Regulatory Role of Glycerol in Candida albicans Biofilm Formation

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ABSTRACT Biofilm formation by Candida albicans on medically implanted devices poses a significant clinical challenge. Here, we compared biofilm-associated gene expression in two clinical C. albicans isolates, SC5314 and WO-1, to identify shared gene regulatory responses that may be functionally relevant. Among the 62 genes most highly expressed in biofilms relative to planktonic (suspension-grown) cells, we were able to recover insertion mutations in 25 genes. Twenty mutants had altered biofilm-related properties, including cell substrate adherence, cell-cell signaling, and azole susceptibility. We focused on one of the most highly upregulated genes in our biofilm proles, RHR2, which specifies the glycerol biosynthetic enzyme glycerol-3-phosphatase. Glycerol is 5-fold more abundant in biofilm cells than in planktonic cells, and an rhr2Δ/Δ strain accumulates 2-fold less biofilm glycerol than does the wild type. Under in vitro conditions, the rhr2Δ/Δ mutant has reduced biofilm biomass and reduced adherence to silicone. The rhr2Δ/Δ mutant is also severely defective in biofilm formation in vivo in a rat catheter infection model. Expression profiling indicates that the rhr2Δ/Δ mutant has reduced expression of cell surface adhesin genes ALS1, ALS3, and HWP1, as well as many other biofilm-upregulated genes. Reduced adhesin expression may be the cause of the rhr2Δ/Δ mutant biofilm defect, because overexpression of ALS1, ALS3, or HWP1 restores biofilm formation ability to the mutant in vitro and in vivo. Our findings indicate that internal glycerol has a regulatory role in biofilm gene expression and that adhesin genes are among the main functional Rhr2-regulated genes.

IMPORTANCE Candida albicans is a major fungal pathogen, and infection can arise from the therapeutically intractable biofilms that it forms on medically implanted devices. It stands to reason that genes whose expression is induced during biofilm growth will function in the process, and our analysis of 25 such genes confirms that expectation. One gene is involved in synthesis of glycerol, a small metabolite that we find is abundant in biofilm cells. The impact of glycerol on biofilm formation is regulatory, not solely metabolic, because it is required for expression of numerous biofilm-associated genes. Restoration of expression of three of these genes that specify cell surface adhesins enables the glycerol-synthetic mutant to create a biofilm. Our findings emphasize the significance of metabolic pathways as therapeutic targets, because their disruption can have both physiological and regulatory consequences.

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quorum-sensing molecules (11, 12). Distinct functions have been
ascribed to each cell type. Yeast cells are released from mature
biofilms and thus can cause disseminated infection (13, 14). Hy-
phae express numerous adhesins and are likely responsible for
biofilm integrity, since every known hypha-defective mutant is
also defective in biofilm production (3).

One approach to understanding key functions in biofilm for-
formation is to identify mutants that either are unable to form bio-
fils or form biofilms with altered properties (15). For C. albicans,
this kind of approach has been implemented with random inser-
tion mutants as well as mutants representing prioritized classes
of gene products (16–22). One prioritization approach uses ex-
pression profiling to examine mutants in genes that are preferentially
expressed in biofilm cells compared to planktonic cells (16, 17,
22). In the foundational C. albicans study of this kind, Bonhomme
et al. (16) relied upon diverse comparisons between biofilm and
planktonic growth conditions to arrive at a core set of biofilm-
related mutants as well as mutants representing prioritized classes
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22). In the foundational C. albicans study of this kind, Bonhomme
et al. (16) relied upon diverse comparisons between biofilm and
planktonic growth conditions to arrive at a core set of biofilm-
related genes (23). Homozygous deletion mutants were con-
structed and screened for a biofilm defect, as assayed by reduced
biofilm biomass (16). Among the 38 genes examined, nine were
required for full biofilm biomass accumulation. Such mutants
hold promise to define new biofilm-specific functions.

We have taken the work of Bonhomme et al. (16) as inspiration
but have modified several features in order to extend the ap-
proach. First, we have used RNA-Seq profiling in order to acquire
a comprehensive view of biofilm-associated gene expression
changes. Second, we used two different sequenced C. albicans clin-
ical isolates, SC5314 and WO-1 (24, 25), in order to focus on
conserved biofilm regulatory responses. Third, we have used a
panel of phenotypic screens to examine several biofilm-related
phenotypes. We find that the majority of biofilm-regulated genes
that we could disrupt influence biofilm properties. We examined
the biofilm-related function of one gene, RHR2, in detail. This
gene specifies glycerol-3-phosphatase, and we confirm the find-
ings of Bonhomme et al. that rhr2Δ/Δ mutants have a mild biofilm
defect when grown in vitro (16). We trace this defect not to a direct
consequence of altered glycerol metabolism but rather to the reg-
ulatory impact of this metabolic pathway. Our findings are partic-
ularly striking because of the severity of the requirement for Rhr2
to form biofilms in vivo, in a catheter model of biofilm infection.
These results emphasize the pivotal role that metabolic pathways
can play, not only in physiology but also in regulation.

RESULTS

Biofilm-responsive gene expression and function. We used gene
expression as a basis to identify genes that may function in biofilm
formation. Two sequenced strains, SC5314 and WO-1 (24, 25),
were analyzed through RNA-Seq profiling. Strain WO-1 can exist
in both white and opaque states (26); we used WO-1 white and
opaque cells as independent inocula. We defined biofilm-
regulated genes as those differentially expressed in biofilm-grown
cells versus planktonic cells, each line of which had been grown for
48 h in Spider medium. Two independent biofilm and planktonic
samples were examined for each type of inoculum (SC5314,
WO-1 white, and WO-1 opaque). We found a total of 180 genes
with significantly altered expression, using a false discovery rate
of <0.05, between biofilm and planktonic samples for all three ino-
cula (Fig. 1A; see also Table S1 in the supplemental material): 127
genes were upregulated and 53 were downregulated. The upregu-
lated genes represented functions in ribosome biogenesis, protein
synthesis, glycerol metabolism, and amino acid transport; down-
regulated genes represented functions in lipid catabolism and
beta-oxidation of fatty acids. We were somewhat skeptical of the
large number of apparently strain-specific gene expression
changes because they were based on only two determinations per
inoculum. However, most of the biofilm-regulated genes that
were shared among our three profiling comparisons have been
defined previously as biofilm regulated (illustrated in Table S2).

We created insertion mutants in order to screen upregulated
genes for functions related to biofilm formation. We selected the
62 most highly upregulated genes for this analysis and obtained
homozygous insertion mutants for 25 genes (see Table S2 in the
supplemental material). The remaining genes may have been es-
sential under our growth conditions or difficult to disrupt for
technical reasons.

The mutants were tested in a panel of assays related to biofilm
formation (see Table S3 in the supplemental material). For many
genes, there were multiple mutant isolates so that consistency of
any phenotypic alteration could be assessed. The mutants were
assayed for overall biofilm formation by visual inspection. They
were also assayed for activation of a cocultured “yeast reporter”
strain. In this assay, a wild-type strain carrying a yeast-phase-
specific YWPI-RFP gene fusion is used to create a mixed biofilm
with each mutant, and expression of the fusion relative to a con-
stitutive TDH3-GFP fusion is used to monitor cell-cell signaling
(11). In addition, the mutants were assayed for initiation of hyphal
production in a germ tube test, because hyphae are a major com-
ponent of C. albicans biofilms. The mutants were assayed quanti-
tatively for two more biofilm-related phenotypes, silicone adher-
ence and sensitivity to fluconazole. The results are summarized in
Table S3. No mutants were defective in germ tube formation.
However, 9 mutants altered yeast reporter strain gene expression,
thus suggesting that the mutations affect production of quorum-
sensing molecules. We also found 14 mutants with altered sensi-
tivity to fluconazole and 11 mutants with altered adherence to
silicone. Overall, these findings suggest that the majority (20/25)
of shared biofilm-regulated genes have a role in biofilm forma-
tion.

Rhr2 function in adherence and biofilm formation. We
found 9 mutants with significantly decreased adherence and 3
mutants with significantly increased adherence compared to the
wild-type strain. Among the adherence-defective mutants, the
rhr2−/− insertion mutant was noteworthy because RHR2 is
among the most highly upregulated biofilm genes (see Table S1 in the
supplemental material). RHR2 encodes the enzyme glycerol-
3-phosphatase, which acts at the terminal step in glycerol biosyn-
thesis (27). The two other glycerol-biosynthetic genes, GPD1 and
GPD2, were also highly induced in biofilms, and mutations in
those genes caused reduced adherence (see Table S3). These find-
ings point toward a role for glycerol and Rhr2 in adherence,
though the basis for this connection is not obvious.

To verify Rhr2 function, we constructed an rhr2Δ/Δ deletion
mutant and an rhr2Δ/Δ+pRHR2 complemented strain. The
rhr2Δ/Δ mutant was defective in adherence, and complementa-
tion rescued the adherence defect (Fig. 2C). Therefore, Rhr2 has
a positive role in adherence.

We quantified glycerol levels in biofilms and planktonic cells to
verify Rhr2 metabolic function. Wild-type biofilms accumulate
glycerol at levels 4.7-fold higher than do planktonic cells (see
Fig. S1 in the supplemental material). The rhr2Δ/Δ mutant had a
FIG 1  Biofilm gene expression and \(RHR2\) function. (A) Transcription profiling comparison. RNA-Seq-based expression ratios for biofilm versus planktonic growth conditions (see Table S1 in the supplemental material) were used to define biofilm-upregulated genes (indicated by \(+\)) and biofilm-downregulated genes (indicated by \(-\)). Common responses among three inocula used in this study, strain SC5314, strain WO-1 white, and strain WO-1 opaque, are indicated by the Venn diagram. An additional 49 genes had divergent responses among the inocula. (B) \(RHR2\) impact on biofilm biomass. Biofilms were grown in Spider or Spider-glycerol medium for 48 h, and the average dry weight was measured (\(n = 5\)). Results are expressed relative to the wild type. The strains used were DAY185 (wild type), JVD005 (\(rhr2\)/\(Δ\)), and JVD006 (\(rhr2\)/\(Δ\)+\(pRHR2\)). (C and D) Confocal imaging of biofilms. Twenty-four-hour biofilms of wild-type, \(rhr2\)/\(Δ\), and \(rhr2\)/\(Δ\)+\(pRHR2\) strains were grown in the media indicated, stained, and imaged. Side-view projections were computed by reslicing the intensity-corrected serial image stack from bottom to top. The resliced stack was then used for maximum-intensity projection. The displayed apical-view projections were pseudocolored to indicate biofilm depth, using the color calibration and scale bar displayed at the top right. The color scale bars correspond to 180 \(µm\) (B) or 101 \(µm\) (C). Biomass and glycerol levels were quantified from 48-h biofilms as described in Materials and Methods. Glycerol levels were normalized to total cell weight.
FIG 2 RHR2-dependent gene expression and function. (A) Genome-wide analysis. RNA-Seq expression data values for rhr2Δ/Δ+pRHR2 complement were divided by expression data values of the rhr2Δ/Δ mutant to calculate fold change values. Genes with fold changes of ≥1.5 or ≤0.67 are shown. For these differentially regulated genes, their fold change values in biofilm versus those under planktonic conditions were obtained. MultiExperimentViewer (MeV v4.6.2) was then used for hierarchical clustering by average linkage clustering based on Manhattan distance and optimized for gene leaf order. Yellow indicates upregulated genes; blue indicates downregulated genes. (B) Glycerol response of gene expression in the rhr2Δ/Δ mutant. Overnight cultures grown in yeast extract-peptone-dextrose and yeast extract-peptone-glycerol were used to inoculate Spider and Spider-glycerol media, respectively, and cells were grown for an additional 8 h for RNA extraction and qRT-PCR determinations. The table shows gene expression in the rhr2Δ/Δ mutant relative to the wild type. (C) Adherence assays. Cell wall adhesin genes were overexpressed using a constitutive TDH3 promoter in the rhr2Δ/Δ background. Substrate adherence was quantified as described in Materials and Methods and the legend to Fig. 1B. An asterisk above a bar indicates a P value of <0.05 with respect to rhr2Δ/Δ. (D) Biofilm formation assays. Adhesin overexpression strains were used to analyze biofilm formation. The biofilm biomass assay and confocal imaging of biofilms were performed as outlined in Materials and Methods and for panel B. The pseudocolor scale bar corresponds to 161 μm.
accumulation (Fig. 1C), though not to the wild-type level, perhaps because of a gene dosage effect. These measurements verify that glycerol accumulates at high levels in biofilms, in parallel with the high-level expression of RHR2.

Prior studies showed that RHR2 is required for efficient biofilm formation, because biofilms produced by an rhr2Δ/Δ mutant had 2-fold-reduced biomass in a minimal medium (16). We confirmed the mutant biomass defect in our standard biofilm medium, Spider medium (Fig. 1B and C). In addition, confocal imaging revealed that the depth of the rhr2Δ/Δ mutant biofilm was greatly diminished compared to that of the wild-type and complemented strains (Fig. 1C). Apical-view images indicate that the mutant biofilm consists primarily of a basal layer of yeast-form cells with few interspersed hyphae (Fig. 1C, lower panels). Therefore, Rhr2 is required for normal biofilm formation.

If a defect in glycerol production is the cause of the mutant biofilm defect, then exogenous glycerol may restore biofilm formation by the mutant. Spider-glycerol medium, in which glycerol replaces mannitol as the carbon source, supported biofilm formation by the wild-type strain, though overall depth and biomass were reduced slightly compared to those of biofilms formed in standard Spider medium (Fig. 1D). These properties likely reflect the lower growth rate in Spider-glycerol than in Spider medium (data not shown). Importantly, the rhr2Δ/Δ mutant strain formed biofilms similar in structure and biomass to those of the wild-type and complemented strains in this medium (Fig. 1B and D). These results confirm that the function of Rhr2 in biofilm formation derives from its role in glycerol synthesis.

The environmental influence on Rhr2 phenotypes led us to ask whether Rhr2 is required for biofilm formation in vivo. We addressed this question with two biofilm infection models, a rat central venous catheter model (29) and a mouse oropharyngeal candidiasis model (30). In the venous catheter model, biofilm formation within the catheter lumen was assessed by scanning electron microscopy. The rhr2Δ/Δ mutant was severely defective in biofilm formation (Fig. 3), yielding a catheter lumen virtually devoid of C. albicans cells. Biofilm formation was restored in the complemented strain. In the mouse oropharyngeal candidiasis model, biofilm formation was assessed by fungal burden on the tongue. In this model, the mutant showed no defect compared to the wild-type and complemented strains (see Fig. S2 in the supplemental material). Therefore, Rhr2 is not required for biofilm infection of the oral mucosa, but it is required for biofilm formation on a central venous catheter. Rhr2 biological function is contingent upon the environment in vivo, as it is in vitro.

Rhr2 impact on biofilm gene expression. It seemed possible that the impact of Rhr2 on adherence and biofilm formation might result from effects on gene expression. To explore that possibility, we compared the mutant and complemented strains through whole-genome expression profiling using RNA-Seq (Fig. 2A; see also Table S1 in the supplemental material), with confirmation by nanoString and quantitative reverse transcription-PCR (qRT-PCR) assays (see Fig. S3). The two strains were grown under planktonic conditions for profiling to avoid indirect effects of differences in biofilm formation ability; two independent cultures of each strain were used for RNA samples. We observed that many genes with functional roles in biofilm formation were downregulated in the rhr2Δ/Δ mutant (Fig. 2A), including the adhesin genes ALS1 and HWP1. A third biofilm adhesin gene, ALS3, was not uniquely detected by RNA-Seq, but its expression defect in the rhr2Δ/Δ mutant was established by nanoString analysis (see Fig. S3) and qRT-PCR (data not shown). Overall, expression of many genes was stimulated both by Rhr2 and by growth under biofilm conditions (Fig. 2A). Providing glycerol to the rhr2Δ/Δ mutant through growth in Spider-glycerol medium led to increased expression of two Rhr2-dependent genes, ALS1 and HWP1, as expected if the mutant’s metabolic defect is the cause of its gene expression defect (Fig. 2B). Taken together, these observations suggest that the high-level expression of RHR2 and glycerol in biofilm cells may be required for a substantial portion of the biofilm-associated gene expression profile.

Functional basis of the rhr2Δ/Δ mutant biofilm defect. The gene expression profile of the rhr2Δ/Δ mutant suggested the simple hypothesis that diminished adhesin gene expression might be the cause of the mutant’s defects in adherence and biofilm formation. To test that hypothesis, we created rhr2Δ/Δ mutant derivatives with restored high-level expression of each adhesin gene—ALS1, ALS3, and HWP1—and assessed their capacity for adherence and biofilm formation. Increased expression of each adhesin improved adherence of the rhr2Δ/Δ mutant to levels comparable to those of the wild-type strain (Fig. 2C). In addition, increased expression of each adhesin restored the biofilm formation ability of the rhr2Δ/Δ mutant in vitro, as determined by both biomass measurements and confocal imaging (Fig. 2D). We also assessed biofilm formation capacity of these strains in vivo with the rat venous catheter biofilm model (Fig. 3). Increased expression of ALS1, ALS3, or HWP1 in the mutant led to biofilm formation in vivo, with increased ALS3 expression causing the most extensive biofilm formation. These findings indicate that Rhr2 is required for biofilm formation in vitro and in vivo because of the regulatory consequences of altered glycerol synthesis.

DISCUSSION
It has been understood for some time that C. albicans biofilm formation depends upon cell surface adhesins (3, 4). There has been considerable progress in identification of the transcription factors that control adhesin gene expression (3, 17, 31). However, the environmental and physiological signals that govern adhesin expression, especially those that function in vivo during infection, are more poorly defined. Our findings here indicate that glycerol biosynthesis is critical for proper expression of numerous biofilm-regulated genes, including three key adhesin genes.

Several prior studies have examined the biofilm transcriptome, using a range of profiling methods and growth platforms (17, 23, 32, 33). In the present study, as in the work of Yeater et al. (33), we have compared biofilm-associated gene expression in different C. albicans strains under similar growth conditions. The strains we used, SC5314 and WO-1, represent different clades (34) and mating types (35, 36). SC5314-derived strains have a very broad transcriptional response to biofilm growth: Garcia-Sánchez et al. found over 300 biofilm-regulated transcripts through a microarray comparison of diverse growth conditions (23); Yeater et al. found roughly 600 biofilm-regulated transcripts though microarray comparison of 48-h samples (33); Nobile et al. found 1,519 biofilm-regulated transcripts through an RNA-Seq analysis (17).
Our data are consistent with an exuberant response by SC5314 (Fig. 1A). For strain WO-1, we used separate white and opaque cell inocula, but because our growth temperature of 37°C induces conversion to white cells (36), we expected the white and opaque biofilms to yield similar expression profiles. Both WO-1 inocula displayed fewer biofilm-regulated transcripts than did SC5314. Day-to-day variability may be the source of some of the differences rather than strain background, given that we used only two samples per inoculum and growth condition. However, our definition of common biofilm-regulated genes among the strains is validated both by comparison to other data sets and by functional analysis.

The overall results of our mutant analysis argue that common upregulated genes function in biofilm development, because 20 insertion mutants among the 25 genes sampled had measurable alterations in biofilm properties. Our findings contrast with the pioneering study by Bonhomme et al. (16), who identified biofilm defects in only 9 of the 38 deletion mutants of biofilm-upregulated genes. The differences between our findings may reflect our gene selection criteria; only 4 genes were disrupted in both studies (RHR2, CAN1, MET3, and orf19.3483). In addition, we have used a larger panel of assays for biofilm-related phenotypes. We acknowledge that differential expression can overlook functionally relevant genes; an example from our data set is HWP1, which clearly functions in biofilm formation (37) and yet is more highly expressed in all of our planktonic samples than in biofilm samples. We value the criterion of differential expression for its positives in the end, and the diversity of genes and phenotypes that we have found invites many future functional studies.

Our focus on RHR2, which specifies glycerol-3-phosphatase, was based on three features. First, it is among the most highly upregulated genes in biofilms compared to planktonic cells in our data sets and in several other profiling studies (23, 33). Second, foundational work from the d’Enfert lab has shown that an rhr2Δ/Δ mutant produces a biofilm with reduced biomass in vitro (16), which we confirmed. Hence, while the mutant defect seems only partial, it is robust. Finally, the mutant had an adherence defect, a phenotype that we have studied in some detail, and yet one with no obvious connection to Rhr2 function in glycerol metabolism. Our analysis reveals that, in one respect, the relationship is fairly simple: Rhr2 is required for RNA accumulation from...
the major adhesin genes ALS1, ALS3, and HWPI. Prior studies have shown that these adhesins are required for biofilm formation in vitro and in vivo (31, 37, 38, 39), and we showed here that increased expression of any one of those adhesins can restore biofilm formation, in vitro and in vivo, in an rhr2ΔA mutant background. These observations argue strongly that Rhr2 is required for biofilm formation primarily to promote expression of key adhesin genes.

The regulatory impact of Rhr2 extends well beyond adhesin gene expression. Under the planktonic growth conditions in which we compared the mutant and complemented strains, almost 400 genes were differentially expressed. Strikingly, the expression alteration in the rhr2ΔA mutant for many of these genes correlates inversely with their expression alteration in response to biofilm growth. These results suggest that glycerol metabolism is a prominent signal that drives biofilm-associated gene expression.

How does glycerol influence gene expression? One hypothesis is related to the well-established role of glycerol in maintaining intracellular osmotic pressure, or turgor (40). In Saccharomyces cerevisiae, turgor is sensed by a phosphorelay system (41, 42) that ultimately activates the mitogen-activated protein (MAP) kinase Hog1 under low-turgor conditions (43). This pathway affects gene expression in numerous fungi (44). A simple model is that loss of Rhr2 mimics the effect of high external osmolarity and causes elevated Hog1 activity, which in turn causes the rhr2ΔA gene expression alterations. Two observations argue against this model. First, Hog1 is constitutively activated by mutation of the phosphorelay gene SLN1, but an sln1Δ/Δ insertion mutation does not cause the adhesive defect predicted by this hypothesis (J. V. Desai, unpublished data). Second, an amino acid substitution in the phosphorelay component Skl1 (DS13K) in C. albicans that causes constitutive Hog1 activation leads to a defect in hypha formation (45, 46). However, the rhr2ΔA mutant has no defect in hypha formation. Therefore, we have no evidence that the Hog1 pathway mediates the biofilm-related defects of the rhr2ΔA mutation.

We favor a second model in which glycerol levels may be sensed by one or several transcription factors that are required for adherence or biofilm formation (17, 47). RHR2 appears to be integrated into the biofilm regulatory network, because most transcription factors that are required for biofilm formation are required for RHR2 RNA accumulation. One biofilm regulatory mutant, tye7ΔA, is largely rescued on glycerol medium (J. V. Desai, unpublished data), as expected if its RHR2 expression defect contributes to the mutant biofilm defect. Biofilm-defective transcription factor mutants that are not rescued on glycerol medium are candidates for glycerol response mediators that act downstream of the glycerol signal.

Why might internal glycerol levels be a regulatory signal that is required for biofilm formation? One possible reason has to do with the need for glycerol in glycosylphosphatidylinositol (GPI) anchor synthesis (48). These glycolipid structures are used to generate the tethers that hold adhesins and other mannoproteins to the cell surface (48). Thus, it may benefit the cell to take inventory of its glycerol stores before embarking on a growth pathway that relies upon functional adhesin biogenesis. A second possible reason has to do with one niche for C. albicans biofilm formation: mucosal surfaces. It is possible that a mucosal biofilm serves as a stepping-stone toward surface invasion. If that is the case, then it may benefit the cell to ensure that glycerol is available to support turgor generation necessary for tissue penetration.

MATERIALS AND METHODS
RNA sample preparation. Biofilm and planktonic cell samples were prepared after growth for 48 h at 37°C. The rhr2ΔA and complemented strains were grown in the media indicated for 8 h at 37°C. Cells were harvested by filtration and stored frozen on filters at −80°C until RNA extraction. RNA was extracted using a RiboPure yeast kit (11, 49).

RNA sequencing and differential expression analysis. For comparison of biofilm and planktonic samples, the RNA-Seq libraries (strand specific, single read) were prepared as described previously (50) and 30 nucleotides (nt) of sequence was determined from one end of each cDNA fragment using the Illumina GA2 platform (51). Twelve samples in total were analyzed, two of each of the following: SC5314 biofilm, SC5314 planktonic, WO-1 white biofilm, WO-1 white planktonic, WO-1 opaque biofilm, and WO-1 opaque planktonic. For the comparison of rhr2ΔA and complemented strains, the RNA-Seq libraries (non-strand specific, paired end) were prepared with the TruSeq RNA Sample Prep kit (Illumina, San Diego, CA) and 100 nt of sequence was determined from both ends of each cDNA fragment using the HiSeq 2000 platform (Illumina, San Diego, CA). Four samples in total were analyzed, two rhr2ΔA cultures and two complemented strain cultures. The sequencing reads were aligned to the C. albicans reference genomes (SC5314 or WO-1) using TopHat (52), allowing up to two mismatches per 30-bp segment and removing reads that aligned to more than 20 locations. The alignment files from TopHat were then utilized to generate read counts for each gene, and a statistical analysis of differential gene expression was performed using the DESeq package from Bioconductor (53). A gene was considered differentially expressed if the false discovery rate for differential expression was less than 0.05.

Additional methods. Additional methods, strain genotypes, primer sequences, and details for the procedures above are provided in Text S1 in the supplemental material. RNA-Seq data is available from GEO under accession number GSE45141.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl doi:10.1128/mBio.00637-12/-/DCSupplemental.

Text S1, DOC file, 0.2 MB.
Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.1 MB.
Table S1, XLS file, 1 MB.
Table S2, XLS file, 0.1 MB.
Table S3, XLS file, 0.7 MB.

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