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 *rhr2*Δ/Δ strain accumulates 2-fold-less biofilm glycerol than does the wild type. Under *in vitro* conditions, the *rhr2*Δ/Δ mutant has reduced biofilm biomass and reduced adherence to silicone. The *rhr2*Δ/Δ mutant is also severely defective in biofilm formation *in vivo* in a rat catheter infection model. Expression profiling indicates that the *rhr2*Δ/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 *rhr2*Δ/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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Most microorganisms exist in surface-associated, matrix-embedded communities called biofilms (1). Biofilms can form on both biotic and abiotic surfaces (2), and their formation on implanted medical devices is a significant source of infection (3). Biofilm cells are resistant to many antimicrobial agents, so device-associated infections may necessitate surgical removal of the device (2, 4). Unfortunately, many patients succumb to these infections (5, 6). An understanding of biofilm development mechanisms may provide strategies for improved therapeutic intervention.

Our focus is on *Candida albicans*, a fungal pathogen that causes device-associated infections (3, 6). *C. albicans* biofilms are commonly found on surfaces of implanted devices such as venous catheters, voice prostheses, dentures, and urinary catheters (2, 6). In addition, *C. albicans* can infect mucosal surfaces, producing a growth state that has similarity to abiotic-surface biofilms in both architecture and genetic control (7, 8).

Biofilm formation is thought to begin with the adherence of individual cells to a surface (3, 4). Growth into a biofilm then requires cell-cell adherence, so that the surface is populated by several layers of cells. As a biofilm matures, the cells display phenotypes that distinguish them from planktonic cells (i.e., cells grown in liquid suspension culture). These biofilm phenotypes include accumulation of extracellular matrix material and acquisition of drug resistance (4, 9). In the case of *C. albicans*, resistance is notable in particular to azole antifungals, which are frontline therapeutics (10). Mature *C. albicans* biofilms also have apparent cell heterogeneity because two major cell types, yeast (blastospores) and hyphae, are present. The balance of yeast and hyphal cells in a biofilm is influenced by diffusible signals in the form of

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). Hyphae 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 formation is to identify mutants that either are unable to form biofilms or form biofilms with altered properties (15). For *C. albicans*, this kind of approach has been implemented with random insertion mutants as well as mutants representing prioritized classes of gene products (16–22). One prioritization approach uses expression 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-induced genes (23). Homozygous deletion mutants were constructed 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 approach. 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* clinical 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 findings of Bonhomme et al. that *rhr2*Δ/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 regulatory impact of this metabolic pathway. Our findings are particularly 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 inocula (Fig. 1A; see also Table S1 in the supplemental material): 127 genes were upregulated and 53 were downregulated. The upregulated genes represented functions in ribosome biogenesis, protein synthesis, glycerol metabolism, and amino acid transport; downregulated 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 essential 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 *YWP1-RFP* gene fusion is used to create a mixed biofilm with each mutant, and expression of the fusion relative to a constitutive *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 component of *C. albicans* biofilms. The mutants were assayed quantitatively for two more biofilm-related phenotypes, silicone adherence 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 sensitivity 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 formation.

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 biosynthesis (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 findings 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 *rhr2*Δ/Δ deletion mutant and an *rhr2*Δ/Δ+pRHR2 complemented strain. The *rhr2*Δ/Δ mutant was defective in adherence, and complementation 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 *rhr2*Δ/Δ mutant had a

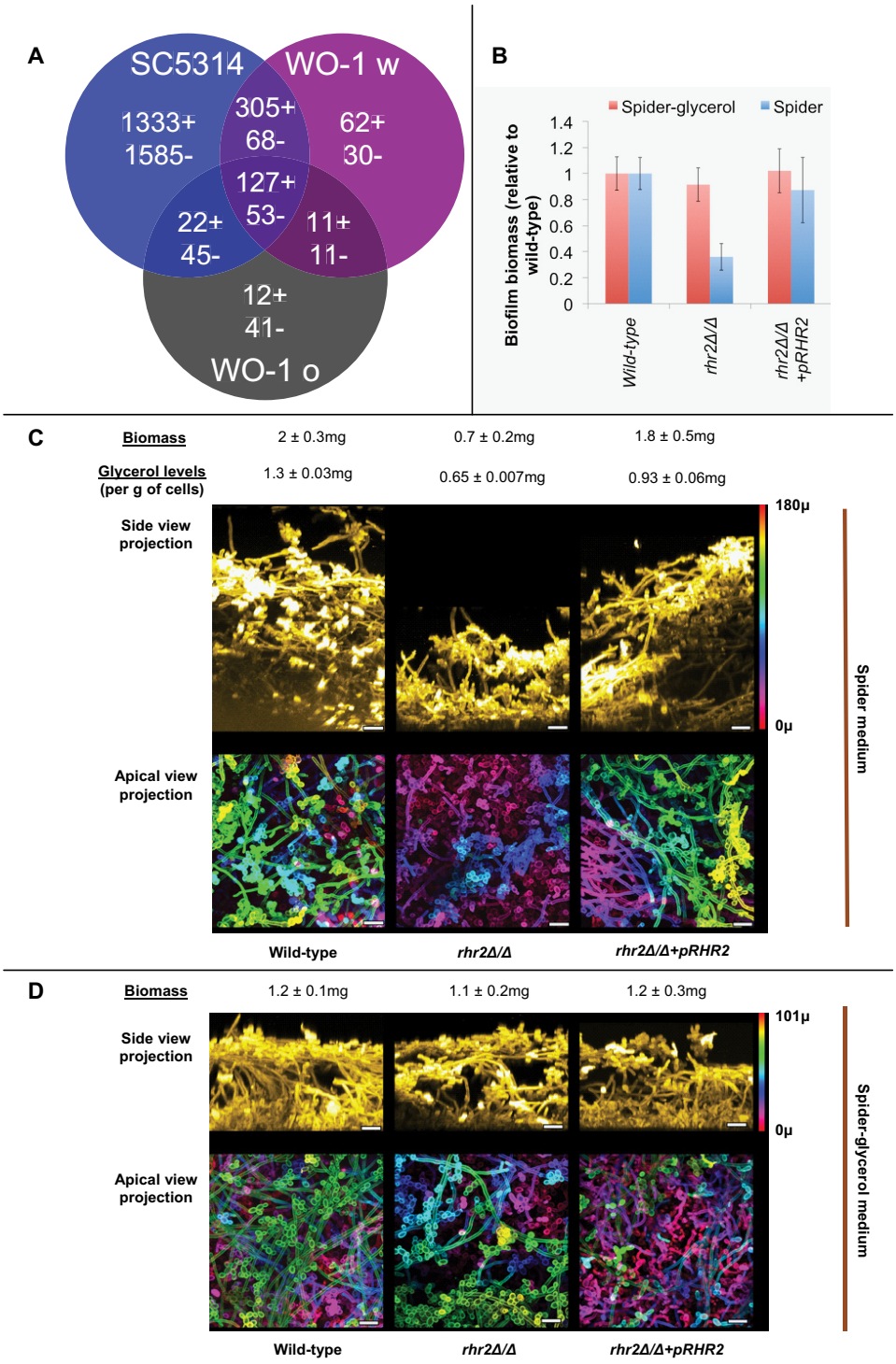
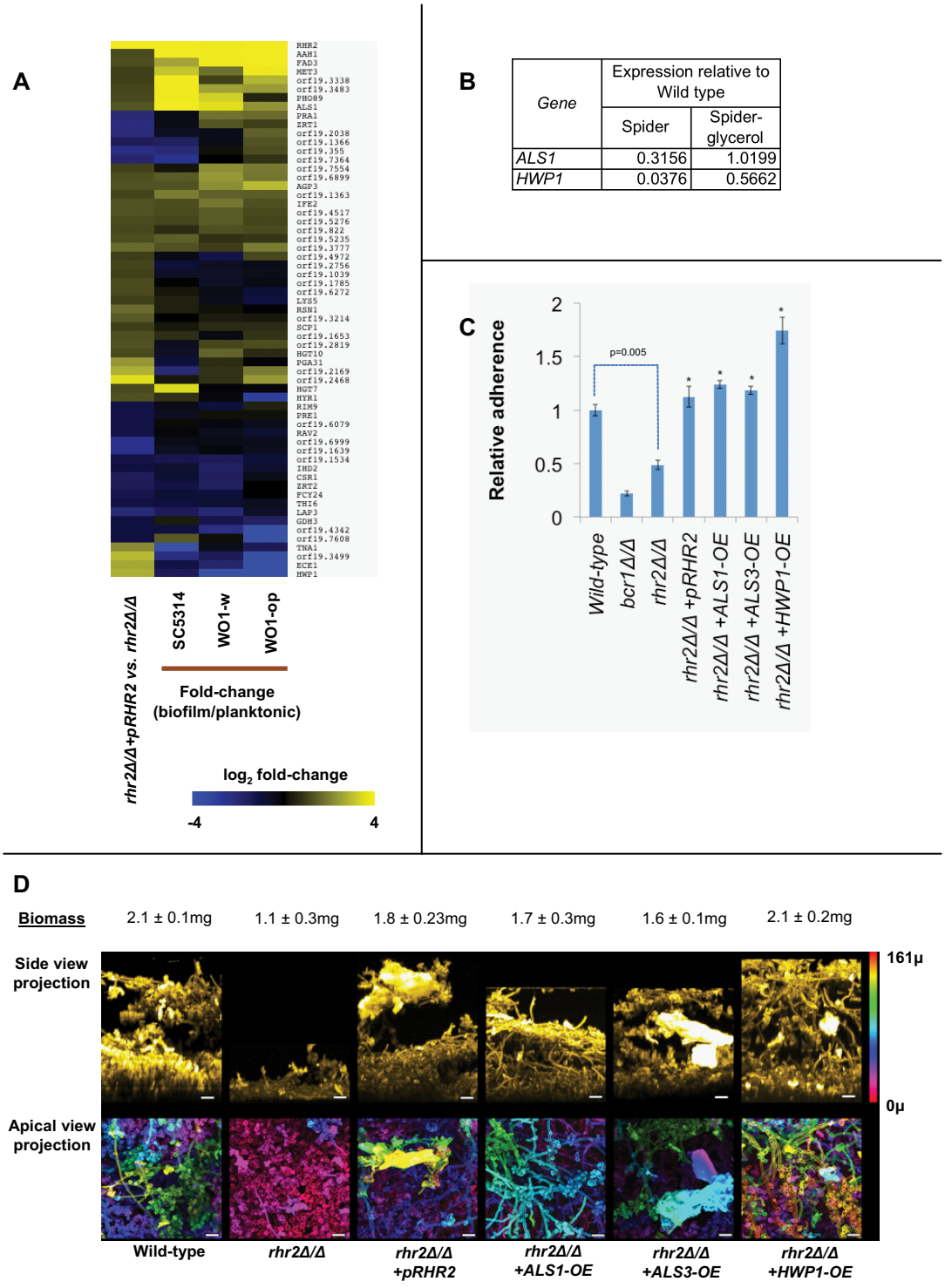


FIG 1 Biofilm gene expression and *RHR2* function. (A) Transcription profiling comparison. RNA-Seq-based expression ratios for biofilm versus planktonic growth conditions (see Table S1 in the supplemental material) were used to define biofilm-upregulated genes (indicated by +) and biofilm-downregulated 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/Δ), and JVD006 (*rhr2*Δ/Δ+pRHR2). (C and D) Confocal imaging of biofilms. Twenty-four-hour biofilms of wild-type, *rhr2*Δ/Δ, and *rhr2*Δ/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.



Gene	Expression relative to Wild type	
	Spider	Spider-glycerol
ALS1	0.3156	1.0199
HWP1	0.0376	0.5662

FIG 2 *RHR2*-dependent gene expression and function. (A) Genome-wide analysis. RNA-Seq expression data values for *rhr2*Δ/Δ+pRHR2 complement were divided by expression data values of the *rhr2*Δ/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 *rhr2*Δ/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 *rhr2*Δ/Δ mutant relative to the wild type. (C) Adherence assays. Cell wall adhesin genes were overexpressed using a constitutive *TDH3* promoter in the *rhr2*Δ/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 *rhr2*Δ/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.

~50% reduction in biofilm glycerol accumulation compared to the wild type (Fig. 1C). (We note that an *S. cerevisiae* glycerol-3-phosphatase defect reduces, but does not abolish, glycerol accumulation [28], as we observed here with *C. albicans*.) Complementation with a wild-type copy of *RHR2* increased glycerol 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 *rhr2*Δ/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 *rhr2*Δ/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 *rhr2*Δ/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 *rhr2*Δ/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 *rhr2*Δ/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 *rhr2*Δ/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 *rhr2*Δ/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 *rhr2*Δ/Δ mutant biofilm defect. The gene expression profile of the *rhr2*Δ/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 *rhr2*Δ/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 *rhr2*Δ/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 *rhr2*Δ/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: García-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).

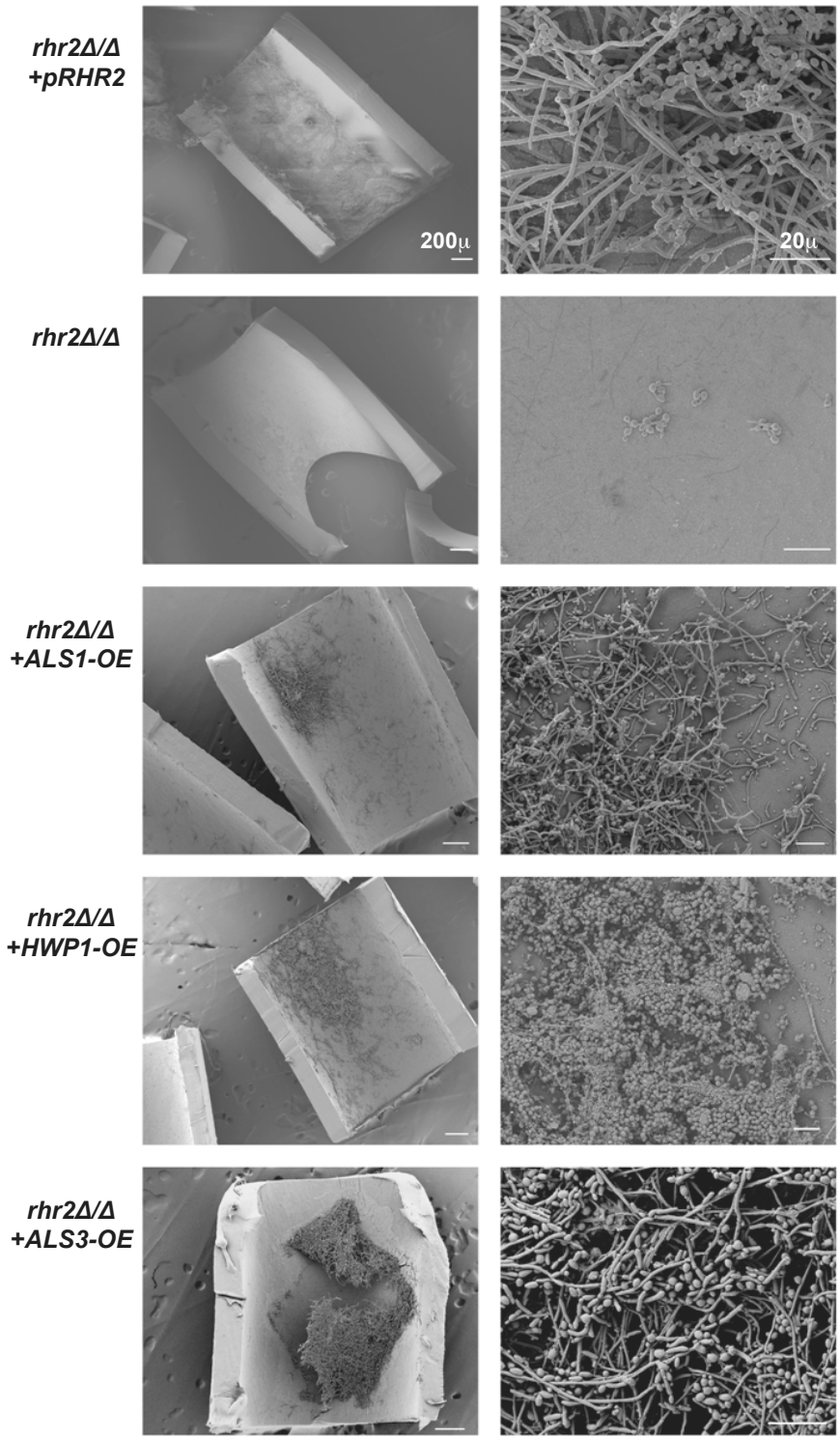


FIG 3 *RHR2* requirement for biofilm formation *in vivo*. Strains indicated were inoculated in the rat venous catheter biofilm model, incubated for 24 h, and imaged using scanning electron microscopy. The images are ×100- and ×1,000-magnification views of the catheter lumens, with scale bars corresponding to 200 μm and 20 μm, respectively. The strains used were JVD006 (*rhr2*Δ/Δ+pRHR2), JVD005 (*rhr2*Δ/Δ), JVD018 (*rhr2*Δ/Δ+*ALS1-OE*), JVD020 (*rhr2*Δ/Δ+*HWP1-OE*), and JVD025 (*rhr2*Δ/Δ+*ALS3-OE*), from top to bottom, respectively.

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° 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 *rhr2*Δ/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 *HWP1*. 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 *rhr2*Δ/Δ 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 *rhr2*Δ/Δ 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 *rhr2*Δ/Δ gene expression alterations. Two observations argue against this model. First, Hog1 is constitutively activated by mutation of the phosphorelay gene *SLN1*, but an *sln1*−/− insertion mutation does not cause the adherence defect predicted by this hypothesis (J. V. Desai, unpublished data). Second, an amino acid substitution in the phosphorelay component Ssk1 (D513K) in *C. albicans* that causes constitutive Hog1 activation leads to a defect in hypha formation (45, 46). However, the *rhr2*Δ/Δ mutant has no defect in hypha formation. Therefore, we have no evidence that the Hog1 pathway mediates the biofilm-related defects of the *rhr2*Δ/Δ 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, *tye7*Δ/Δ, 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 *rhr2*Δ/Δ 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 *rhr2*Δ/Δ 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 *rhr2*Δ/Δ 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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