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

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The ability of *Candida parapsilosis* to form biofilms on indwelling medical devices is correlated with virulence. To identify genes that are important for biofilm formation, we used arrays representing approximately 4,000 open reading frames (ORFs) to compare the transcriptional profile of biofilm cells growing in a microfermentor under continuous flow conditions with that of cells in planktonic culture. The expression of genes involved in fatty acid and ergosterol metabolism and in glycolysis, is upregulated in biofilms. The transcriptional profile of *C. parapsilosis* biofilm cells resembles that of *Candida albicans* cells grown under hypoxic conditions. We therefore subsequently used whole-genome arrays (representing 5,900 ORFs) to determine the hypoxic response of *C. parapsilosis* and showed that the levels of expression of genes involved in the ergosterol and glycolytic pathways, together with several cell wall genes, are increased. Our results indicate that there is substantial overlap between the hypoxic responses of *C. parapsilosis* and *C. albicans* and that this may be important for biofilm development. Knocking out an ortholog of the cell wall gene RB7T1, whose expression is induced both in biofilms and under conditions of hypoxia in *C. parapsilosis*, reduces biofilm development.

*Candida parapsilosis* is closely related to the major human fungal pathogen *Candida albicans*: both are members of the Saccharomycotina CTG clade and translate CTG as serine (16). Whereas *C. albicans* is the most common cause of candidiasis, other species are isolated with increasing frequency. *C. parapsilosis* is now responsible for 8 to 10% of *Candida* bloodstream infections (45). The incidence of *C. parapsilosis* infection has been associated with the increased use of caspofungin (17), most likely because *C. parapsilosis* and closely related species are less susceptible to treatment with echinocandins due to a polymorphism in the target protein Fks1 (β-1,3-glucan synthase) (19).

Unlike other *Candida* species, *C. parapsilosis* is often isolated from the hospital environment and, in particular, from the hands of health care workers (12, 30, 34, 60). There have been several reports of infection outbreaks, from hospitals in the southern United States (12, 48) to neonatal intensive care units in Finland (51) and Taiwan (60). Children are particularly susceptible, and *C. parapsilosis* is responsible for approximately 20% of *Candida* infections in infants less than 1 year old (3, 13).

Infection with *C. parapsilosis* is strongly correlated with its ability to grow as biofilms on indwelling medical devices (29). Although there have been relatively few studies with *C. parapsilosis*, biofilm development by *C. albicans* has been well characterized (8). Cells first adhere to a surface (such as a catheter) and then build up a layer through cell-to-cell contact. At some point following adherence, the cells undergo a switch from yeast to hyphal growth, which is strongly correlated with the development of a structured biofilm (5, 47). The mature biofilm is surrounded with an extracellular matrix and is much less susceptible to antifungal drugs. Several groups have determined the transcriptional changes that occur during biofilm growth of *C. albicans* (20, 38, 64). In general, there is an increase in levels of expression of genes involved in glycolysis and amino acid and lipid metabolism.

There are considerable differences between the biofilms generated by *C. parapsilosis* and those generated by *C. albicans*. Most reports indicate that *C. parapsilosis* biofilms are smaller and less complex (25, 29), although this depends on the concentration of glucose available (53) and the colony morphology (32). *C. parapsilosis* does not form hyphae, and its biofilms are composed of yeast and pseudohyphal cells only (15, 18, 29). Biofilm production is inhibited in the presence of exogenous farnesol (32, 50), as in *C. albicans* (46), but unlike *C. albicans*, *C. parapsilosis* does not secrete farnesol (62). However, there are also many similarities between biofilms in the two species, in particular the fact that the transcription factor Bcr1 is a major regulator of biofilm formation in both of them (15, 40, 41). In *C. albicans*, Bcr1 regulates the expression of several cell wall and adhesion genes, and at least some targets are conserved in *C. parapsilosis* (15).

We describe here the use of partial genomic microarrays to determine the transcriptional changes that occur during biofilm development in *C. parapsilosis*. We observed an upregulation in the expression of genes involved in glycolysis, fatty
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

#### Strains and media.
All experiments were carried out using C. parapsilosis CLIB214 and derivatives (Table 1). For the biofilm microarray studies, cells were grown 37°C in SD medium (0.67% yeast nitrogen base) with 2% dextrose supplemented with methionine (20 mg/μl), arginine (50 mg/μl), and histidine (20 mg/μl) where required. For pH experiments, SD medium was buffered 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 workstaton) with 1% O2.

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 of yeast growing overnight in an A600 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. C. albicans cells were grown in SD medium supplemented with 2% dextrose. The supernatant (planktonic and nonadherent cells) was then removed, and adherent cells were grown in SC medium supplemented with 2% dextrose at 37°C under normoxic (atmospheric-oxygen) conditions. Two 100-ml aliquots were removed after 3 h, and the cells were collected by centrifugation and resuspended in preconditioned medium (incubated under hypoxic conditions for 12 h before use). One flask was incubated under normoxic conditions (21% O2) in a standard orbital shaker, and the other was incubated under hypoxic conditions (1% O2 and 99% N2) in a dedicated chamber (In Vivo, 400 workstation). Both cultures were incubated at 37°C and at 200 rpm for 2 h.

RNA was isolated using an RNeasy kit (Qiagen) for the biofilms and a Ribopure kit (Ambion) for the hypoxia cultures according to the manufacturers’ 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). Four independent biological replicates were compared; in two replicates, the biofilm sample was labeled with Cy5, and in the other two replicates, the biofilm sample was labeled with Cy3.

For the hypoxia 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, wash, and scanning protocols were the same as those used for the biofilm arrays. Six independent biological replicates were compared; in four replicates, the hypoxic samples were labeled with Cy5 and in two replicates, the samples were 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 the proteins were assigned to 2,727 genes on 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
TABLE 2. Biological processes enriched in biofilms

| GO term                                      | Significance value |
|----------------------------------------------|--------------------|
| Alcohol metabolic process                    | 6.60E+10           |
| Monocarboxylic acid metabolic process        | 4.20E+08           |
| Protein folding                              | 6.30E+07           |
| Monosaccharide catabolic process             | 2.40E+06           |
| Fatty acid metabolic process                 | 7.50E+06           |
| Acetyl coenzyme A biosynthetic process       | 5.70E+04           |
| Ergosterol metabolic process                 | 8.70E+04           |
| Glycolysis                                   | 1.25E+03           |

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.

qRT-PCR. For the biofilm experiments, a supplementary DNase treatment (Turbo kit; Ambion) was carried out with purified RNA to ensure the absence of DNA, which was verified by RT-PCR. cDNAs were synthesized using 2 μg of RNA mixed with 0.5 μg of oligo(dT) (Invitrogen) by incubation at 85°C for 5 min, 65°C for 20 min, and 42°C for 10 min. The final mix was added to a volume of 20 μl containing RT buffer and 150 U Superscript II (Invitrogen), 15 U of RNaseOut (Invitrogen), and 3 mM of deoxynucleoside triphosphate. RT was performed at 42°C for 60 min followed by 10 min at 70°C. Quantitative PCR were performed with the Power SYBR green master mix kit (Applied Biosystem) on an ABI Prism 7000 apparatus (Applied Biosystem) in a 20-μl reaction mixture containing 5 μl of cDNA. The results shown are an average of five or six replicates from two independent biological samples. For the hypoxic experiments, real-time PCR was carried out as described previously (37). For all qRT-PCR experiments, primers were designed using Primer 3.0 Plus (http://www.ambioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), and qRT-PCR experiments, primers were designed using Primer 3.0 Plus (http://www.ambioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The final mix was added to a volume of 20 μl containing RT buffer and 150 U Superscript II (Invitrogen), 15 U of RNaseOut (Invitrogen), and 3 mM of deoxynucleoside triphosphate. RT was performed at 42°C for 60 min followed by 10 min at 70°C. Quantitative PCR were performed with the Power SYBR green master mix kit (Applied Biosystem) on an ABI Prism 7000 apparatus (Applied Biosystem) in a 20-μl reaction mixture containing 5 μl of cDNA. The results shown are an average of five or six replicates from two independent biological samples. For the hypoxic experiments, real-time PCR was carried out as described previously (37). For all qRT-PCR experiments, primers were designed using Primer 3.0 Plus (http://www.ambioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

TABLE 3. Differentially expressed genes identified by comparing the levels of gene expression of cultures of six independent replicates of C. parapsilosis CLIB214 cells exposed to normoxic (21% O2) or hypoxic (1% O2) environments for 2 h

| GO term                                      | Significance value |
|----------------------------------------------|--------------------|
| Alcohol metabolic process                    | 6.60E+23           |
| Ergosterol biosynthetic process              | 6.40E+13           |
| Glycolysis                                   | 4.80E+07           |
| Galactose metabolic process                  | 8.60E+07           |
| Fatty acid metabolic process                 | 1.30E+04           |
| Heme biosynthetic process                    | 3.40E+04           |
| Pyruvate metabolic process                   | 8.90E+04           |
| O-linked mannosylation                       | 3.31E+05           |

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 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 Thermonox 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 FBPI 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-phase growth to ensure that the observed differences are not an artifact of the culture conditions of the control. The levels of expression of the ergosterol pathway genes ERG1 and ERG11 and the glycolytic genes PGK1 and PFK2 are increased in biofilms compared to those in planktonic cells in both exponential and stationary phases (Fig. 1B). Similarly, we also confirmed that the level of expression of the cell wall gene 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 PHRI 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
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 in stationary-phase growth. A large increase in levels of expression of glycolytic genes in C. albicans, particularly during early biofilm development, was also reported (64). 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 postquasene 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* biofilms.
C. 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 F1-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 rbtl 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 rbtl 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 *Tecl*, major regulators of hyphal growth, are also important for biofilm development (41, 47). *Tecl* regulates the expression of *BCRI*, which is required for biofilm development in both *C. albicans* and *C. parapsilosis* (15, 40, 41). The level of expression of *CPF2* (which regulates the expression of *TECI* 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 *TECI* 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). Noble 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* (uroorphyrinogen 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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