Correlation between Biofilm Formation and the Hypoxic Response in *Candida parapsilosis*†‡

Tristan Rossignol,¹,²‡ Chen Ding,²‡ Alessandro Guida,³ Christophe d’Enfert,¹ Desmond G. Higgins,³ and Geraldine Butler²*†

Institut Pasteur, Unité Biologie et Pathogénicité Fongiques, INRA USC2019, 25 rue du Docteur Roux, 75724 Paris, France;¹ UCD School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland;² and UCD School of Medicine and Medical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland³

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acid metabolism, and ergosterol synthesis. Some of these changes are similar to those observed in C. albicans cells grown under low-oxygen conditions (52). We therefore also profiled C. parapsilosis cells growing in a hypoxic environment, and we characterized the effect of deleting RBT1, a gene induced both in biofilms and under conditions of hypoxia.

MATERIALS AND METHODS

Biofilms on 96-well plates and silicone squares. Ninety-six-well polystyrene microtiter plates (catalog number 167008 from Nunc or from Techno Plastic Products AG) were inoculated with 100 μl per well of a C. parapsilosis culture grown overnight diluted to an A₅₀₀ of 0.05 in either SD medium supplemented with 50 mM dextrose or RPMI 1640 with Glutamax medium (Invitrogen) buffered with 50 mM HEPES and incubated at 37°C for 1 h to allow adherence. Hypoxic experiments were carried out in a dedicated chamber (In Vivo 400 workstation) with 1% O₂.

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RNA was isolated using an RNeasy kit (Qiagen) for the biofilms and a Ribopure kit (Ambion) for the hypoxia cultures according to the manufacturer’s instructions. The quality and concentration of the isolated RNA were analyzed using an Agilent 2100 Bioanalyzer. For the biofilm experiments, RT, cDNA labeling, and probe purification were carried out using an Atlas PowerScript fluorescent labeling kit (Takara) according to the manufacturer’s instructions, starting with 5 μg of total RNA. Partial genomic microarrays representing 3,849 putative ORFs described previously by Rossignol et al. (50) were used. Hybridization, washing, and scanning were carried out as described previously by Rossignol et al. (50). For the hypoxic experiments, 24 μg of total RNA was labeled as described previously (50). The experimental and control samples were mixed and applied to genomic microarrays representing 5,834 ORFs (manufactured by Agilent Technologies, design 015742). The hybridization, washing, and scanning protocols were the same as those used for the biofilm arrays. Six independent biological replicates were compared; in four replicates, the biofilm samples were labeled with Cy5, and in the other two replicates, the biofilm sample was labeled with Cy3, and the other two replicates, the biofilm sample was labeled with Cy3.

Data analysis. The data from both the biofilms and the hypoxic conditions were statistically analyzed using the LIMMA package from the Bioconductor project (54). The data sets were preprocessed using Loess normalization and no background correction (as suggested in reference 65). Probes with a change lower than 1.5-fold were discarded. For the biofilm arrays, probes with adjusted P values of greater than 0.05 were discarded, resulting in 185 genes that are differentially expressed. In the hypoxia experiments, probes with adjusted P values higher than 0.005 were excluded. For this data set, only genes with three or four significant probes were included. The final list contained 341 differentially expressed genes.

GO analysis. The Bioconductor package topGO was used to identify enrichment of Gene Ontology (GO) terms in both data sets. C. parapsilosis orthologs in C. albicans were first identified using gene family and reciprocal BLAST analysis (16). For the biofilm experiment, 3,573 (94.3%) of the spotted probes and 185 (94.6%) of the differentially expressed genes had identifiable orthologs in C. albicans. For the hypoxia experiment, 5,091 (87.3%) orthologs of the entire C. parapsilosis gene set and 300 (88%) orthologs of the differentially expressed genes were identified. The most recent GO annotation (version 1.493, 13 May 2008) for C. albicans was downloaded from the Candida Genome Database (http://www.candidagenome.org). GO terms associated with probes were assigned to 300 (88%) of the biofilm arrays and 3,017 genes on the hypoxia arrays. For the differentially expressed genes, GO terms were assigned to 121 genes in the biofilm experiment and 174 genes in the hypoxia experiment. Enrichment of categories was determined using two statistical approaches, classic (Fisher’s exact test compares the number of observed
The SAT1 gene was deleted using the ApaI flipper cassette as previously described (15). Oligonucleotides Rbt1Apa5 and Rbt1int_Apa, which both introduce ApaI recognition sites, were used to amplify a 587-bp fragment from the region downstream of SAT1. An 8-kb PvuI/SacI fragment encompassing SAT1 and the SAT1 nourseothricin marker was introduced into C. parapsilosis CLIB214. Nourseothricin-resistant colonies were selected, and integration at the rbt1 deletion was confirmed using oligonucleotides Rbt1up and Rbtmid, which generate a 1.0-kb PCR product (CDR1, CDR210, and CDR311), which is absent from the wild-type strain (CLIB214) and recycled strains (CDR1, CDR212, and CDR88). The presence of at least one intact RBT1 gene was tested using oligonucleotides Rbtup and Rbtmid, which generate a 1.0-kb product from the wild-type allele only (CLIB214, CDR1, CDR14, CDR311, and CDR88). Oligonucleotides RbtKpn and RbtSfi were used to confirm recycling of the SAT1 flipper cassette from both alleles. The wild-type allele generates a 3.0-kb PCR product (CDR14 and CDR210) and recycled strains generate a 1.0-kb PCR product (CDR1, CDR210, and CDR121).

An intact RBT1 allele was reconstituted by amplifying the entire ORF (including 190 bp of promoter sequence and 40 bp of termination sequence) using oligonucleotides Rbt1Ap5 and Rbt1int_Apa, which both introduce ApaI restriction sites. The PCR fragment was cloned into plasmid pCD39, which contains the SAT1 cassette and a 587-bp fragment from the region downstream of RBT1. An 8-kb PvuI/SacI fragment encompassing RBT1 and the SAT1 nourseothricin marker was introduced into C. parapsilosis CLIB214. Nourseothricin-resistant colonies were selected, and integration at the rbt1 deletion was confirmed using oligonucleotides Rbt1up/Rbtmid and RbtSfi/Bat237, which generate fragments of 1.0 kb and 850 bp, respectively. The SAT1 marker was then removed by inducing recombination.

The constructs were also confirmed by Southern hybridization using a probe amplified from the cassette using oligonucleotides Rbt1int and Rbt1Ap5. Labeling and hybridization were carried out using DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche) according to the manufacturer’s instructions. Genomic DNA was isolated from a 5-ml culture grown overnight and was digested using NcoI and XmaI overnight at 37°C. A 4.8-kb NcoI fragment (representing the wild-type RBT1 allele) was detected in CLIB214, CDR1, and CDR14. A 3.8-kb NcoI/XmaI fragment was detected after integration at one RBT1 allele in strains CDR1 and CDR210. Recycling of the integration cassette produces a 2.7-kb NcoI fragment, which is present in strains CDR14, CDR210, and CDR212. Reintroducing the RBT1 allele (CDR88) generates a 5.0-kb fragment (4.8 kb from the wild-type allele plus a 200-bp duplication generated during construction of the vector). We also observed a nonspecific fragment around 3.0 kb, which is present in all strains.

Fluorescence microscopy. Fluorescence microscopy was performed using biofilms grown for 50 h in the same medium and conditions as those used for the microarray experiments. The biofilms were stained in a 5-ml solution of 50 μg ml−1 Alexa Fluor 594 conjugate of concanavalin A (Invitrogen) for 1 h in the dark and observed without washing. Images were obtained with an Upright Wide Field Microscope Axiosplan (Zeiss) and Axiovision 4.5 (Zeiss) software, with a 40× immersion objective. An HBO mercury short arc lamp was used with a XF 43 filter (excitation wavelength of 563 to 587 nm and emission wavelength of 615 to 645 nm; Omega Optical), z-stack images were acquired at 1-μm step intervals. Image deconvolution was performed using Huygens software (SVI), and three-dimensional reconstruction was done with Imaris software (Bitplane).

Microarray data accession numbers. The transcriptional data and a description of the arrays used here have been deposited in the Gene Expression Omnibus database under accession numbers GPL7693, GSE13717, GSE13722, and GSE13832.

<table>
<thead>
<tr>
<th>GO term</th>
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</tr>
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<tbody>
<tr>
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</tr>
<tr>
<td>Monocarboxylic acid metabolic process</td>
<td>4.20E−08</td>
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</tr>
<tr>
<td>Ergosterol metabolic process</td>
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</tr>
<tr>
<td>Glycolysis</td>
<td>1.25E−03</td>
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</tbody>
</table>

a RNA was extracted from C. parapsilosis biofilms after 50 h, and the transcriptional profile was compared to that of planktonic culture for independent replicates. GO enrichment analysis was carried out using topGO (2) to determine GO classes that are overrepresented in the differentially expressed data. The GO terms listed are sorted by adjusted P values using the classic (Fisher’s exact test) approach and are also significantly different using the adjusted P values from the elim (GO term hierarchy) approach. The full gene list is available in Table S2 in the supplemental material, and the GO enrichment analysis is available in Table S4 in the supplemental material.

<table>
<thead>
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<th>GO term</th>
<th>Significance value</th>
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<td>O-linked mannosylation</td>
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a GO enrichment analysis was carried out as described in the legend of Fig. 1 and Table 1. The full gene list is available in Table S3 in the supplemental material, and the GO enrichment analysis is available in Table S5 in the supplemental material.
RESULTS

Gene expression in biofilms. There have been numerous studies of biofilms formed by *C. albicans* on various surfaces including plastic and glass (reviewed in references 8 and 39). For this study, we used the microfermentor model described previously by Garcia-Sanchez et al. (20). This allows the development of large quantities of biofilm on plastic Theranox slides under continuous flow with unlimited nutrients and under aerobic conditions. To facilitate comparisons with *C. albicans* biofilms, which were developed over 48 h (20), RNA was extracted from *C. parapsilosis* biofilm cells after 50 h of growth, and gene expression was compared to that of planktonic cultures in exponential-phase growth.

Transcriptional profiling was carried out using microarrays representing 3,849 ORFs from *C. parapsilosis*, which were based on a genome sequence survey (35). One hundred eighty-five genes showed reproducible changes in gene expression: 122 genes with increased levels of expression in biofilms and 63 genes with reduced levels of expression (see Table S2 in the supplemental material). Where possible, the *C. albicans* orthologs were identified using reciprocal BLAST analysis, and these were assigned to GO processes. Enrichment of specific GO categories in the differentially expressed data was determined using topGo within the Bioconductor package (21). Two measurements of statistical reproducibility were used, the classic Fisher’s exact test, which compares the number of observed incidences with the number expected, and elim, which compensates for overlapping categories by removing genes mapped to significant GO terms from higher-level GO terms (2). GO categories that are significantly enriched include metabolism of ergosterol, fatty acids, and glucose (Table 2). The levels of expression of ergosterol pathway and glycolytic genes in particular are increased, whereas levels of expression of the gluconeogenic enzymes *FBP1* and *PCK1* are decreased (see Table S2 in the supplemental material).

Changes in levels of expression of several genes were confirmed by quantitative RT-PCR (Fig. 1A and B). Expression in biofilms was compared to that in planktonic cells in exponential and in stationary growth phases to ensure that the observed differences are not an artifact of the culture conditions used (Table S2). The level of expression of some of our target genes. Figure 1B shows that the level of expression of *PHR1*, *PHR2*, and *RBT1* is increased in biofilms.

In addition, changes in levels of expression of a number of genes associated with pH regulation (*PUT1*, *PUT2*, *PHR1*, *CCP1*, and *RIM101*) led us to investigate the role of pH in the planktonic cultures. In unbuffered medium, the pH of the planktonic culture drops to 3.8, whereas it remains stable at pH 5.4 in the continuous-flow fermentor system. We therefore determined if the difference in pHs under planktonic and biofilm conditions could have contributed to the variation of levels of expression of some of our target genes. Figure 1B shows that buffering of the medium has no effect on the expression of the ergosterol pathway genes or on the expression of *PGK1*. The level of expression of *PFK2*, however, is increased at pH 5.4 relative to that at pH 3.8. The level of expression of *PHR1* is also strongly increased at pH 5.4, whereas the level of expression of *PHR2* is unchanged, as expected. The expression of *RBT1* is known to be regulated by pH, at least in *C. albicans* (6), but levels of expression in *C. parapsilosis* are essentially the same at pH 5.4 and pH 3.8 under the conditions used (P value of 0.067 by t test).

Gene expression in hypoxia. The increase in levels of expression of genes involved in ergosterol metabolism and in glycolysis is reminiscent of gene expression changes reported previously for *C. albicans* cultures during growth under conditions of low oxygen (52). We therefore determined the gene expression changes that occur when *C. parapsilosis* is grown under hypoxic (low-oxygen) conditions. For these experiments, we utilized the emerging whole-genome sequence of *C. parapsilosis* (http://www.sanger.ac.uk/sequencing/Candida/parapsilosis/). Microarrays were manufactured by Agilent, representing 5,834 genes identified in an in-house annotation of the genome sequence.

The response of *C. parapsilosis* to hypoxic conditions was first investigated by comparing the growth on YPD medium in 21% oxygen with that on YPD medium in 1% oxygen (Fig. 2A). Low oxygen has a dramatic effect on growth and colony size in three different *C. parapsilosis* isolates, whereas the growth of *C. albicans* under the same conditions is only slightly reduced. To measure changes in gene expression that are related to hypoxia rather than reduced growth, we restricted the incubation time of *C. parapsilosis* cultures under low-oxygen conditions. Cells were grown to exponential phase in atmospheric oxygen in SD medium at 37°C. The cultures were then split and incubated for 2 h in either atmospheric oxygen or a hypoxia chamber at 1% oxygen, and RNA was isolated. Analysis of enrichment of specific GO categories was done as described above for the biofilm experiments. We once again observed an overrepresentation of genes involved in ergosterol metabolism and glycolysis (Table 3; see Table S3 in the supplemental material). In addition, the expression levels of genes required for heme synthesis are increased. Changes in levels of expression of selected genes were confirmed using qRT-PCR (Fig. 2B). The level of expression of ergosterol pathway genes (*ERG1* and *ERG11*) was increased four- to fivefold. The levels

![FIG. 1. Transcriptional profile of *C. parapsilosis* biofilm cells.](http://www.sanger.ac.uk/sequencing/Candida/parapsilosis/)
of expression of glycolytic genes (PFK2 and PGK1) were similarly affected. The level of expression of the cell wall gene RBT1 was also increased, by approximately 30-fold. One of the genes with the highest induction in expression in the array experiments (CPAG_05061) has no ortholog in any other Candida species and, indeed, no obvious similarity to any sequenced gene. qRT-PCR confirmed that expression is induced (by approximately 40-fold) under hypoxic conditions, but we do not know the function of this gene.

In total, 60 genes with differential expression were shared between the biofilm and hypoxic experiments, 57 with the same patterns (i.e., expression levels increased or decreased both in biofilms and under conditions of hypoxia) (Fig. 2C; see Table S6 in the supplemental material). The vast majority of upregulated genes in both experiments are involved in fatty acid or ergosterol synthesis or in glycolysis (Fig. 3; see Tables S2 and S3 in the supplemental material).

**RBT1 is required for biofilm development.** Setiadi et al. (52) previously observed that hypoxia induces the expression of several hypha-specific genes in C. albicans, including HWP1. C. parapsilosis does not generate true hyphae, but the genome does include several members of the Hwp1 family. The level of expression of one of these members, an ortholog of *C. albicans* RBT1, is increased both in biofilms (Fig. 1) and under conditions of hypoxia (Fig. 2B). We therefore tested the effect of knocking out this gene on biofilm development. Both RBT1 alleles were deleted using an SAT1 flipper cassette (15), and in one construct, the RBT1 gene was reintroduced at the same location (Fig. 4). The wild-type and *rbt1* knockout isolates were incubated on Thermax slides in the fermentor system, and the structure of the resulting biofilms was determined using fluorescent imaging (Fig. 4C). Whereas the wild type generates biofilms of approximately a 300-µm depth, this is reduced to less than 40 µm in the *rbt1* knockout. Biofilm mass (measured using an FDA assay) was also reduced when cells are grown in 96-well plates (Fig. 4D). However, there was no obvious difference in biofilms generated on silicone squares (not shown). The heterozygous strains CDR14 and CDRbt8 that have only one allele of *RBT1* have slightly reduced biofilms (Fig. 4D), although the growth rate is the same for all the strains, including the wild type and the double-knockout mutant (not shown). Two RBT1 alleles are therefore required for full biofilm development, at least on some surfaces. Deleting *rbt1* has no observed effect on the hypoxic growth of *C. parapsilosis* (not shown).

**ALS genes.** Increased levels of expression of the ALS adhesin family have been associated with biofilm formation in *C. albicans* (11, 20, 23, 44, 64). We identified little change in levels of expression of the family in *C. parapsilosis* biofilms in the array experiments. However, not all family members were represented on the arrays, and there is likely to be some cross-hybridization due to sequence similarities. We identified five members of the ALS family in *C. parapsilosis*, and we measured levels of expression in biofilms at 24 h and 50 h using qRT-PCR (Fig. 5). Levels of expression of CPAG_05314, CPAG_00368, and CPAG_00369 were essentially unchanged. The level of expression of CPAG_05054 was induced approximately threefold at 24 h but not at 50 h, and the level of expression of CPAG_05056 was slightly increased in 50-h biofilms. The changes in levels of expression are much lower than those observed for RBT1 (which is not a member of this family) in 50-h biofilms. The expression of the ALS family is also not greatly affected by hypoxia; the level of expression of CPAG_05056 is reduced, and that of CPAG_05314 is slightly increased (Fig. 5; see Table S3 in the supplemental material). It is, however, possible that levels of expression of gene families (such as the ALS genes) may vary in different isolates of *C. parapsilosis*, which we have not yet tested.
DISCUSSION

Transcriptional profiling has made major contributions to our understanding of biofilm formation, particularly by bacteria (4). In C. albicans, array analysis led to the identification of the role of the transcription factor Gcn4 and the role of sulfur metabolism in biofilm development (20, 38, 64). Our analyses of gene expression changes reveal substantial overlaps during biofilm growth in C. parapsilosis and that in C. albicans. At least four glycolytic genes have increased levels of expression in biofilm versus that in planktonic cells in C. parapsilosis. This was confirmed for PFK2 and PGK1 using qRT-PCR, where we showed that the level of expression is higher in biofilms than in planktonic cells in exponential- or stationary-phase growth. Levels of expression of glycolytic genes also increase during planktonic growth, but the difference is greater in biofilms (64). In contrast, levels of expression of glycolytic and ergosterol metabolism genes are decreased in stationary-phase cells in C. albicans (59), suggesting that the changes observed in biofilm cells do not result from reaching stationary-phase growth. Changes in carbohydrate metabolism are therefore important for biofilm development in both species.

Surprisingly, we did not observe any change in sulfur amino acid metabolism or, indeed, in general amino acid metabolism in C. parapsilosis biofilms. This is a feature of most of the C. albicans-profiling experiments reported to date (20, 38, 64). Our experimental conditions are generally comparable to those reported previously by Garcia-Sanchez et al. (20), who identified a pivotal role for the amino acid regulator Gcn4 in C. albicans biofilms. Our biofilm experiments measured levels of expression of only a portion (3,789 ORFs) of the C. parapsilosis gene repertoire, but this does include most of the amino acid biosynthetic genes. In addition, we did not observe major changes in genes required for protein synthesis reported in C. albicans (20, 64). This result suggests that the growth stage (or the protein synthesis needs) under the two conditions tested here (planktonic exponential phase and biofilm) are similar, in contrast to that observed for C. albicans. The difference between the expression profiles in the two species may be linked to the growth rate; we observed that C. parapsilosis grows approximately twofold slower than does C. albicans. The biofilms generated are therefore unlikely to be mature, even after

FIG. 3. Gene expression changes in biofilms and hypoxia. C. albicans orthologs of C. parapsilosis genes with altered expression in biofilms or under hypoxic conditions were identified as described in Materials and Methods. GO terms with significant enrichment were determined using GeneSet enrichment analysis. The figure shows selected GO processes and the associated genes that are enriched in the biofilm and hypoxia arrays. C. albicans gene names are used, and genes highlighted in gray are common to both experiments. Several genes are associated with more than one process. CoA, coenzyme A. (The structure of the figure is based on a similar diagram in reference 1.)
50 h, and the biomass increases with longer incubation times (not shown). It is therefore likely that there are some significant differences between the metabolic profiles of *C. parapsilosis* and *C. albicans* biofilms.

We also observed an increase in levels of expression in ergosterol genes in the *C. parapsilosis* biofilms. Altered expression of the ergosterol pathway in *C. albicans* biofilms has been associated with increased antifungal resistance (36), and the level of expression of *ERG10* is increased in the early stages (64). However, we observed changes in levels of expression of several genes, including *ERG1*, *ERG11*, *ERG25*, and *ERG5*, that act in the oxygen-dependent postqualene part of the ergosterol biosynthesis pathway (61). When we compared the upregulated genes to data from several *C. albicans* experiments (using List-to-List at http://candida.bri.nrc.ca/l2l), the closest match was to the data set with increased levels of expression under conditions of hypoxia (from reference 52). This prompted us to compare the transcriptional responses of *C. parapsilosis* and *C. albicans* to hypoxia.

**FIG. 4.** Construction of an *rbt1* deletion of *C. parapsilosis*. (A) The *RBT1* gene was disrupted as described previously (15). Briefly, upstream and downstream sequences from the *RBT1* gene were amplified using primer pairs Rbt1kpn/Rbt1apa and Rbt1sII/Rbt1sI and inserted at either side of the *SAT1* flipper cassette in plasmid pCD8, which contains a nourseothricin resistance gene and an FLP (flippase recombination enzyme), surrounded by two FRT (flippase recognition target) sites. The entire fragment was used to replace one *RBT1* allele in *C. parapsilosis* CLIB214 by homologous recombination. The *SAT1* cassette was removed by inducing the expression of the recombinease, used again to delete the second *RBT1* allele, and the cassette was finally recycled. A reconstituted strain was generated by cloning a wild-type copy of *RBT1* upstream from the *SAT1* flipper cassette. The entire fragment was used to replace one *rbt1* allele by homologous recombination, and the cassette was recycled by inducing recombination between the FRT sites. (B) All steps in the construction were confirmed using PCR (top and middle) and by Southern hybridization (bottom). The order of the strains for each reaction shown at the top is as follows: lane 1, CLIB214; lane 2, CDR1 (integration at the first allele); lane 3, CDR14 (first recycle); lane 4, CDR210 (integration at the second allele); lane five, CDR212 (second recycle). In the middle, lanes 1 and 2 are CDR311 (reconstituted strain), and lanes 3 and 4 are CDRbt8 (reconstituted strain following recycling of the cassette). M, marker. (C) Fluorescence microscopy of biofilms on Thermanox slides. Biofilms were grown for 50 h and stained with a fluorescent conjugate of concanavalin A. z-stack images were acquired at intervals of 1 μm, and a three-dimensional reconstruction was generated using Imaris software. (CLIB214, wild type; CDR212, *rbt1*/H9004/*rbt1*/H9004). (D) Biofilm quantification in 96-well plates. Cells were first inoculated at an *A*$_{600}$ of 0.05 for 1 h, wells were washed two times with PBS, and fresh RPMI 1640 with Glutamax medium (Invitrogen) buffered with 50 mM HEPES was added. The plates were then incubated for 24 h at 37°C. Cells were washed with PBS, and biofilms were quantified using the FDA assay. (CLIB214, wild type; CDR14, *RBT1/rbt1*/Δ; CDR212, *rbt1*/Δ/*rbt1*/Δ; CDRbt8, *RBT1/rbt1*/Δ:*RBT1).
parapsilosis biofilms to gene expression changes that occur under low-oxygen conditions.

C. parapsilosis appears to be more susceptible to low-oxygen conditions than C. albicans, as colonies grown on YPD plates in 1% oxygen are much smaller than C. albicans colonies grown under the same conditions (Fig. 2A). The transcriptional response of C. parapsilosis following short-term exposure to hypoxia is, however, very similar to that observed for C. albicans. We determined the hypoxic profile of C. parapsilosis cells grown in SD medium at 37°C to mimic the conditions used for biofilm development, and we also analyzed the response of cells growing in YPD medium at 30°C to facilitate a direct comparison with previously published results for C. albicans (52). In both species, irrespective of the media and growth temperature used, low oxygen induces the expression of fatty acid and ergosterol metabolism, glycolysis and fermentation, heme biosynthesis and iron metabolism, and cell wall genes (see Table S3 in the supplemental material) (52). Similar pathways respond to anaerobiosis in Saccharomyces cerevisiae (31). We compared the genes that are upregulated under conditions of hypoxia in C. parapsilosis to gene lists generated from several profiling experiments with C. albicans (http://candida.bri.nrc.ca/l2l/). The most similar profiles (found in C. parapsilosis cultures grown in both SD and YPD media under conditions of hypoxia) are C. albicans genes downregulated in deletions of efg1 (24), ace2 (37), and pmt6 (10) and genes upregulated in hypoxia (from reference 52). Efg1, Ace2, and Pmt6 are all required for the expression of glycolytic genes during normoxia, although Efg1 at least is not required for hypoxic induction (10, 37, 52).

In both C. albicans and S. cerevisiae, exposure to low-oxygen in rich medium (YPD medium at 30°C) results in decreased levels of expression of genes in the tricarboxylic acid cycle and in the electron transport chain (31, 52). We did not observe similar changes in expression in C. parapsilosis cultures grown in SD medium at 37°C in 1% oxygen, and in fact, levels of expression of several cytochrome c oxidase (COX) genes were increased (see Table S3 in the supplemental material). However, when C. parapsilosis cultures were grown in YPD medium at 30°C, there was a decrease in levels of expression in most of the tricarboxylic acid enzymes (LSC1, LSC2, CIT1, IDH1, IDH2, IDP2, FUM12, and MDH1) and in components of the F_{1}-ATP synthase (ATP7 and ATP14) (data not shown). The profile of downregulated genes in C. parapsilosis cells grown in YPD medium under conditions of hypoxia most closely resembles the profile of C. albicans genes downregulated under conditions of hypoxia, whereas there is little obvious similarity between the profile of downregulated genes in C. parapsilosis cultures grown in SD medium and any other C. albicans profile. A reduction in respiration in low oxygen during growth on rich medium is therefore a conserved response across several fungal species, but the medium used (and perhaps the growth temperature) can have a major effect.

One important feature of the hypoxic response in S. cerevisiae is the induction of the seripaurin family of mannoproteins (14). There is no expansion of this family in Candida species, but hypoxia does induce the expression of other cell wall genes in C. albicans, including RBT5 and other members of the CFEM family and HWP1, a member of the RBT1 family (52, 55). Surprisingly, the expression of the CFEM family is not induced by hypoxia in C. parapsilosis either in the array experiments (see Table S3 in the supplemental material) or when measured by RT-PCR (not shown).

One of the closest relatives of HWP1 in the C. parapsilosis genome is CPAG_00831, an ortholog of C. albicans RBT1, which is induced both under conditions of hypoxia and in biofilms. The expression in biofilms is unlikely to be attributable to the difference in pH between the planktonic and biofilm cultures (Fig. 1). Knockout analysis confirms that RBT1 is important for biofilm development (Fig. 4). RBT1 is induced during filamentation in C. albicans (9). An rbt1 mutant in C. albicans is defective in virulence in a rabbit cornea model (9) and is partially attenuated in a mouse cornea model (27). However, there is no effect on hyphal formation in C. albicans, suggesting that the reduction in the virulence of rbt1 is not associated with a defect in hyphal formation. RBT1 has not been associated with biofilm growth in C. albicans, but HWP1 is required (42). The Hwp1/Rbt1 family is therefore implicated in biofilm development, and possibly in virulence, in both species.
Several genes that are important for biofilm formation in *C. albicans* have been identified. Ace2, which regulates the expression of cell wall genes, is important for adherence (28). Mutations in some genes (such as *SUV3, NUP85, MDS3, and KEM1*) cause defects in hyphal development that may be important for biofilm formation (49). EfG1 and Tec1, major regulators of hyphal growth, are also important for biofilm development (41, 47). Tec1 regulates the expression of BCR1, which is required for biofilm development in both *C. albicans* and *C. parapsilosis* (15, 40, 41). The level of expression of CPH2 (which regulates the expression of *TEC1* in *C. albicans*) is increased both in biofilms and under conditions of hypoxia in *C. parapsilosis* (see Tables S2 and S3 in the supplemental material). The expression of *TEC1* is also induced under conditions of hypoxia (see Table S3 in the supplemental material). It is therefore likely that the Cph2/Tec1/Bcr1 pathway plays a conserved role in biofilm development in *Candida* species independent of the yeast/hyphal transition. However, there are also distinct differences. The Bcr1-dependent regulation of ALS genes is important for biofilm formation in *C. albicans* (40), but Bcr1 in *C. parapsilosis* plays no obvious role in regulating the expression of ALS genes (15). We identified five members of the ALS family in *C. parapsilosis* (compared to seven members in *C. albicans*), but we observed only minor changes in the levels of expression of two of these members in *C. parapsilosis* biofilms (Fig. 5). Nobile et al. (43) recently demonstrated that Als1, Als3, and Hwp1 act as redundant adhesins in biofilm formation in *C. albicans*. It is possible that Rbt1 in *C. parapsilosis* plays a role similar to that of Hwp1, but determining if the ALS family has any function will require more investigation.

There is not complete overlap between the gene sets induced in biofilms and those induced under hypoxic conditions (Fig. 3). Levels of expression of heme biosynthesis enzymes are increased under conditions of hypoxia but are not obvious in biofilms. The expression of genes requiring molecular oxygen (HEM13 and HEM14) and also the expression of earlier steps in the pathway, including HEM1 (5-aminolevulinate synthase) and HEM4 (uroporphyrinogen III synthase), are induced (see Table S3 in the supplemental material). The expression of heme biosynthesis is also induced under conditions of hypoxia in *C. albicans* and *S. cerevisiae* (31, 52, 57). In *S. cerevisiae*, decreasing of levels of heme biosynthesis is at least one method used to sense lowering oxygen concentrations.

Oxygen availability is important for biofilm development by bacterial species. In biofilms formed by *Pseudomonas aeruginosa*, low-oxygen conditions result in decreased protein synthesis, and under aerobic conditions, the concentration of available oxygen decreases across the biofilm (63). Growth in low oxygen inhibits adhesion and biofilm formation by *Escherichia coli* (33). Several bacterial species respond to lower oxygen levels in biofilms by altering the expression of the respiratory pathway (reviewed in reference 4). *C. albicans* regularly forms biofilms in low-oxygen environments, such as on dentures, but biofilm development is generally reduced compared to that in aerobic environments (7, 58). Some *Candida* species appear to form more biofilms under anaerobic conditions (58). Ours is the first study to our knowledge that suggests that the hypoxic environment of biofilms results in an altered transcriptional response in *Candida* species, at least for *C. parapsilosis*.

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