted proteins or as cell wall or plasma membrane components. Some of them are indeed proteins related to cell wall construction and/or to the maintenance of its integrity (i.e., Chr1p, Chr2p, Wsc3p, Wsc2p, PstIp, and Kre1p). Alternatively, they are structural cell wall component proteins (Pir3p). Most of these proteins (24 clones) display a clear signal peptide. An additional group of 13 clones correspond to \textit{C. albicans} proteins whose homologues are located in the secretory pathway. Some of them are involved in the secretory mechanism that allows other proteins to reach the cell surface. For example, Scj1p, Mdp1p, and Hdr3p are involved in protein folding or are required when unfolded or misfolded proteins are introduced into the endoplasmic reticulum (ER), while Erv25p and Emp24p form a complex constituent of the COPII coat of certain ER-derived vesicles. Some proteins also located in the secretory pathway are involved in the N-glycosylation process of mannosproteins (Ost3p or Swp1p) or in GPI anchor synthesis (Gpi16p).

For 11 of the selected clones, the \textit{C. albicans} fragment displays homology with novel proteins of unknown function, mainly identified through sequencing projects. These are summarized in Table 3. Interestingly, we cloned a homologue of the \textit{S. cerevisiae} \textit{PRY1} gene—similar to genes encoding plant pathogenesis-related proteins—as well as two clones (Cep211 and Cep91) corresponding to proteins encoded by \textit{YAL053w} and \textit{YGL139w}, which are close homologues of each other. Although some of these proteins have been designated putative, it can be assumed that the \textit{C. albicans} homologue can at least be expressed in this heterologous host.

**(iii) New \textit{C. albicans} proteins without known homologues.
Five cloned fusions contain *C. albicans* gene fragments that encode proteins without homologues in public databases. These proteins, which are completely novel and which, to date, have been detected only in this fungus, are listed in Table 4. In silicio analysis indicated that all of them do have a signal peptide, while ORF 6.701 (Cep79) and ORF 6.7284 (Cep106) encode proteins that seem to be GPI anchored. This result suggested their membrane or cell wall location. Furthermore, ORF 6.7284 encodes a serine- and threonine-rich protein (a feature characteristic of many cell wall proteins). The location of the other three proteins in Table 4 is also predicted to be extracellular.

Table 5 includes 15 clones with a *C. albicans* sequence that is present at the Stanford *Candida* Genome Center (Assembly 6 Contig Index) but for which no ORF was identified. For all of them, a peptide encoded by a sequence in frame with the corresponding *suc2m* allele was detected, and in some cases a signal peptide was predicted. Further work is needed to determine whether they really encode a functional signal peptide for a *C. albicans* secreted protein.

**DISCUSSION**

The availability of the *C. albicans* genome is having a profound impact on the way in which biological research is being carried out with this fungal pathogen, especially in view of the difficulties involved in *C. albicans* genetic studies (19, 63). While the amount of information is significant and while several new genes are being identified through global sequencing programs (9,168 ORFs in the Stanford Assembly 6 Contig Index of the *C. albicans* sequence), there is a significant need to define the functionality of the encoded proteins, especially from the perspective of finding novel cell wall components. Given the ability of several *C. albicans* genes to be expressed in a heterologous host (63), the complementation of specific *S. cerevisiae* mutants altered in cell wall functions is an attractive...
and feasible approach. Global functional strategies have been carried out with *S. cerevisiae* (for example, using a bacterial β-galactosidase to monitor both gene expression and subcellular localization) (10, 70); however, for *C. albicans*, only one recent report, using an antisense approach, has addressed the identification of essential functions (20) in this organism. In the present work, by making use of *S. cerevisiae* genetic tools, we carried out a genetic screening with the primary goal of identifying *C. albicans* secretory protein-encoding genes and/or export domains within them. We used the invertase encoded by the *GSC1* gene of *C. albicans* to find new secretory protein-encoding genes, indicating their involvement in processes specific to *C. albicans* biology, such as dimorphism or virulence.

A different but important group of cloned genes encode proteins located in the secretory machinery. This finding was expected, since these proteins have a signal peptide responsible for their entry into the ER and for reaching their final cellular location. The homologues of most of the proteins found are located in the ER. This finding can be explained in terms of the notion that they are isolated as truncated chimeras and are therefore devoid of the four amino acids that contain the ER carboxy-terminal retention signal (HDEL) (62). Finding this signal can be explained in terms of the notion that they are isolated as truncated chimeras and are therefore devoid of the four amino acids in the sequence that contain the ER carboxy-terminal retention signal (HDEL) (62).

As expected, we selected a significant number of genes encoding extracellular proteins (39 clones, Tables 1, 2, and 3; Fig. 2 shows localization by description, homology, or in silicio analysis). Some of the proteins have already been shown to be located in the fungal cell wall (eight clones) and to be involved either in the biosynthesis of the major cell wall components glucan and chitin or in the maintenance of this cellular structure. Proteins whose final destination is the cytoplasmic membrane (11 clones) or that are destined for secretion (9 clones) were also identified, since these are apparently able to enter the secretory pathway. They include membrane receptors and transporters and extracellular enzymes. In some cases, the true subcellular location of the identified protein is not well defined, although it is known or predicted to be the cell surface (11 clones). The five new *C. albicans* proteins described in Table 4 are included in this group. Identification of these proteins, which, to date, have been considered only putative in the *C. albicans* sequencing project at the Stanford Genome Technology Center, would be one of the more relevant results of the screening. We observed that the putative ORFs encode expressed proteins, at least in *S. cerevisiae*. The fact that these proteins have no homologues in databases suggests that they are species-specific proteins, indicating their involvement in processes specific to *C. albicans* biology, such as dimorphism or virulence.

A different but important group of cloned genes encode proteins located in the secretory machinery. This finding was expected, since these proteins have a signal peptide responsible for their entry into the ER and for reaching their final cellular location. The homologues of most of the proteins found are located in the ER. This finding can be explained in terms of the notion that they are isolated as truncated chimeras and are therefore devoid of the four amino acids in the sequence that contain the ER carboxy-terminal retention signal (HDEL) (62). Finally, the clones in Table 5 did not correspond to any ORF identified at the Stanford Candida Genome Center (Assembly 6 Contig Index). Among the possible explanations are that they arise from mistakes in the Stanford database, that they are ORFs able to encode proteins shorter than 100 amino acids, or even that they are spliced genes.

It should be stressed that the assignment of proteins or functions between even closely related microorganisms is risky. The assignment of homology in Tables 2 and 3 was made based on computer algorithms and, in some cases, the *C. albicans* protein may not represent the counterpart of the described protein. For example, the best result in the homology search with Cep101 was *UTH1*. However, the protein showed homology with all members of the SUN family (56). This family has four members with many different cellular functions. Sun4p, one of its members, has been described as a cell wall protein

### Table 1. *C. albicans* selected proteins described previously

| Category                                | Clone | Gene | aa | Protein description                      | Localization | Signal peptide | Reference(s) or source |
|-----------------------------------------|-------|------|----|-----------------------------------------|--------------|---------------|------------------------|
| Related to cell wall, membrane, and secreted proteins | Cep8  | LIP4 | 24 | Secretory lipase 4                      | Secreted     | Yes           | 38                     |
|                                         | Cep27 | CHT1 | 30 | Hydrolysis of chitin                    | Cell surface | Yes           | 49                     |
|                                         | Cep33 | PLB1 | 102| Phospholipase B                         | Secreted     | Yes           | 49                     |
|                                         | Cep58 | XOG1 | 80 | Exo-β,1,3-glucanase                     | Secreted     | Yes           | 16                     |
|                                         | Cep74 | GSC1 | 28 (Met648) | Catalytic subunit, (fragment) | Plasma membrane, integral | No (fragment: Yes) | 51                     |
|                                         | Cep123| SAP9 | 307| Aspartyl protease, GPI anchored         | Cell wall or plasma membrane | Yes           | 53                     |
|                                         | Cep168| KRE9 | 38 | β,1,6-Glucan synthesis                  | Cell surface | Yes           | 48                     |
|                                         | Cep213| MNN9 | 94 | N-glycosylation of secreted and/or cell wall mannoproteins | Type II membrane protein, Golgi complex | Secreted | 79                     |
|                                         | Cep246| SAP10| 48 | Secretory aspartyl proteinase           | Secreted     | Yes           | AAF66711f             |
| Related to morphological changes         | Cep18 | ECE1 | 39 | Expression in cell elongation           | ND           | Yes           | 7                      |
|                                         | Cep68 | OPS4 | 95 | Opaque-phase specific                  | ND           | Yes           | 55                     |

* aa, number of amino acid residues in the fusion that are derived from the *C. albicans* ORF.

* Determined experimentally or predicted by in silicio analysis, ND, not determined.

* Described in the reference cited or in the corresponding SwissProt database or YPD entry, unless otherwise indicated.

* The signal peptide was predicted by in silicio analysis (see Materials and Methods).

* The fragment of *GSC1* in clone Cep74 beginning at Met648.
| Category                              | Clone | ORF (Stanford) | aa$^c$ | C. albicans signal peptide$^b$ | Gene homologous to | Species$^c$ | E value$^c$ | Protein description or function                                                                 | Localization$^c$ | Homologue signal peptide$^c$ | Reference |
|--------------------------------------|-------|----------------|--------|-------------------------------|-------------------|-----------|--------|-----------------------------------------------------------------------------------------------|-----------------|-----------------------------|-----------|
| Related to cell wall, membrane, and secreted proteins | Cep2  | 6.1639         | 353    | Yes                           | CRH2              | Sc        | 1.3e$^{-103}$ | Cell wall construction                                                                  | Cell wall       | Yes                         | 68        |
|                                      | Cep3  | 6.8192         | 90     | Yes                           | FET3              | Ca        | 1.6e$^{-154}$ | Ceroid oxidase of the multicopper oxidase family                                            | Plasma membrane | Yes                         | 25        |
|                                      | Cep6  | 6.4841         | 121    | Yes                           | STA1              | Sc        | 3.1e$^{-88}$  | Glucoamylase precursor                                                                  | Extracellular    | Yes                         | 89        |
|                                      | Cep10 | 6.1769         | 22     | Yes                           | Lustrin A         | Hr        | 7.4e$^{-18}$  | Matrix protein from shell and pearl nacre, modular structure                               | Extracellular    | Yes                         | 76        |
|                                      | Cep32 | 6.3505         | 31     | Yes                           | CRH1              | Sc        | 2.1e$^{-67}$  | Cell wall construction                                                                  | Cell wall       | Yes                         | 68        |
|                                      | Cep64 | 6.5059         | 92     | Yes                           | WSC3              | Sc        | 3.1e$^{-16}$  | Maintenance of cell wall integrity and stress response                                   | Plasma membrane | Yes                         | 86        |
|                                      | Cep69 | 6.3299         | 32     | Yes                           | HYRI              | Ca        | 1.5e$^{-93}$  | Cell wall construction                                                                  Cell wall | Yes                        | 3         |
|                                      | Cep77 | 6.3113         | 22     | Yes                           | AGAI              | Sc        | 2.3e$^{-31}$  | Cell adhesion receptor, anchor subunit of a-agglutinin                                   Cell wall | Yes                        | 71        |
|                                      | Cep78 | 6.3600         | 184    | Yes                           | PIR3              | Sc        | 3.7e$^{-58}$  | Structural cell wall protein                                                             Cell wall       | Yes                         | 57        |
|                                      | Cep103| 6.2855         | 114    | Yes                           | ADP1              | Sc        | 1.4e$^{-26}$  | Membrane transporter (ATP-binding cassette superfamily)                                 Plasma membrane, integral | Yes                        | 22        |
|                                      | Cep111| 6.857          | 325    | No                            | HYRI              | Ca        | 5.1e$^{-171}$ | Hypophyllally regulated                                                                  Cell wall       | Yes                         | 3         |
|                                      | Cep117| 6.848          | 220    | Yes                           | MID1              | Sc        | 3.5e$^{-72}$  | Required for Ca$^{2+}$ uptake and mating                                                  Plasma membrane, integral | Yes                        | 39        |
|                                      | Cep118| 6.5424         | 315    | Yes                           | YFW1              | Sc        | 4.4e$^{-42}$  | Serine and threonine rich, homologue of pathogenesis-related proteins                    Membrane       | Yes$^d$                      | 88        |
|                                      | Cep119| 6.5099         | 56     | Yes                           | PHO2              | Yl        | 4.2e$^{-130}$ | Acid phosphatase precursor                                                              Secreted        | Yes                         | 84        |
|                                      | Cep125| 6.1768         | 145    | Yes                           | WSC2              | Sc        | 1.2e$^{-11}$  | Cell wall integrity and stress response component 2                                      Plasma membrane | Yes                        | 86        |
|                                      | Cep131| 6.1832         | 150    | Yes                           | PHO3              | Sc        | 8.7e$^{-108}$ | Acid phosphatase with thiamine-binding activity                                             Periplasmic space | Yes                        | 60        |
|                                      | Cep142| 6.4807         | 277    | No                            | mtr               | Nc        | 1.8e$^{-65}$  | Amino acid permease                                                                      Plasma membrane | No                         | 80        |
|                                      | Cep156| 6.4005$^a$     | 163    | Yes                           | SCW4              | Sc        | 3.1e$^{-73}$  | Potential glucanase                                                                      Cell wall       | Yes                        | 13        |
|                                      | Cep170| 6.3969         | 361    | Yes                           | PST1              | Sc        | 1.4e$^{-59}$  | Cell wall generation                                                                     Cell surface     | Yes                        | 61        |
|                                      | Cep221| 6.1240         | 83     | Yes                           | SSP120             | Sc        | 2.0e$^{-46}$  | Secretory protein SSP120                                                                 Secreted        | Yes                        | 77        |
|                                      | Cep231| 6.4725         | 90     | Yes                           | HYRI              | Ca        | 1.3e$^{-103}$ | Hypophyllally regulated                                                                  Cell wall       | Yes                         | 3         |
|                                      | Cep242| 6.5009         | 44     | Yes                           | FET3              | Ca        | 2.0e$^{-202}$ | Ferredoxinase of the multicopper oxidase family                                          Plasma membrane | Yes                        | 25        |
|                                      | Cep243| 6.3942         | 90     | Yes                           | ALX2              | Sc        | 3.6e$^{-103}$ | Required for axial budding pattern                                                         Plasma membrane | Yes                        | 69        |
|                                      | Cep244| 6.8973         | 76     | Yes                           | KREI              | Ca        | 1.3e$^{-12}$  | Beta-1,3-glucan synthesis                                                                Cell surface     | Yes                        | 9          |
|                                      | Cep247| 6.7473         | 66     | Yes                           | YPS1              | Sc        | 1.4e$^{-20}$  | GPI-anchored aspartyl protease                                                           Plasma membrane | Yes                        | 1          |
| Gene  | E-value | Sec      | Exp     | Description                                                                 | Location          |
|-------|---------|----------|---------|-----------------------------------------------------------------------------|-------------------|
| Cep258| 6.6914  | 120      | Yes     | CSA1 Ca 2.5e−20               | Mycelial surface antigen precursor, may function in host interaction |
| Cep1  | 6.6467  | 88       | Yes     | SCJ1 Sc 1e−74                 | Chaperone, protein folding |
| Cep37 | 6.8569  | 68       | Yes     | ERV25 Sc 7.7e−81               | Component of the COPII coat of ER-derived vesicles, complexes with Emp24p |
| Cep38 | 6.7851  | 30       | Yes     | SLS1 Yl 5.6e−30               | Involved in the protein translocation process |
| Cep61 | 6.230   | 324      | Yes     | CPY1CA Ca 3.1e−148             | Carboxypeptidase Y |
| Cep65 | 6.7407  | 234      | Yes     | OST3 Sc 4.4e−42                | Oligosaccharyl transferase gamma subunit necessary for N-glycosylation |
| Cep71 | 6.5557  | 270      | Yes     | GPII6 Sc 2.4e−112              | Protein subunit of GPI transamidase complex |
| Cep85 | 6.5860  | 122      | Yes     | EMP24 Sc 5.8e−63               | Component of the COPII coat of ER-derived vesicles, complexes with Erv25p |
| Cep86 | 6.2244  | 33       | Yes     | EPS1 Sc 7.3e−72                | Protein disulfide isomerase-related protein |
| Cep107| 6.7797  | 125      | Yes     | SWP1 Sc 8.7e−21                | Oligosaccharyl transferase delta subunit involved in N-glycosylation |
| Cep109| 6.4628  | 256      | Yes     | HRD3 Sc 3.6e−58                | Protein degradation and translocation |
| Cep114| 6.4218  | 58       | Yes     | CWH41 Sc 4.2e−60               | Glucosidase I involved in N-glycan processing |
| Cep132| 6.8096  | 288      | Yes     | PRBI Sc 7.3e−159               | Cerevisin precursor (protease B) |
| Cep223| 6.4056  | 225      | Yes     | MDP1 Sc 1.2e−48                | Suppresses the loss of protein disulfide isomerase |
| Other |         |          |         |                               |                   |
| Cep101| 6.7977  | 200      | Yes     | UTH1 Sc 2.0e−10                | Aging protein also involved in the regulation of mitochondrial biogenesis |
| Cep250| 6.9117  | 263      | Not clear | PMP47B Cb 1.3e−55             | Probably encodes a substrate carrier |

**Notes:**
- See Table 1, footnote a.
- The signal peptide was predicted by in silicio analysis (see Materials and Methods).
- Ca, C. albicans; Cb, Candida boidinii; Nc, Neurospora crassa; Hr, Haliotis rufescens; Sc, S. cerevisiae; Yl, Yarrowia lipolytica.
- The expected value (E-value) is a parameter that describes the number of hits that one can expect to see just by chance when searching a database of a particular size.
- See Table 1, footnote b.
- Described in the reference cited or in the corresponding SwissProt database or YPD entry, unless otherwise indicated.
- The signal peptide was predicted by in silicio analysis (see Materials and Methods).
- The S fragment isolated in clone Cep156 is nearly the same as orf6.4005 found in assembly 6 of the *C. albicans* sequence, but it has three repeats of an 18-bp sequence instead of the two repeats found in this ORF.
- The 5' fragment isolated in clone Cep156 is nearly the same as orf6.4005 found in assembly 6 of the *C. albicans* sequence, but it has three repeats of an 18-bp sequence instead of the two repeats found in this ORF.

*See Table 1, footnote b.*
TABLE 3. C. albicans proteins homologous to unknown or putative proteins

| Clone | ORF (Stanford) | aa\(^a\) | C. albicans signal peptide\(^b\) | Gene homologous to:\(^c\) | Species\(^d\) | E value\(^e\) | Protein description or function\(^f\) |
|-------|----------------|---------|-------------------------------|------------------|------------|-----------|-------------------------------------|
| Cep39 | 6.2232         | 22      | Yes                           | YPR157w           | Sc         | 4.4e\(^{-98}\) | Unknown function                     |
| Cep90 | 6.1449         | 185     | No                            | IMB2 (fragment)   | Sp         | 1.6e\(^{-25}\) | Importin-like protein (fragment)     |
| Cep91 | 6.2324         | 54      | Yes                           | YGL139w           | Sc         | 1.6e\(^{-187}\) | Strong similarity to YPL221w and YAL053w proteins |
| Cep102| 6.6369         | 82      | No                            | YHL017w           | Sc         | 2.5e\(^{-78}\) | Unknown function                     |
| Cep104| 6.2554         | 90      | Yes                           | YBR220c           | Sc         | 2.9e\(^{-137}\) | Similar to acetyl coenzyme A transporters |
| Cep112| 6.9066         | 258     | Yes                           | YHR151c           | Sc         | 9.1e\(^{-44}\) | Unknown function                     |
| Cep113| 6.3650         | 127     | Yes                           | YOR154w           | Sc         | 1.1e\(^{-76}\) | Unknown function                     |
| Cep130| 6.8185         | 706     | Yes                           | CG3376            | Dm         | 3.1e\(^{-50}\) | High homology to several sphingomyelin phosphodiesterases |
| Cep144| 6.975          | 144     | Yes                           | PRY1              | Sc         | 3.2e\(^{-39}\) | Similar to plant pathogenesis-related proteins |
| Cep211| 6.1174         | 65      | Yes                           | YAL053w           | Sc         | 1.1e\(^{-193}\) | Strong similarity to YOR365c, YGL139w, and YPL221w proteins |
| Cep254| 6.6421         | 15      | Yes                           | YDL072c           | Sc         | 4.6e\(^{-38}\) | Unknown function                     |

\(^a\) See Table 1, footnote \(^a\).
\(^b\) See Table 2, footnote \(^b\).
\(^c\) Dm, Drosophila melanogaster; Sc, S. cerevisiae; Sp, Schizosaccharomyces pombe.
\(^d\) Data were obtained from databases.

with a clear signal peptide, as was also observed for the protein corresponding to the C. albicans clone identified in this screening (Cep101). Therefore, a complete functional analysis may change this in silico assignment. In addition, the subcellular location of any given protein may vary from host to host.

There are some limitations to this screening. One is that it relies on heterologous expression, and although C. albicans genes are normally expressed in S. cerevisiae (63), the level and/or pattern of the time dependence of expression of these genes may differ between the organisms, resulting in a lack of complementation. A few modifications to this screening might expand the numbers of putative exported proteins identified. For example, larger genomic fragments could be selected to avoid the cloning of genes for small peptides (such as the clones in Table 5), which would hinder interpretation of the results. The construction of new genomic libraries (in which genomic DNA is digested with different restriction enzymes or even randomly) should allow the cloning of more new genes. Expression libraries could also be used. In this case, the entire protein, with most of the location domains (except for C-terminal domains such as HDEL or the GPI anchor), would be expressed.

Despite these limitations, the screening carried out here is extremely useful for several reasons. First, it allowed the selection of 5 novel interesting ORFs from among more than 9,000 ORFs in the C. albicans genome. Second, it is a broad-range screening; a similar screening for secretory protein-encoding genes of S. cerevisiae based on the functional selection of fusions with a gene reporter (PHO5) was reported several years ago (77). In this case, the authors described the isolation of five unique sequences and the complete SSPI20 gene. Significantly, the C. albicans SSPI20 homologue was also obtained in our approach. Third, the SUC2 system has afforded new data about the localization of previously known proteins relevant to different physiological processes but whose localization (measured as the ability to export the intracellular invertase) was not known. These include some proteins relevant to the dimorphic transition, such as Ece1p, the sequence of which was cloned by using a cDNA differential screening (7), or phenotypic switching, such as Ops4, a protein differentially expressed.

TABLE 4. New C. albicans proteins without known homologues

| Clone | ORF (Stanford) | aa\(^a\) | C. albicans signal peptide\(^b\) | GPI anchor\(^c\) | Localization\(^d\) |
|-------|----------------|---------|-------------------------------|------------------|---------------------|
| Cep22 | 6.7675         | 87      | Yes                           |                  | Extracellular (including cell wall) |
| Cep60 | 6.4379         | 50      | Yes                           |                  | Extracellular (including cell wall) |
| Cep79 | 6.701          | 65      | Yes                           | Yes              | Plasma membrane or extracellular (including cell wall) |
| Cep106| 6.7284         | 188     | Yes                           | Yes              | Plasma membrane or extracellular (including cell wall) |
| Cep248| 6.8516         | 13      | Yes                           |                  | Extracellular (including cell wall) |

\(^a\) See Table 1, footnote \(^a\).
\(^b\) See Table 2, footnote \(^b\).
\(^c\) Seems to be GPI anchored (predicted by the PSORTII program at the PSORT World Wide Web server).
\(^d\) Most likely location (predicted by the PSORTII program at the PSORT World Wide Web server).
TABLE 5. N-terminal fragments of new putative Candida albicans proteins

| Clone | N-terminal sequence in fusiona | aa b | Signal peptidec |
|-------|--------------------------------|------|----------------|
| Cep7  | MMITLLTTTAAMTHHFLLLIAQYCHLN  | 27   | Yesd           |
| Cep9  | MIVLLYLLFLNKSFK               | 18   | Yes            |
| Cep14 | MYLYPIPLILGML                 | 13   | Not clear      |
| Cep20 | MLKLIQIKTNLCLVYLILLEEP        | 23   | Yes            |
| Cep63 | MSIIQYLLLRLSCHLTLGELACGQ     | 28   | Yes            |
| Cep80 | MLSRFALVTNFICKSIDMNRR         | 24   | Yesd           |
| Cep82 | MIWIEILSLLVAMI                | 14   | Yes            |
| Cep84 | MYLCFYCSYLYLGFL              | 16   | Yesd           |
| Cep88 | MLFSLLHMWMMFMVQIE            | 20   | Yesd           |
| Cep92 | MLDRTKVEQFIIWIGSLLGIQLNQFR   | 28   | No             |
| Cep229| MLIFYVQWLSMLCYFLLRSCOEI      | 27   | Yes            |
| Cep230| MKVFKIATSLSLAVAIMSMMYMKIG    | 27   | Yesd           |
| Cep241| MIRSNIGKMSVGLKKPSTPTCLIGIYI  | 41   | Mitochondrial  |
| Cep249| MOILFKYLLAACASNYVK            | 18   | Mitochondrial  |
| Cep251| MIIGMLIOLQPLVAVVVGGLI         | 24   | Yesd           |

a N-terminal sequence of the putative peptide encoded in frame with the suc2 allele, as determined with PCGENE software.
b Number of amino acid residues in the peptide encoded in frame.
c The signal peptide of the peptide encoded in frame was predicted by in silicio analysis.
d Result of the prediction with the iPSORT WWW Service when this result was not clear with the SignalP program (see Materials and Methods).

in the opaque phase (55). Finally, the screening was able to select proteins that showed no obvious signal peptide in their amino-terminal regions but which were indeed extracellular proteins. The presence of this kind of protein—such as glycolytic enzymes—within the fungal cell wall has remained controversial for some time (26, 75), although it was recently shown that yeast enolase, encoded by the ENO2 gene, can be efficiently incorporated into regions external to the plasma membrane (61). The use of this system for other genetic elements, such as transposons, may provide an additional useful tool for C. albicans genetic studies.

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