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Mitochondrial Two-Component Signaling Systems in *Candida albicans*

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Two-component signal transduction pathways are one of the primary means by which microorganisms respond to environmental signals. These signaling cascades originated in prokaryotes and were inherited by eukaryotes via endosymbiotic lateral gene transfer from ancestral cyanobacteria. We report here that the nuclear genome of the pathogenic fungus *Candida albicans* contains elements of a two-component signaling pathway that seem to be targeted to the mitochondria. The *C. albicans* two-component response regulator protein Srr1 (stress response regulator 1) contains a mitochondrial targeting sequence at the N terminus, and fluorescence microscopy reveals mitochondrial localization of green fluorescent protein-tagged Srr1. Moreover, phylogenetic analysis indicates that *C. albicans* Srr1 is more closely related to histidine kinases and response regulators found in marine bacteria than are other two-component proteins present in the fungi. These data suggest conservation of this protein during the evolutionary transition from endosymbiont to a subcellular organelle. We used microarray analysis to determine whether the phenotypes observed with a *srr1*Δ/Δ mutant could be correlated with gene transcriptional changes. The expression of mitochondrial genes was altered in the *srr1*Δ/Δ null mutant in comparison to their expression in the wild type. Furthermore, apoptosis increased significantly in the *srr1*Δ/Δ mutant strain compared to the level of apoptosis in the wild type, suggesting the activation of a mitochondrion-dependent apoptotic cell death pathway in the *srr1*Δ/Δ mutant. Collectively, this study shows for the first time that a lower eukaryote like *C. albicans* possesses a two-component response regulator protein that has survived in mitochondria and regulates a subset of genes whose functions are associated with the oxidative stress response and programmed cell death (apoptosis).

Two-component signaling systems (TCSS) are widely used for signal transduction by bacteria, eukaryotic microorganisms, and plants. To date, TCSS have not yet been identified in animals and are absent in the human genome (1). Earlier studies have demonstrated the role of the two-component signal proteins in the pathogenesis of *Candida albicans* in a mouse model of hematogenously disseminated candidiasis, survival in human neutrophils *in vitro*, adherence to human esophageal tissue, quorum sensing, and adaptation to stress (2–10). These features make two-component proteins very attractive targets for antifungal drug discovery.

Two-component signal transduction pathways originated in prokaryotes and are thought to have been inherited by eukaryotes through various horizontal gene transfer events from ancestral cyanobacteria (11). Among eukaryotes, only plants, slime molds, and fungi are reported to contain two-component signal transduction pathways (12). The term "two-component" reflects a requirement for two proteins, the first of which is a sensor histidine kinase, usually a transmembrane protein, and the second a response regulator (RR), which typically acts as a transcription factor in bacteria to adapt cells to the environmental signal (13). The basic chemistry of phosphotransfer reactions is essentially similar in both prokaryotes and eukaryotes. However, a major difference between prokaryotes and lower eukaryotes, such as yeasts, is that the latter generally require an intermediate protein, a histidine phosphotransfer protein, which shuttles phosphate from histidine kinase to response regulator proteins (1, 13).

A two-component phosphorelay system in fungi includes a membrane-bound sensor histidine kinase protein, which is autophosphorylated on a histidine residue in the histidine kinase domain and then transfers the phosphoryl group to an internal receiver domain. The phosphoryl group is then shuttled through a histidine phosphotransfer protein, Ypd1, to a terminal RR. When stress signals are detected by yeast cells, the RR protein is dephosphorylated and is able to activate the downstream mitogen-activated protein kinase (MAPK) pathway to adapt cells to stress (1, 13). The genome of *C. albicans* has been reported to encompass three histidine kinases, two RRs, and one histidine phosphotransfer protein, Ypd1 (1, 14). We recently discovered that *C. albicans* possesses an additional RR gene, *SRR1* (stress response regulator 1), besides the previously reported two RRs, *SSK1* and *SKN7* (10). *SRR1* is unique to the fungi belonging to the CUG clade of Saccharomycotina (15). This is a group of fungi that uses an alternative genetic code in which the CUG codon is translated as serine instead of leucine. Using bioinformatics tools, we predicted that Srr1 is located in the mitochondria, and fluorescence microscopy confirmed the mitochondrial localization of green fluorescent protein (GFP)-tagged Srr1. Furthermore, phylogenetic analysis of Srr1 indicated that *C. albicans* Srr1 is more closely related to RRs found in marine bacteria than are other response regulator proteins present in the fungal kingdom. These data suggest conservation of this protein during the evolutionary transition from an

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TABLE 1 List of *C. albicans* strains used in the present study

Strain	Genotype and/or description[a]	Reference or source
NC1 (wild type; *SRR1*/*SRR1*)	SN148, CIp20-Leu2 (His1 Ura3 Leu2)	10
NC2 (*srr1*Δ/Δ)	*srr1*Δ::*CmLEU2*/*srr1*Δ::*CdHIS1 ura3*Δ-*iro1*Δ::*imm*^{434}/*ura3*Δ-*iro1*Δ::*imm*^{434} *his1*Δ/*his1*Δ *arg4*Δ/*arg4*Δ *leu2*Δ/*leu2*Δ *CIp10* (*URA3*)	10
NC3 (*srr1*Δ/Δ+*SRR1*)	*RP10-SRR1*::*CaURA3*:: *srr1*Δ::*CmLEU2*/*srr1*Δ::*CdHIS1 ura3*Δ-*iro1*Δ::*imm*^{434}/*ura3*Δ-*iro1*Δ::*imm*^{434} *his1*Δ/*his1*Δ *arg4*Δ/*arg4*Δ *leu2*Δ/*leu2*Δ	10
NC5 (*srr1*Δ/Δ+*SRR1*-GFP)	*RP10-ACT1-SRR1*-GFP::*CaURA3*:: *srr1*Δ::*CmLEU2*/*srr1*Δ::*CdHIS1 ura3*Δ-*iro1*Δ::*imm*^{434}/*ura3*Δ-*iro1*Δ::*imm*^{434} *his1*Δ/*his1*Δ *arg4*Δ/*arg4*Δ *leu2*Δ/*leu2*Δ	This study
NC6 (*srr1*Δ/Δ+*SRR1*-TAP)	*RP10-ACT1-SRR1-TAP*::*CaURA3*:: *srr1*Δ::*CmLEU2*/*srr1*Δ::*CdHIS1 ura3*Δ-*iro1*Δ::*imm*^{434}/*ura3*Δ-*iro1*Δ::*imm*^{434} *his1*Δ/*his1*Δ *arg4*Δ/*arg4*Δ *leu2*Δ/*leu2*Δ	This study

[a] *Cm*, from *C. maltosa*; *Cd*, from *C. dubliniensis*; *Ca*, from *C. albicans*.

endosymbiont to a subcellular organelle. Based on these observations, we hypothesize that Srr1 plays an important role in mitochondrial function. A variety of key events take place in mitochondria, such as oxidative metabolism, signal transduction, and apoptosis (16). Recent data have also led to recognition of the importance of mitochondria as key contributors to fungal pathogenesis (17). The data presented herein provide a link between the regulation of mitochondrial functions, such as apoptosis, and two-component signal transduction pathways in *C. albicans*.

MATERIALS AND METHODS

***C. albicans* strains, plasmids, and growth conditions.** The *C. albicans* strains and plasmids used in the present study are listed in Table 1 and in Table S1 in the supplemental material, respectively. All *Candida* strains were maintained as frozen stocks and grown on YPD agar (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar). The *C. albicans* strains were grown routinely in liquid YPD medium at 30°C in an incubator shaker overnight prior to use in the experiments. For drop plate assays, overnight cultures of *C. albicans* cells were harvested by centrifugation, washed with phosphate-buffered saline, and enumerated with a hemacytometer prior to use.

Construction of *SRR1*-GFP strains. To determine its subcellular localization, the coding sequence of *SRR1* was fused in frame with yeast enhanced green fluorescent protein (yEGFP) (18). Two different expression cassettes, containing either the native *SRR1* promoter or the *ACT1* promoter, were constructed to investigate the subcellular localization of the Srr1-GFP fusion protein. The first construct, pSRR1-GFP-CIp10 (native promoter construct) was generated by subcloning yEGFP3 (codon-optimized GFP for *C. albicans*) into the SpeI and BamHI sites of plasmid CIp10 (19) to generate CIp10-yEGFP3 (the plasmid pYGFP3 containing codon-optimized GFP for *C. albicans* was a generous gift from Brendan Cormack, Johns Hopkins University). Next, a 1-kb region (SRR1 promoter) was PCR amplified from CAF2-1 genomic DNA as the template and cloned upstream from yEGFP3 between the XbaI and SpeI sites in CIp10-yEGFP3, resulting in the construct CIp10-SRR1-Promoter-yEGFP3. Finally, the *SRR1* open reading frame (ORF) without the stop codon was amplified and cloned into the SpeI site between the *SRR1* promoter and yEGFP3 to generate the construct pSRR1-GFP-CIp10. This construct was linearized with BglII prior to transformation into the *C. albicans srr1*Δ/Δ (*ura*) null mutant strain. The second *C. albicans SRR1*-GFP fusion construct, pACT1-*SRR1*-GFP, was generated by PCR amplifying the *SRR1* ORF by using high-fidelity Phusion DNA polymerase (NEB). The PCR-amplified product was cloned in the HindIII site of plasmid pACT1-GFP (20) to generate pACT1-*SRR1*-GFP. This construct utilizes the *ACT1* promoter for the expression of the *SRR1*-GFP gene fusion. The recombinant plasmid containing the *SRR1*-GFP gene fusion was linearized by using StuI and transformed into the *C. albicans srr1*Δ/Δ (*ura*) null mutant strain, and transformants were selected by uracil prototrophy. All constructs were verified by sequencing to rule out any point mutations due to PCR amplification reactions.

Oxidative stress assays. The sensitivities of *C. albicans* wild-type (*SRR1*/*SRR1*), *srr1*Δ/Δ, and gene-reconstituted (*srr1*Δ/Δ+*SRR1*) strains and a strain expressing Srr1-GFP (*srr1*Δ/Δ+*SRR1*-GFP) to oxidative stress were assayed by spotting dilutions of 5×10^5 to 5×10^1 cells (each in a total volume of 5 μl) from an overnight culture of yeast cells grown in YPD broth at 30°C onto YPD agar plates containing 7 mM hydrogen peroxide (H_2O_2). The growth of each strain was examined after 48 h.

Fluorescence microscopy. Srr1 contains an N-terminal mitochondrial targeting sequence. Consequently, it is predicted by several algorithms to be located in the mitochondria. To determine its subcellular localization, we performed bright-field and fluorescence microscopy of GFP-tagged Srr1. Briefly, a *C. albicans* strain expressing *SRR1*-GFP was grown at 30°C overnight and then diluted to a starting OD_{600} of 0.1 in 50 ml of YPD broth. Cells were grown at 30°C and, upon reaching log phase, were stained with 250 nM MitoTracker red (Molecular Probes) for 45 min. The cells were washed and stressed with H_2O_2 for 15 min and then photographed by using a wide-field fluorescence microscope equipped with a cooled charge-coupled device (CCD) camera, appropriate filter sets, a high numerical aperture (>1.3) 100× objective, and image acquisition software. For 4′,6′-diamidino-2-phenylindole (DAPI) staining, log-phase cells were fixed with 4% formaldehyde for 10 min, washed twice with phosphate-buffered saline (PBS), and resuspended in PBS. One microliter of Prolong gold DAPI (Invitrogen) was added to the cells on the slide, and then they were covered with a coverslip and the samples were imaged as described above.

Construction of *SRR1*-TAP strain. The tandem affinity purification (TAP) method consists of two different tags arranged in tandem separated by a tobacco etch virus (TEV) protease cleavage site. The two tags are a protein A-binding site and a calmodulin binding peptide. The plasmid pJN74 (kindly provided by Jeremy Brown, University of New Castle, United Kingdom) used in the current study allow for C-terminal tagging of the Srr1p. This cassette utilizes the *ACT1* promoter to obtain maximum expression of the tagged gene. The integration of this plasmid occurs at the RPS10 locus. The plasmid pJN74-SRR1 was created by PCR amplifying the full-length *SRR1* ORF from *C. albicans* CAF2-1 genomic DNA and cloning it between the XmaI and PstI restriction sites in the polylinker of this plasmid. The SRR1-TAP-tagged plasmid was digested with NcoI prior to transformation into the *srr1*Δ/Δ (Ura^-) *C. albicans* strain. The transformants were selected by uridine prototrophy and verified by PCR to ensure the identification of the correctly aligned TAP tags.

Isolation and purification of mitochondria. Mitochondria were isolated as described in detail elsewhere (21). Briefly, a *C. albicans* strain expressing Srr1-TAP and a *Saccharomyces cerevisiae* strain expressing Pos5-TAP were grown in YPD broth supplemented with raffinose (1% yeast extract, 2% peptone, 0.5% glucose, 2% raffinose) to an optical density at 600 nm (OD_{600}) of 1.5 and harvested by centrifugation. Cells were suspended in 0.1 M Tris-SO_4, pH 9.4, containing 10 mM dithiothreitol (DTT) (~5 to 6 ml/g of cells) and incubated at 30°C for 10 min. After centrifugation, the cell pellets were resuspended in spheroplast buffer (20 mM potassium phosphate, pH 7.4, 1.2 M sorbitol) and incubated with

TABLE 2 List of *SRR1*-dependent genes

ORF	Gene	Molecular function	Fold change (H_2O_2 treated vs untreated) in indicated expt with strain of genotype:			
			Wild type (*SRR1*/*SRR1*)		*srr1*Δ/Δ (*srr1*/*srr1*)	
			1	2	1	2
orf19.697		Unknown	5.0	4.6	1.8	2.0
orf19.847	*YIM1*	Oxidoreductase activity	11.2	15.2	4.4	4.8
orf19.916		Apoptosis	5.9	8.9	2.0	2.9
orf19.1149	*MRF1*	Oxidoreductase activity	9.6	12.4	2.7	2.6
orf19.1360		Unknown	2.0	3.8	0.5	1.0
orf19.2175		Apoptosis	5.0	3.7	1.5	2.0
orf19.3782		Putative acetyl-coenzyme A transporter	2.0	3.4	0.8	0.4
orf19.3818	*GOA1*	Oxidative stress, apoptosis	5.6	5.2	1.2	1.3
orf19.4392	*DEM1*	Unknown	2.1	2.6	0.8	0.9
orf19.4577		Unknown	2.3	3.5	0.9	1.1
orf19.4733	*YMC2*	Unknown	2.4	2.2	0.9	0.6
orf19.4864		Unknown	3.6	3.8	0.8	1.1
orf19.5599	*MDL2*	Putative transporter	2.9	2.6	1.2	1.1
orf19.7187	*MAM33*	Unknown	4.6	5.2	1.7	2.0
orf19.7281	*PDK2*	Putative sensor kinase	2.8	2.4	0.5	0.5
orf19.7365		Apoptosis	3.4	3.0	1.0	0.9
orf19.7634	*MCD1*	Apoptosis	6.7	7.1	2.2	2.9

Zymolyase 100T (1 mg/g of cells) at 30°C for 1 h. The spheroplasts were washed twice with spheroplast buffer and then suspended in ice-cold buffer A (20 mM HEPES-KOH, pH 7.5, 0.6 M sorbitol, 0.1% bovine serum albumin [BSA], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF]) and homogenized in a Dounce homogenizer on ice. The homogenate was diluted with an equal volume of ice-cold buffer A and centrifuged at 1,500 × *g* for 5 min at 4°C. Supernatants were combined and centrifuged (1,500 × *g* for 5 min at 4°C) to remove cell debris. A crude mitochondrial fraction was obtained by centrifugation (10,000 × *g* for 10 min at 4°C) of the supernatant. To further purify mitochondria, the crude mitochondrial pellet was resuspended in buffer A, loaded on top of a Percoll gradient, and centrifuged in a Beckman SW28 rotor (70,000 × *g* for 30 min at 4°C). Mitochondria were collected from the interface between 20 and 40% Percoll, diluted 10-fold with buffer A, and centrifuged (10,000 × *g* for 10 min at 4°C). The purified mitochondrial pellet was resuspended in buffer A or frozen at −80°C until further use.

Western blotting. The Western blot assay followed our protocol as described previously (10). Equal amounts (200 μg) of purified mitochondria prepared from the *C. albicans* strain expressing TAP-tagged Srr1 and the *S. cerevisiae* strain expressing TAP-tagged Pos5 were separated by SDS-PAGE. The electrophoresed proteins were then transferred to nitrocellulose membranes and probed with an affinity-purified rabbit IgG antibody (1:1,000). As a positive control, another blot was reacted with anti-Mir1 antibody (1:1,000). The blots were then developed as recommended by the manufacturer (GE Healthcare).

Assays for growth on alternate carbon source. The sensitivities of *C. albicans* wild-type (*SRR1*/*SRR1*), *srr1*Δ/Δ (*srr1*/*srr1*), and gene-reconstituted (*srr1*Δ/Δ+*SRR1*) strains to alternate carbon sources, such as glycerol, lactate, and ethanol, were assayed by spotting dilutions of 5×10^1 to 5×10^5 cells (each in a total volume of 5 μl) from an overnight culture of yeast cells grown in YPD broth at 30°C onto YPD agar and YPG (4% glycerol) agar plates. The growth of each strain was examined after 24 h.

Growth conditions for microarray analysis. Overnight cultures of *C. albicans* wild-type (*SRR1*/*SRR1*), *srr1*Δ/Δ (*srr1*/*srr1*), and gene-reconstituted (*srr1*Δ/Δ+*SRR1*) strains were grown in YPD broth at 30°C. These overnight cultures were then diluted to an initial optical density (OD_{600}) of 0.1 in YPD broth (50 ml), grown at 30°C until logarithmic growth phase, and then treated with H_2O_2. The concentration of stressor used was equivalent to 1× the MIC for each strain. The time point chosen for array analysis was 30 min after treatment with oxidative stressor. At that time, RNA was extracted from cells by the hot phenol method (22) and prepared for array analysis.

Gene expression profiling and analysis. Gene expression profiles were obtained by hybridizing labeled cRNAs generated from *C. albicans* total RNA onto Affymetrix *C. albicans* custom expression arrays (CAN07, 49-5241 array format), which have been described elsewhere (23). Total RNA was isolated using the hot phenol method, and subsequent cRNA synthesis and labeling were performed as previously described (23). Subsequent hybridization of cRNAs, washes, staining, and scanning of the stained array chips were performed as previously described (23). The scanned images were analyzed using software resident in the GeneChip operating system, version 2.0 (Affymetrix). Sample loading and variations in staining were standardized by scaling the average of the fluorescence intensities of all genes on an array to a constant target intensity. The signal intensity for each gene was calculated as the average intensity difference, represented by [Σ(PM − MM)/(number of probe pairs)], where PM and MM denote perfect match and mismatch probes.

The scaled gene expression values from the GeneChip version 2.0 operating system software were generated using the MAS5.0 algorithm. The value of each probe set was generated using Tukey's biweight computation. The algorithm considers the contribution of each match and mismatch probe corrected for the background of the average of the probe set. A weighted mean is then calculated for each probe pair. The raw signal values are then $\log_2$ transformed, and the array is normalized to this value. Probe sets were deleted from subsequent analysis if they were determined to be absent by the Affymetrix criterion. Pairwise comparison of gene expression levels was performed for each matched experiment. Among direct comparisons between strains, genes were considered to be differentially expressed if their change in expression was 2-fold or greater (for upregulated genes) or 0.5-fold or less (for downregulated genes) in both independent experiments of each comparison. Genes were defined as Srr1 dependent in response to oxidative stress (Table 2) if the fold change in expression between peroxide-treated wild-type cells and untreated wild-type cells was at least 2-fold greater in both experiments than the fold change in expression between peroxide-treated *srr1*Δ/Δ mutant cells and untreated *srr1*Δ/Δ mutant cells.

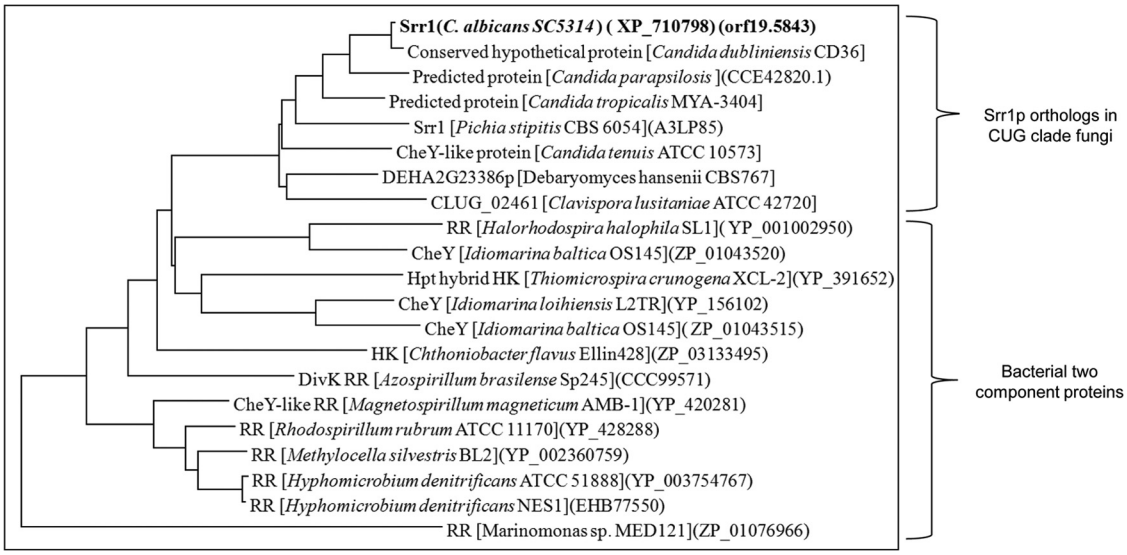

FIG 1 Phylogeny of *C. albicans* Srr1. Phylogenetic analysis was conducted in MEGA5 (43). The analysis involved amino acid sequences of Srr1 and related bacterial and eukaryotic two-component signaling proteins. The evolutionary history was inferred using the neighbor-joining method (44). The optimal tree is drawn to scale with the sum of branch length of 17.98984977; the branch lengths depict the evolutionary distances. The evolutionary distances were computed using the Poisson correction method (45) and are in units of the number of amino acid substitutions per site. GenBank accession numbers are shown in parentheses.

Real-time reverse-transcription (RT)-qPCR. Total RNA was extracted as described above from wild-type (*SRR1*/*SRR1*), *srr1*Δ/Δ (*srr1*/*srr1*), and gene-reconstituted (*srr1*Δ/Δ+*SRR1*) strains grown in YPD broth at 30°C, and cDNA synthesized by using the SuperScript indirect cDNA synthesis kit (Invitrogen) following the manufacturer's instructions. Primers used in all PCR amplifications are indicated in Table S2 in the supplemental material. Real-time PCR experiments were performed on a Stratagene Mx3005P quantitative PCR (QPCR) system by using the Clontech SYBR advantage qPCR kit according to the manufacturer's instructions. The levels of expression of differentially expressed genes were normalized to the expression of the actin control and expressed as relative expression units.

Immunofluorescence and apoptosis assay. *C. albicans* cells from a culture grown overnight in YPD broth at 30°C were fixed on glass coverslips and permeabilized in 70% ethanol for 40 min at −20°C. The cells were incubated in terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) reaction mixture (Roche, Mannheim, Germany) at 37°C for 1 h, rinsed three times in PBS, and incubated in blocking solution (5 mM EDTA, 1% fish gelatin, and 1% essentially immunoglobulin-free BSA) for 30 min at room temperature. The cells were then incubated in primary antibody (anti-COX-4 antibody at a concentration of 1:800 [Abcam, Cambridge, MA]) overnight at 4°C. After a 1-h wash in PBS at room temperature, the fixed cells were washed four times with PBS, incubated with fluorescein isothiocyanate (FITC)-conjugated, Cy3-conjugated sheep anti-rabbit IgG antibody (1:300) for 1 h at room temperature, followed by another wash in PBS for 1 h. Coverslips were then mounted using Prolong Gold with DAPI (Invitrogen) and examined by using confocal microscopy (Leica Microsystems, Wetzlar, Germany). Specificity was confirmed by replacing the primary antibody with a nonspecific myeloma protein of the same isotype. The percentage of apoptotic yeast cells was determined by double immunofluorescence staining (TUNEL and DAPI). The numbers of yeast cells were counted, as well as the numbers of cells that were TUNEL positive, in 10 fields per coverslip, and the data were expressed as the percentage of apoptotic yeast as determined by NIS Elements Advance Research (Nikon, Japan).

Statistical analysis. In order to determine the statistical significance of the differences in the expression of select genes determined by qRT-PCR, we performed Student's *t* test. A *P* value of <0.05 was considered statistically significant.

Microarray data accession number. Data files for each scanned microarray chip were submitted to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/). The GEO accession number for this series is GSE45438.

RESULTS

Conservation of Srr1 during evolutionary transition. We recently discovered a new response regulator gene, *SRR1*, a key missing piece of the two-component signaling network in *C. albicans* (10).The *SRR1* gene is unique to the pathogenic fungi belonging to the CUG clade of Saccharomycotina (15). Phylogenetically, Srr1 and other two-component signaling proteins present in *C. albicans* are quite distinct. Phylogenetic analysis indicates that *C. albicans* Srr1 clusters more closely with histidine kinases/RRs found in marine bacteria than with other *C. albicans* two-component proteins (Fig. 1). This intriguing observation hints at a prokaryotic origin of Srr1 and provides important clues to the origin of biological pathways in present-day eukaryotic organisms from their prokaryotic ancestors.

Identification of mitochondrial targeting sequence in Srr1. The majority of mitochondrial proteins are synthesized in the cytosol. These proteins are then imported into mitochondria with the help of an amino-terminal sequence. The mitochondrial sequence prediction was performed by using MitoProt predictor software (http://psort.hgc.jp/form2.html). A canonical mitochondrial targeting signal sequence was predicted at the N terminus of Srr1 (Fig. 2) by this software. A mitochondrial localization signal at the N terminus is usually found in proteins destined for import into the mitochondrial matrix (24). Based on these *in silico* data, we hypothesize that Srr1 is located in the *C. albicans* mitochondria. In addition, a stretch of 20 amino acids (amino acid 78 to amino acid 97) was identified as aspartate rich (Fig. 2). How-

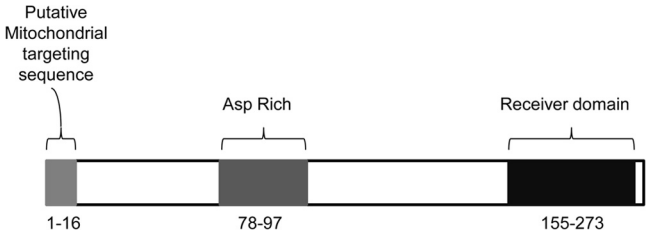

FIG 2 Schematic representation (size not to scale) of Srr1 (282 amino acids), depicting the mitochondrial targeting sequence at the N terminus, a stretch of aspartate-rich region (amino acids 78 to 97), and a conserved receiver domain at the C terminus. The receiver domain prediction was done by using SMART (http://smart.embl-heidelberg.de/). Mitochondrial target sequence prediction was performed by using MitoProt (http://psort.hgc.jp/form2.html), and the aspartate-rich region was identified using UniProt (http://uniprot.org).

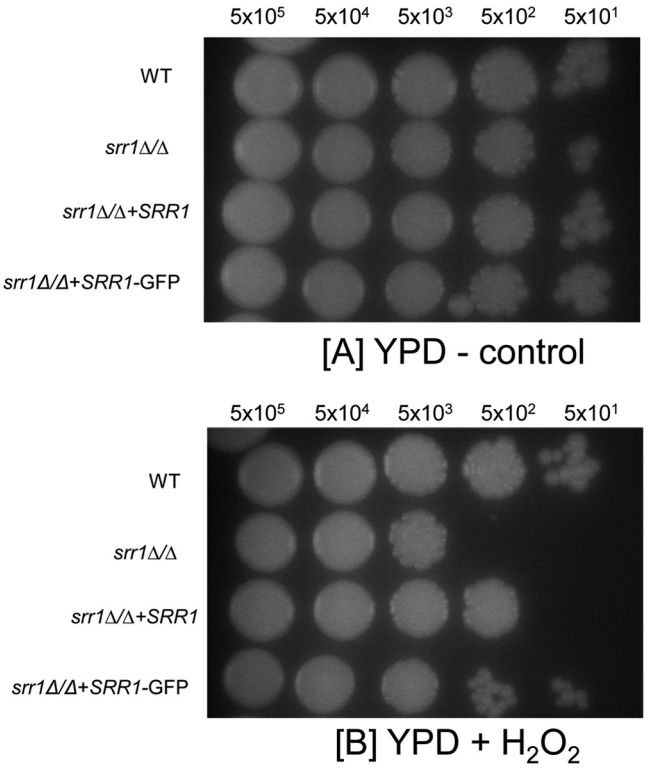

FIG 3 Growth of wild-type (WT) (*SRR1*/*SRR1*), *srr1*Δ/Δ, *srr1*Δ/Δ+*SRR1*, and *srr1*Δ/Δ+*SRR1*-GFP strains of *C. albicans* at 30°C for 48 h on YPD agar (control) (A) and YPD agar containing 7 mM H_2O_2 (B). Five-microliter cell dilutions (5×10^5 to 5×10^1 cells) were spotted onto each plate.

ever, whether or not this aspartate-rich compositional bias has any biological significance remains to be elucidated.

Srr1-GFP fusion is functional. We have shown previously that the *srr1*Δ/Δ strain is sensitive to oxidative stress (10). We took advantage of this phenotype to determine whether the Srr1-GFP fusion protein is functional. In a complementation assay, the *C. albicans* strain expressing Srr1-GFP, the wild-type strain (*SRR1*/*SRR1*), the null mutant strain *srr1*Δ/Δ, and the gene-reconstituted strain (*srr1*Δ/Δ+*SRR1*) were incubated at 30°C for 48 h on YPD agar plates containing hydrogen peroxide (H_2O_2). As reported above, we found that the *srr1*Δ/Δ strain was more sensitive to H_2O_2, while the sensitivity of the GFP fusion strain to H_2O_2 was equivalent to that of the wild-type strain, suggesting that the GFP fusion protein restores the functional activity of Srr1 after loss of the endogenous protein. Thus, these data confirm that the Srr1-GFP fusion protein complements the loss of Srr1 (Fig. 3).

Srr1 is located in the mitochondria. To determine the subcellular localization of GFP-tagged Srr1, we performed fluorescence microscopy (Fig. 4). The *C. albicans* strain expressing *SRR1*-GFP was grown to log phase and stained with MitoTracker red (Molecular Probes) before treatment with hydrogen peroxide. Unstressed cells were used as a control. We observed that GFP-tagged Srr1 was located in the mitochondria under both unstressed and oxidative-stress growth conditions. This was evident from the presence of mitochondrion-like tubular structures in cells transformed with GFP-tagged Srr1 when compared to the diffused fluorescence observed in the control strain transformed with empty plasmid vector (Fig. 4). The Srr1-GFP signal matched perfectly with the MitoTracker red signal. In DAPI-stained cells, we again observed tubular networks of mitochondria due to the green fluorescence emanating from mitochondria showing expression of Srr1-GFP (Fig. 4d). For fluorescence microscopy, we utilized *SRR1* (native), as well as the *ACT1* (overexpressed) promoter for the expression of GFP-tagged Srr1. No differences were observed in the mitochondrial localization of Srr1, irrespective of the promoter. To further confirm the fluorescence microscopy results and, also, to determine that the presence of a fluorescent tag did not alter the localization of Srr1, we performed Western blot analysis of purified mitochondria from the *C. albicans* strain expressing TAP-tagged Srr1 (Fig. 5). We used affinity-purified rabbit IgG antibody to detect TAP-tagged Srr1. As a positive control, a previously characterized mitochondrial protein from *S. cerevisiae* Pos5 (25) was included in the same blot. A reactive band corresponding to ~60 kDa, indicating the presence of TAP-tagged Srr1, was observed in purified mitochondria from *C. albicans*. TAP-tagged Pos5 from *S. cerevisiae* gave an expected band of ~70 kDa. We also probed the same mitochondrial preparation from both strains with antibody to another known mitochondrial protein, Mir1 (26). A reactive band of ~30 kDa was observed in both *C. albicans* and *S. cerevisiae* mitochondria. Collectively, the data from fluorescence microscopy and the Western blotting experiments confirm that Srr1 is localized to mitochondria, and that finding led us to hypothesize that Srr1 has a role in mitochondrial function.

Srr1 is not required for growth on alternate carbon sources. Since Srr1 is localized in the mitochondria, we wanted to investigate its role in the utilization of carbon sources other than glucose. Interestingly, no differences were observed in the growth of the wild type versus the *srr1*Δ/Δ mutant strain when grown on alternate carbon sources, such as glycerol, lactate, or ethanol (data not shown). Evidence from the published literature suggests that yeast mitochondrial mutant strains defective in growth on glycerol are usually respiration incompetent (27). Because *srr1*Δ/Δ does not show any defect in the utilization of alternate carbon sources, we hypothesize that Srr1 does not participate in the regulation of pathways required for the generation of energy (ATP).

Mitochondrial genes are downregulated in the *srr1*Δ/Δ mutant strain. In order to further investigate how Srr1 contributes to mitochondrial function and regulates genes that are crucial to mitochondrial homeostasis, we performed microarray analysis

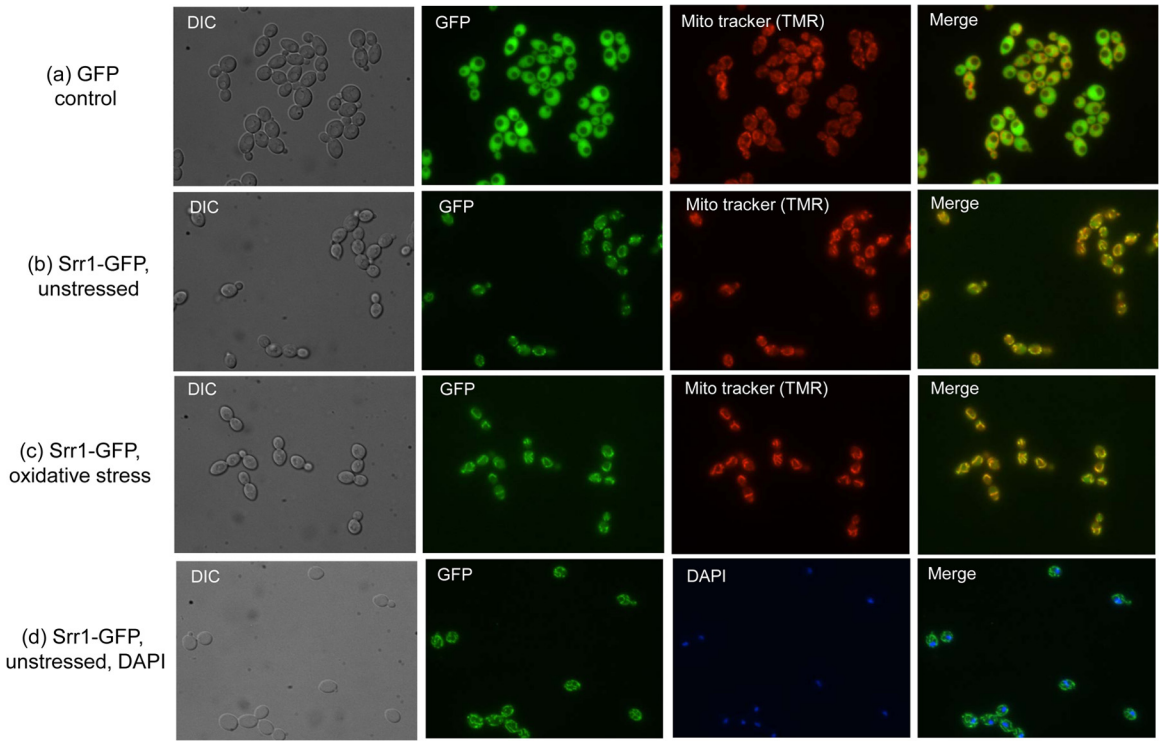

FIG 4 Subcellular localization of Srr1-GFP. All images have similar contrast. GFP was imaged in the FITC channel with a 2-s exposure, and MitoTracker was imaged in the tetramethylrhodamine (TMR) channel using a 1-s exposure. Cells expressing GFP or Srr1-GFP were treated as indicated. DIC, differential interference contrast.

using RNA preparations from the wild-type and *srr1*Δ/Δ mutant strains. Each strain was compared for differential expression of genes in cells grown in YPD broth until logarithmic growth phase, followed by treatment with H_2O_2. One of the major goals of the transcriptional profiling experiments was to identify *SRR1*-dependent genes. These are genes that increase in expression by at least 2-fold in response to H_2O_2 in wild-type cells but whose expression in response to H_2O_2 is diminished by at least 50% (2-fold) in the *srr1*Δ/Δ mutant compared to the response of wild-type cells to H_2O_2. Genes that fit these criteria were interpreted as dependent on *SRR1* in order to achieve maximum expression in an oxidative-stress-inducing environment. We hypothesized that

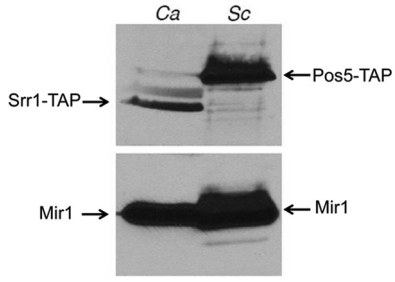

FIG 5 Western blot analysis of purified mitochondria from *C. albicans* strain expressing TAP-tagged Srr1 (*Ca*) and *S. cerevisiae* strain expressing TAP-tagged Pos5 (*Sc*). Western blots were performed using either an anti-IgG or an anti-Mir1 antibody. The anti-IgG antibody binds to protein A present in the TAP-tagged Srr1 and Pos5, while the anti-Mir1 antibody is specific for the Mir1 protein found in the mitochondria of both *C. albicans* and *S. cerevisiae*.

genes that are significantly downregulated in the *srr1*Δ/Δ mutant could be Srr1 regulated and, thus, critical to the identification of its downstream effector proteins. The complete list of genes that were differentially expressed in the *srr1*Δ/Δ mutant and the wild-type strain is presented in Tables S3 and S4 in the supplemental material. As anticipated, several oxidative stress genes were induced in the mutant (see Table S4). Data from the transcriptional profiling experiments revealed that the majority of genes that were downregulated in the *srr1*Δ/Δ mutant were annotated as uncharacterized or of unknown function on the *Candida* genome database (www.candidagenome.org) website (28). Interestingly, two gene families were significantly affected in the mutant, including those associated with stress adaptation and genes whose putative functions are related to mitochondrial function (Table 2). Among the most strongly downregulated genes in the *srr1*Δ/Δ mutant were *MRF1* (orf19.1149), orf19.2175, *GOA1* (orf19.3818), *MAM33* (orf19.7187), and orf19.7365 (Table 2). Mrf1 (putative mitochondrial respiratory protein) and Mam33 (putative mitochondrial acidic matrix protein) are uncharacterized but are predicted to be mitochondrial, and based on the functions of their orthologs in *S. cerevisiae*, these proteins are predicted to have functions related to regulation of the oxidative stress response. Goa1 has been shown to be localized in *C. albicans* mitochondria and provides important functions related to resistance to oxidative stress and apoptosis (27, 29). orf19.2175 and orf19.7365 are both uncharacterized. We noted that the putative functions of orf19.2175 and orf19.7365 are related to apoptosis, just as for *GOA1*. We pursue the hypothesis that Srr1 functions in apoptosis below. The altered expression of genes in the *srr1*Δ/Δ strain was

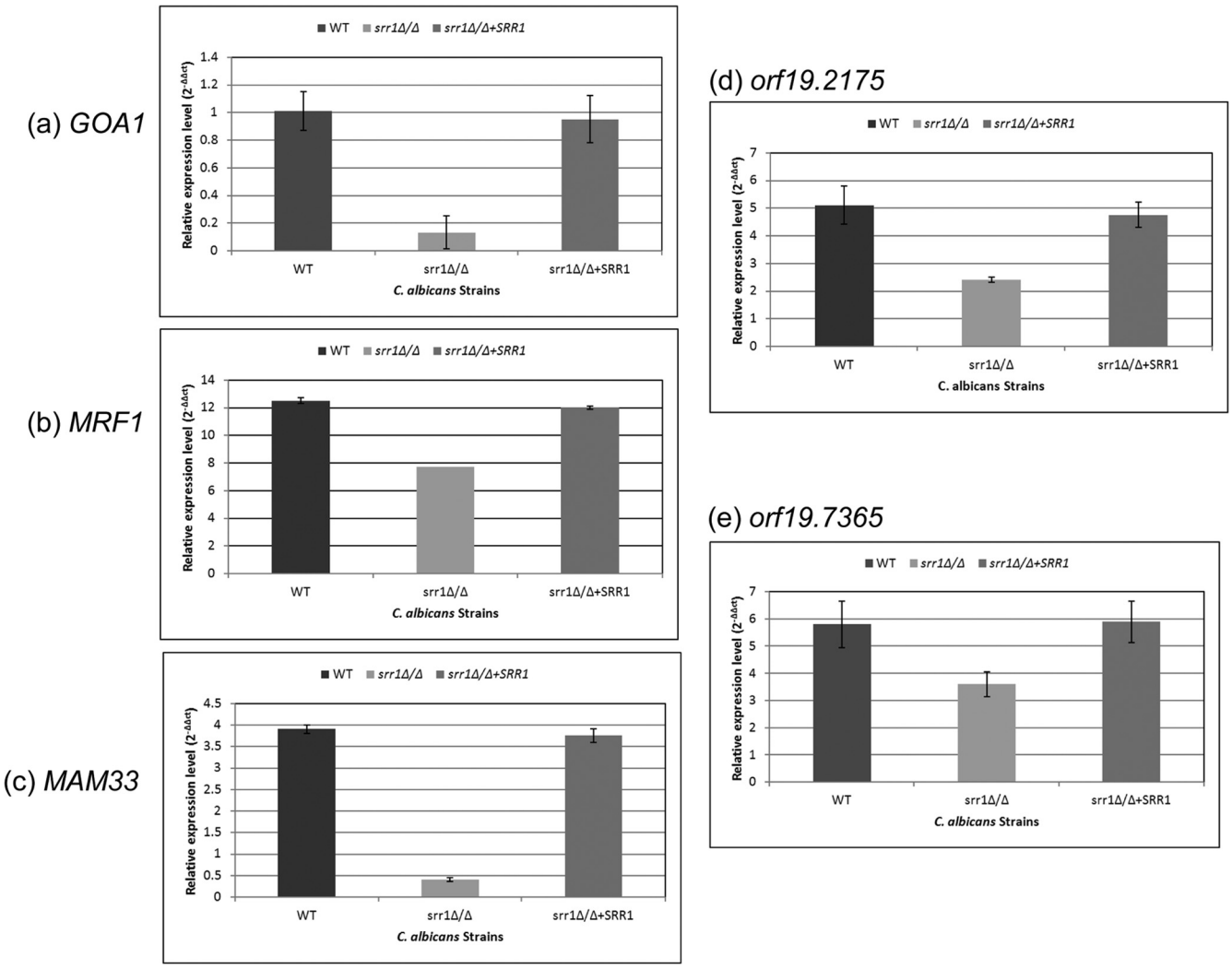

FIG 6 Expression of *SRR1*-dependent genes. *C. albicans* strains were grown to log phase in YPD broth at 30°C and treated with H_2O_2. qPCR was done to determine the RNA levels of *GOA1* (a), *MRF1* (b), *MAM33* (c), orf19.2175 (d), and orf19.7365 (e). Each gene's expression level was normalized to that of the actin control and expressed as relative expression units compared to each strain. Error bars show standard deviations.

validated by qPCR and was compared to their expression in the wild-type and gene-reconstituted strains (Fig. 6). The transcriptional changes of *GOA1* and *MAM33* in the *srr1*Δ/Δ mutant versus their expression levels in the wild-type or gene-reconstituted strain, validated by qPCR, were statistically significant (P value $<$ 0.001). Importantly, the microarray data correlate well with our observation that Srr1 is located in the mitochondria and with at least one previously reported phenotype of the *srr1*Δ/Δ mutant, i.e., increased susceptibility to oxidative stress (10).

Deletion of *SRR1* triggers apoptosis in *C. albicans*. The data from the microarray experiments demonstrate that a number of genes that were downregulated in the *srr1*Δ/Δ mutant had functions related to redox metabolism, including apoptosis. To determine the role of *SRR1* in apoptosis, we performed a TUNEL (terminal deoxynucleotidyltransferase dUTP-biotin nick end labeling) assay. Our data indicated an approximately 40-fold increase in apoptosis in the *srr1*Δ/Δ mutant compared to the level in the wild-type strain (Fig. 7a and b). Reintroduction of a wild-type copy of *SRR1* in a gene-reconstituted strain restored apoptosis to levels similar to the levels in the wild type (Fig. 7a). Apoptosis in a living cell can be triggered by either extrinsic or intrinsic factors. Our observation of increased apoptosis in the *srr1*Δ/Δ mutant in the absence of any external stimuli suggests the activation of the cell death pathway in this mutant. In the presence of hydrogen peroxide, we observed an expected significant increase in apoptosis in wild-type cells (Fig. 7b). When treated with H_2O_2, 100% of *srr1*Δ/Δ cells were apoptotic (data not shown). Next, we sought to examine the integrity of the mitochondria by measuring the release of mitochondrial cytochrome *c* (CytC) into the cytoplasm (Fig. 7a). The CytC levels were measured by using Cox4 antibody (Abcam Cambridge, MA). No changes in CytC release were observed in any of the *C. albicans* strains tested, suggesting that mitochondrial integrity is not compromised in the *srr1*Δ/Δ strain. Taken together, the data from these experiments support the hypothesis that Srr1 is critical to cell survival. Differential expression of mitochondrial genes predicted to have apoptosis-related functions offers clues to the likely role of downstream effector proteins that may enable the Srr1 to carry out its functions.

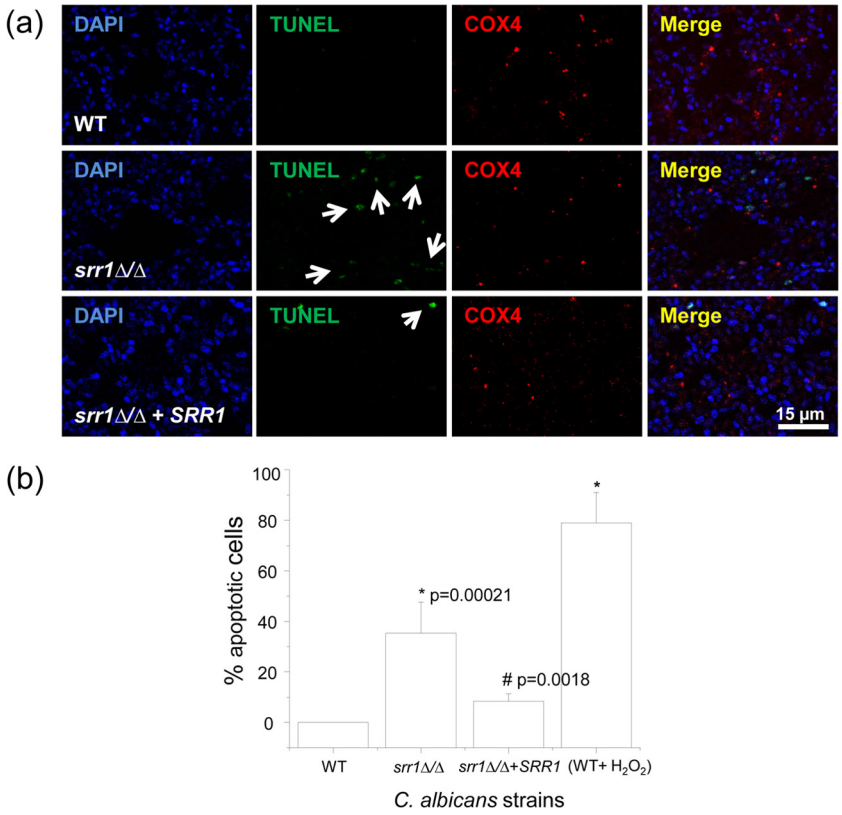

FIG 7 Apoptosis measurements in strains of *C. albicans*. Cells of each strain were grown overnight in YPD medium. (a) TUNEL assay was performed by using an *in situ* cell death detection kit (Roche). The number of cells with green fluorescence is a direct measurement of TUNEL-positive cells. Arrows indicate TUNEL-positive cells. (b) Quantitation of the percentage of apoptosis in strains of *C. albicans* as revealed by TUNEL assay. The number of TUNEL-positive cells in the *srr1*Δ/Δ mutant versus the numbers in the wild-type ($P = 0.00021$) and gene-reconstituted ($P = 0.0018$) strains was statistically significant. Error bars show standard deviations. Wild-type (*SRR1*/*SRR1*) cells treated with H_2O_2 were included as a positive control.

DISCUSSION

Mitochondria and chloroplasts originated as bacterial symbionts (30–32). Mitochondria are known to play vital roles in metabolism and energy production in all living eukaryotic cells (33, 34). It is estimated that approximately 750 to 800 proteins are present in the *C. albicans* mitochondria (35, 36). During the course of evolution, most of the genes contained in the mitochondrial genome were either moved to the eukaryotic host or were lost (37). Consequently, in spite of having their own genome, mitochondria rely heavily on nuclear genes for their function and biogenesis. The photosynthetic organisms *Arabidopsis thaliana* and *Zea mays* (maize) contain genes coding for histidine kinases and response regulators that are located in the chloroplasts or mitochondria (37). Furthermore, there is a growing body of evidence to suggest that, in photosynthetic plants, transcriptional control of photosynthesis is governed by TCSS present in chloroplasts (38–40). Similarly, the eukaryotic red alga *Porphyra purpurea* contains plastid-encoded proteins homologous to histidine kinases from members of the cyanobacterial genus *Synechocystis* (41). If chloroplasts and mitochondria were responsible for the acquisition of TCSS by eukaryotes, it is of interest to determine whether these organelles themselves retain TCSS from their bacterial ancestors. Currently, there are no data in the literature which link functions of two-component signal transduction proteins with fungal mitochondria. We demonstrate here that the nuclear genome of *C. albicans* contains elements of a two-component signaling pathway (*SRR1*) that are targeted to the mitochondria. Phylogenetic analysis and the sequence homology of Srr1 to bacterial two-component proteins suggest conservation of function during the evolutionary transition of these proteins from prokaryotes to eukaryotes.

Previous data from our laboratory and another group have shown that Srr1 is critical for adaptation to oxidative stress (10, 42). However, there was a notable difference between the two studies concerning the role of Srr1 in virulence. This discrepancy in virulence results could be due to differences in the inoculum size used to infect animals in the two studies. In addition, Bruce et al. reported that Srr1 is localized to the nucleus (42). In contrast to their results, we observed that Srr1 is located in the mitochondria. These experiments were repeated several times, using both the native and the actin promoter; at least in our hands, we failed to detect nuclear localization of Srr1.

The microarray data presented in the manuscript correlate well with the oxidative stress phenotype observed with the *srr1*Δ/Δ mutant strain (10). Oxidative stress in *C. albicans* and the model yeast *S. cerevisiae* is reported to be processed through the Hog1 MAPK pathway (13). The requirement of an upstream two-component signaling pathway for activation of the Hog1 pathway in the oxidative stress response was demonstrated in an earlier study, in which it was shown that the Ssk1 response regulator protein is

indispensable for the phosphorylation of Hog1 MAP kinase (8). Similar experiments with *srr1*Δ/Δ did not reveal any role for Srr1 in Hog1 phosphorylation (N. C. Chauhan, personal communication).

The *SRR1*-dependent genes identified by the transcriptional profiling experiments were among those whose putative function was associated either with oxidative stress or the regulation of mitochondrial function. *GOA1* of *C. albicans* was one such gene that was downregulated in the *srr1*Δ/Δ strain. Goa1 is a recently identified mitochondrial protein. The major phenotypes observed with the *goa1*Δ/Δ mutant include reduced resistance to oxidative stress and increased apoptosis compared to these phenotypes in the wild-type parent strain (27, 29). Along with *GOA1*, the other major downregulated genes identified by microarray experiments in the *srr1*Δ/Δ mutant were *MRF1*, *MAM33*, orf19.2175, and orf19.7365. None of these genes are functionally characterized and described on the *Candida* Genome Database as genes encoding putative mitochondrial proteins with function(s) associated with oxidative stress and apoptosis. Some of the *SRR1*-dependent genes, such as *MRF1*, encode several of the putative oxidoreductases present in *C. albicans*. The major function of oxidoreductases is to catalyze the oxidation-reduction reaction and retain cells in the reduced state during oxidative metabolism. These proteins have important roles in countering the toxic effects of the reactive oxygen species (ROS) generated as a consequence of normal cellular metabolism. Thus, the set of target genes identified by transcriptional profiling experiments provide a basis for our understanding of downstream effector proteins that may enable the Srr1 to carry out its functions.

In summary, we show here for the first time that Srr1, a two-component response regulator protein, is localized to the mitochondria of *C. albicans*. The gene encoding this protein has in all probability moved during evolution from ancestral cyanobacteria to the nuclear genome of eukaryotes. Srr1 is synthesized in the cytosol and imported into the mitochondria. Based upon data presented in this paper, we hypothesize that *SRR1* plays critical roles in cell survival by regulating the mitochondrial apoptotic pathway. We also hypothesize that Srr1 is the evolutionary link between mitochondria and the expression of genes that are vital to cellular homeostasis in *C. albicans*.

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