Divergent Targets of *Candida albicans* Biofilm Regulator Bcr1 *In Vitro* and *In Vivo*

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*Candida albicans* is a causative agent of oropharyngeal candidiasis (OPC), a biofilm-like infection of the oral mucosa. Biofilm formation depends upon the *C. albicans* transcription factor Bcr1, and previous studies indicate that Bcr1 is required for OPC in a mouse model of infection. Here we have used a nanoString gene expression measurement platform to elucidate the role of Bcr1 in OPC-related gene expression. We chose for assays a panel of 134 genes that represent a range of morphogenetic and cell cycle functions as well as environmental and stress response pathways. We assayed gene expression in whole infected tongue samples. The results sketch a portrait of *C. albicans* gene expression in which numerous stress response pathways are activated during OPC. This one set of experiments identifies 64 new genes with significantly altered RNA levels during OPC, thus increasing substantially the number of known genes in this expression class. The *bcr1Δ*Δ mutant had a much more limited gene expression defect during OPC infection than previously reported for *in vitro* growth conditions. Among major functional Bcr1 targets, we observed that ALS3 was Bcr1 dependent *in vivo* while HWP1 was not. We used null mutants and complemented strains to verify that Bcr1 and Hwp1 are required for OPC infection in this model. The role of Als3 is transient and mild, though significant. Our findings suggest that the versatility of *C. albicans* as a pathogen may reflect its ability to persist in the face of multiple stresses and underscore that transcriptional circuitry during infection may be distinct from that detailed during *in vitro* growth.

*Candida albicans* is a major invasive fungal pathogen of humans and can cause both mucosal and disseminated infections. Infections of the oral mucosa in particular, called oropharyngeal candidiasis (OPC), affect HIV patients (41), diabetes patients (51), and head and neck cancer patients receiving radiation therapy (1, 39, 48). Our goal is to define the attributes of *C. albicans* that make it a successful oral pathogen.

Mucosal infections may be considered biofilms, in that the pathogen adheres to a surface and produces an extracellular matrix (15, 39). This analogy has prompted investigations that test the hypothesis that genes required for biofilm formation *in vitro* may be required for mucosal infection as well. Findings from these studies have underscored the utility of this perspective, in that there are several common genetic requirements for the formation of abiotic surface biofilms and mucosal infections (10, 11, 15, 19).

One of the central regulators of biofilm formation is the zinc finger transcription factor Bcr1. It was identified in screens for mutants defective in biofilm formation on abiotic surfaces (33–35) and in adherence to a silicone substrate (14). Bcr1-dependent genes have been defined under *in vitro* growth conditions (14, 33–35). Many are cell surface protein genes, including ALS1, ALS3, and HWP1. These three genes are major functional Bcr1 targets, in that they are required for abiotic surface biofilm formation, and their overexpression restores biofilm formation in bcr1ΔΔ mutant backgrounds (33, 34).

Transcription factors like Bcr1 have long been used to define the functional basis of pathogenicity traits (8, 27, 42). One strength of this approach comes from the fact that virulence potential may arise from expression of gene families or other gene sets with overlapping functions. Because transcription factors often control functionally related target genes, a single transcription factor defect can abolish a function that is carried out by redundant genes. One weakness of this approach comes from the fact that the spectrum of transcription factor target genes may be contingent upon environmental conditions. Thus, the gene expression impact of a transcription factor in an infection setting may be different from its impact *in vitro*. In fact, there are now several examples in which a target gene is expressed in colonization or infection samples independently of a transcriptional regulator that was defined by *in vitro* assays (24). Moreover, this limitation is not restricted to transcription factors, since almost any genetic perturbation has gene expression consequences (22) that may contribute to a mutant phenotype.

Clearly it is critical to assess pathogen gene expression *in vivo* during infection, and several prior studies have done so (2, 18, 21, 29, 36, 38, 47, 49, 52, 54). Relevant to oral *C. albicans* infection, there has been a quantitative reverse transcription (QRT)-PCR analysis of *C. albicans* gene expression in a gnotobiotic pig OPC model (50), which revealed that ECE1 RNA accumulated at very high levels during infection. There have also been microarray analyses of both reconstituted human epithelial (RHE) infection and OPC patient samples (31, 54). The most highly upregulated genes in these contexts, compared to *in vitro* yeast extract-peptone dextrose (YPD)-grown cells, included the cell surface protein genes ALS3 and HWP1. All three of these genes—ECE1, ALS3, and HWP1—require Bcr1 for expression under *in vitro* growth conditions (35). These findings suggested that Bcr1 may be required for...
TABLE 1 *Candida albicans* strains used in this study

| Strain name | Genotype | Reference |
|-------------|----------|-----------|
| BWP17       | ura3Δ::imm434 arg4::hisG his1::hisG | 53        |
| DAY185      | ura3Δ::imm434 his1::hisG ARG4::URA3 arg4::hisG | 53        |
| CAI4-URA3   | ura3Δ::imm434 ARG4::pARG4-URA3 | 40        |
| CJN698      | ura3Δ::imm434 arg4::hisG his1::hisG pPHS1::BCR1 | 35        |
| CJN702      | ura3Δ::imm434 arg4::hisG his1::hisG pPHS1 | 35        |
| CAH7-1A1E2  | hwp1::hisG eno1::URA3 | 45        |
| CAHR3       | ura3Δ::imm434 hwp1::hisG | 45        |
| CAYF178U    | ura3Δ::imm434::URA3::IRO1 als3::ARG4 his1::hisG | 33        |
| CAQTP178U   | ura3Δ::imm434::URA3::IRO1 als3::ARG4::ALS3 his1::hisG | 33        |

OPC. Indeed, a recent analysis in a murine OPC model has shown that Bcr1 is required for infection (11). In that context, Bcr1 seemed to act through *HWP1*, because overexpression of *HWP1* significantly improved virulence of the *bcr1Δ/Δ* mutant. These findings were consistent with previous biochemical and functional analysis of *Hwp1*, which has shown that it is required for OPC in transgenic immunocompromised mouse models and that it is an epithelial adhesin (44, 45). Overexpression of *ALS3* also improved virulence by qualitative assay (11), though quantitative measures did not reach significance thresholds. The overall results suggested that Bcr1 is required for both *ALS3* and *HWP1* expression during OPC, a functional relationship similar to that described in *vitro* (35).

Here we have looked at gene expression during OPC to understand the basis for the *bcr1Δ/Δ* mutant phenotype. Our study has been aided by new technology, the nanoString nCounter, which has sensitivity on par with that of real-time RT-PCR yet is capable of profiling over 100 genes per sample (17). We expected that this assay platform may be useful for infected tissue samples, because it enables selective assay of pathogen gene expression despite the presence of abundant host RNA. This issue has previously been shown to enable selective assay of pathogen gene expression despite the overwhelming dominance of host RNAs. The overall results suggested that Bcr1 is required for both *ALS3* and *HWP1* expression during OPC, a functional relationship similar to that described in *vitro* (35).

### MATERIALS AND METHODS

**Media and strain construction.** *C. albicans* strains were grown on YPD (2% Bacto peptone, 2% dextrose, 1% yeast extract) or in Spider medium (1% nutrient broth [BD Difco], 1% D-mannitol [Sigma], 0.2% K2HPO4 [Sigma]). *C. albicans* strains used in this study are listed in Table 1.

**Mouse model of oropharyngeal candidiasis.** To assess the virulence of the various strains of *C. albicans*, the OPC mouse model was used (6, 37). This study was approved by the Animal Use Committee of the Los Angeles Biomedical Institute in compliance with NIH guidelines for the ethical treatment of animals. Male BALB/c mice (National Cancer Institute) weighing approximately 20 g were immunosuppressed with cortisone acetate (Sigma-Aldrich) at a dose of 225 mg/kg of body weight administered subcutaneously on days −1, +1, and +3 relative to the day of infection. To induce OPC, the mice were anesthetized with xylazine and ketamine (both from Phoenix pharmaceuticals) administered intraperitoneally. Next, calcium alginate urethral swabs (Type 4 Calgiswab; Puritan Medical Products Company LLC) were saturated with *C. albicans* by placing them in Hanks balanced salt solution (HBSS) containing 10^5 organisms per ml. The swab was placed sublingually in the anesthetized mice for 75 min. Each strain of *C. albicans* was inoculated into 7 to 9 mice per experiment. The virulence of the *bcr1Δ/Δ* mutant was compared to that of the wild-type and *bcr1Δ/Δ::BCR1* complemented strains in multiple experiments involving a total of 20 to 30 mice per strain. The virulence of the *als3* and *hwp1* mutants was tested in single experiments. After the mice recovered from anesthesia, they were given food and water *ad libitum*. The mice were sacrificed after either 1 or 5 days of infection, after which their tongues and adjacent sublingual tissue were excised. The tissue was weighed, homogenized, and quantitatively cultured on Sabouraud dextrose agar containing chloramphenicol. Differences in oral fungal burden among mice infected with different strains were analyzed using the Wilcoxon rank sum test.

**RNA isolation.** Immediately after being harvested, the tongues were cut into small pieces on ice and then immersed in RNAlater (Ambion). The saturated swabs were placed sublingually in the anesthetized mice for 75 min. Each strain of *C. albicans* was inoculated into 7 to 9 mice per experiment. The virulence of the *bcr1Δ/Δ* mutant was compared to that of the wild-type and *bcr1Δ/Δ::BCR1* complemented strains in multiple experiments involving a total of 20 to 30 mice per strain. The virulence of the *als3* and *hwp1* mutants was tested in single experiments. After the mice recovered from anesthesia, they were given food and water *ad libitum*. The mice were sacrificed after either 1 or 5 days of infection, after which their tongues and adjacent sublingual tissue were excised. The tissue was weighed, homogenized, and quantitatively cultured on Sabouraud dextrose agar containing chloramphenicol. Differences in oral fungal burden among mice infected with different strains were analyzed using the Wilcoxon rank sum test.

**NanoString analysis of gene expression.** Samples containing 100 ng of *C. albicans* total RNA (from samples grown in Spider media) or 5 to 10 µg of *C. albicans*-infected mouse tissue RNA were mixed with custom-designed probe code set and incubated at 65°C overnight (12 to 18 h). The hybridized samples were then processed on a nanoString prep station using the manufacturer’s default program. The resultant cartridges were then transferred to the nanoString digital analyzer and scanned for 600 fields per sample. The raw counts were first adjusted for technical variability using the positive and negative controls of irrelevant RNA sequences included in the code set. The technically adjusted counts were then normalized for total input *C. albicans* RNA using the housekeeping gene *TDH3*. The normalized counts were used to compare gene expression levels among different samples.
We included control genes for high expression, gene, responsive to adherence regulators (14). We included a control specific cell cycle phases (7). We also included genes that were highly expressed in response to conditions included azole treatment (26), biofilm formation in vitro (16), biofilm formation in vitro (32), general stress (13), hyphal growth (4), hypoxia (46), iron limitation (43), kidney infection (49), oxidative stress (12), and caspofungin treatment (3) and during specific cell cycle phases (7). We also included genes that were highly responsive to adherence regulators (14). We included a control gene, TDH3, for normalization among samples and backup control genes for high expression, ACT1, and for low-to-moderate expression, ARP3 and YRA1. These control genes varied little in numerous microarray studies and in vitro nanoString studies from our lab (see Data File S1, Gene Selection Tab, in the supplemental material). Thus, our panel of genes presented the opportunity to relate anticipated findings to well-studied pathways and responses. In addition, we had likely enriched for reporter genes that were regulated at the level of RNA accumulation.

Gene expression was assayed during in vivo OPC infection using infected mouse tongue tissue as a source of RNA. We compared RNA levels detected by our probes among tissue samples from three animals (Fig. 1; see also Data File S1 in the supplemental material). The nanoString platform detected C. albicans RNA transcript levels from in vivo samples even though the total RNA sample contained less than 0.1% Candida RNA, as estimated from C. albicans TDH3 RNA levels. The relative expression levels of our reporter genes were highly reproducible. For example, for the two in vivo infection samples presented in Fig. 1, there was a 10-fold difference in the quantity of C. albicans RNA, as seen by comparison of the x and y axes. Nonetheless, signals were well above background. For example, the ratio of median signal counts to maximum background counts was roughly 50 (400 counts/8 counts). Most importantly, the relative expression levels among the genes were consistent in the two samples (Fig. 1). Similar agreement was evident among other comparisons of biological replicates (see Fig. S1 and S2 in the supplemental material).

The genes most highly expressed in murine OPC samples (Fig. 1) included ALS3, HWP1, and ECE1, in keeping with prior studies of piglet OPC infection and RHE infection (50, 54), and SAP5 and SAP6, as expected from studies of patient samples (30). These results, along with high-level expression of other hyphal genes (IHD1 and HYR1), fit well with the observation that hyphae are prominent in infected tissue samples (23). Genes repressed by Hap43 or high iron levels (5, 20, 43) were also highly expressed (orf19.670.2, UCE1, and CAT1), which is consistent with the finding that the iron-repressed CFL2 and FRE4 transcripts are highly expressed in human patient samples (54). These results suggest that iron is limiting during OPC infection in this murine model.

We also sought to define gene expression relationships between infection and in vitro growth. To do so, we compared OPC infection RNA measurements to previous RNA measurements on
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TABLE 2 Classes of genes that are upregulated during OPC infection

| Response pathway | Representative genes |
|------------------|----------------------|
| General stress   | DMR48, orf19.675, SOD5 |
| Alkaline pH      | CAT1, ECM331, GCA1, GNPI1, IHDI1, PHR1, PHR2, PRA1, SCW4 |
| Oxidative stress | CAI1, CAT1, orf19.6816, TRR1, TRXI, SOD4 |
| Nitrosative stress| ALS2, ARO9, CAT1, CTT1, ECM331, orf19.4094, orf19.5982, orf19.6702, orf19.675, PKC1, PGA48, PHR2, RNR2, STF2, TRX1, UCF1, YHB1 |
| Iron limitation  | ECE1, ECI1, HWP1, IHD1, IHDI1, orf19.6702, PCK1, SAP4, SAP5, SAP6, TEC1, UME6 |
| Zinc limitation  | CSH1, GCA1, GC2A, IFD6, orf19.1534, PRA1, ZRT1, ZRT2 |
| Hypoxia          | ECM331, orf19.2030, orf19.6702, orf19.675, orf19.6702, PKC1 |
| Hyphal and virulence | ALS1, ALS3, ECE1, FAU2, HWP1, YHR1, IHDI1, orf19.6702, PCK1, SAP4, SAP5, SAP6, TEC1, UME6 |
| Other            | ADH1, ALS7, BP1, GCN4, HGT6, KIP4, orf19.2125, orf19.3460, orf19.4174, orf19.4706, orf19.6329, orf19.6578, PGA13, PGA17, PGA25, PGA34, PLB5, RCT1, SGA1, UGP1 |

Response pathways, defined in in vitro studies, are pathways whose activation results in upregulation of the genes listed.

The C. albicans genes listed were upregulated in OPC infection samples compared to in vitro growth in Spider medium at 37°C. Note that some genes are activated by multiple pathways and thus may be listed more than once.

Gene identified as OPC-upregulated gene in prior studies.

Gene trended toward upregulation but had a p-test P value of <0.1 and >0.05. All other genes listed displayed significant upregulation, reflecting a p-test P value of <0.05.

not evident in prior RHE infection studies (54). Many iron and zinc limitation genes were also upregulated, as expected if these nutrients are limiting during infection. Surprisingly, despite the fact that our comparison of OPC samples was to hyphal cells, many hyphal genes were expressed at significantly higher levels in the OPC samples. Induction of these genes during OPC infection was greater than in many serum-containing media as well in our preliminary studies (see Data File S2 in the supplemental material). Finally, a useful outcome of gene expression analysis is the identification of new candidate genes that may have functional roles during infection. Although our probe selection was biased toward genes of known function, we note that several OPC-upregulated genes are uncharacterized (orf19.4174, orf19.2125, orf19.4706, orf19.3460, and orf19.6329) and may be interesting for future analysis. All told, our findings argue that the success of C. albicans as an oral pathogen may reflect its ability to mount multiple stress responses. Conversely, if we view C. albicans as a bio-sensor, then we infer that the oral cavity is an inhospitable environment, even after immunosuppression.

It seemed possible that our experiments might only verify the microarray-based identification of C. albicans genes upregulated in RHE infection and OPC patient samples (54). Indeed, we did verify upregulation during OPC infection of the highly expressed genes ALS3, ECE1, HWP1, IHDI, PCK1, PHR1, SAP5, SOD5, STF2, and YHB1. However, we also defined 62 new genes whose RNA levels are regulated during OPC. If YPD 30°C cell samples are used as the comparator, this number increases to 83 new OPC-regulated genes. These findings probably reflect the sensitivity of the nanoString, because the previously known OPC-upregulated genes all have RNA levels above the median in our experiments.

Bcr1-responsive gene expression in vivo. In order to understand Bcr1-dependent gene expression in vivo, we analyzed RNA from tongues of three mice that had been infected with a bcr1ΔΔ strain. For comparison to in vitro-grown cells, we used our recently published data set (14) of bcr1ΔΔ cells grown in Spider medium at 37°C. In vivo and in vitro measurements were normalized for each gene to the mean wild-type expression levels under comparable growth conditions (Fig. 3). The data for the bcr1ΔΔ strain were highly reproducible among biological replicates (Fig. 3; see Fig. S1 in the supplemental material). The data set for in vitro-grown cells correlated well for previously reported Bcr1-dependent genes (including ALS1, ALS3, HWP1, CHT2, ECE1, and RBT5) from in vitro RNA microarrays (35) and RNAseq studies (34). Several additional Bcr1-dependent genes (including SOD5, IFE2, PGA7, and PHO89) were detected only in nanoString (14) and RNAseq (34) data sets. These results indicate that Bcr1-responsive genes defined under in vitro growth conditions are highly reproducible, extending across different detection methods and laboratories.

In contrast, the spectrum of Bcr1-responsive genes from in vivo OPC infection samples was quite different from that in vitro (Fig. 3; see Data File S1 in the supplemental material). A core set of genes had significantly altered expression in the mutant both in...
vivo and in vitro, including downregulated genes ALS1, ALS3, and IFE2 and upregulated genes RBR1, SAP4, PGA17, and PGA25. Some upregulated genes are repressed by RIM101 (PHR2, RBR1, and RBR2 [28]). This response may reflect the facts that Bcr1 activates expression of RIM8 (34) and Rim8 is an upstream member of the Rim101 pathway (9). However, most striking were the many examples of genes that were Bcr1 responsive only in vitro or in vivo. Prominent in this class were many hyphal genes, including HWP1 and ECE1 (Fig. 3). Our results indicate that environmental conditions can alter the spectrum of Bcr1-responsive genes considerably. Although there are previous reports of other transcription factors whose function is bypassed in vivo for specific target genes (38, 50; reviewed in reference 24), the possibility that most targets of a transcription factor are altered in vivo has not previously been investigated. Our findings are in keeping with Kumamoto’s suggestion that several of the known hyphal gene regulators may have overlapping functions in vivo (24), though recent discoveries have expanded the number of hyphal gene regulators dramatically (14, 34). One might imagine that the differential regulation separates direct and indirect Bcr1 target genes: the more stringent in vivo growth conditions might result in only direct Bcr1 target genes showing Bcr1-dependent expression. However, based on genome-wide chromatin immunoprecipitation (ChIP) data from Nobile et al. (34), this hypothesis seems incorrect. For example, IFE2 and ALS3 are indirect Bcr1 target genes (regulated by Bcr1 but not bound by Bcr1), yet these are Bcr1 dependent in vitro and in vivo. In contrast, RBT5 and CHT2 are direct Bcr1 targets yet show Bcr1-dependent expression only in vitro. Given the extensive upregulation of C. albicans stress response pathways during OPC infection, perhaps one or more stress response pathways may override Bcr1 dependence for many target genes.

**Functional analysis of** BCR1, ALS3, and HWP1 in vivo. We
selected the adhesin genes ALS3 and HWP1 for functional analysis, with the specific goal of assessing virulence potential during OPC infection of null mutants under uniform assay conditions. HWP1 has been implicated in OPC based on its overexpression (11) and on in vivo null mutant assays in a transgenic mouse model (44). A role for ALS3 in OPC is uncertain (11). We first verified that Bcr1 is required for OPC infection. We observed that a bcr1Δ/Δ mutant produced a significantly lower fungal burden than the wild-type strain at days 1 and 5 of infection (Fig. 4). Parallel assays indicated that Als3 is required only early in infection: an als3Δ/Δ mutant had significantly lower fungal burden than the wild type at day 1 of infection but had no defect at day 5 (Fig. 4). The overall role of Als3 seemed modest. Hwp1 has a prominent role at all times: an hwp1Δ/Δ strain yielded significantly reduced fungal burden at days 1 and 5 of infection (Fig. 4). These results verify that Bcr1, Als3, and Hwp1 have roles in OPC infection despite their altered relationship based on expression analysis above. In addition, the fact that an als3Δ/Δ strain has a milder and more transient OPC defect than a bcr1Δ/Δ strain helps to explain why overexpression of ALS3 does not fully rescue the bcr1Δ/Δ phenotype (11); there must be additional Bcr1-dependent genes that contribute to the bcr1Δ/Δ OPC defect.

Our gene expression data provide only a limited view of OPC infection, but they improve our understanding of OPC in several ways. The most important point is that infecting cells express targets of numerous stress response and nutrient limitation pathways. Our findings extend what was known from pioneering microarray profiling studies of RHE and patient samples (54). Thus, the versatility of this pathogen may reflect its ability to endure multiple stresses. A second important point is that many new genes that are highly expressed or highly upregulated during OPC infection have been identified. While many of these genes have functional roles that can be deduced from homology, many others do not, and they are thus candidates for future functional studies. Finally, our results suggest a new twist on the idea that C. albicans transcriptional regulatory circuits have been rewired (25). It is generally appreciated that evolutionary forces have reshaped the features of transcriptional circuits in C. albicans, compared to Saccharomyces cerevisiae. Our studies, as well as those of Kumamoto and colleagues (50), underscore the fact that transcriptional circuits we study in vitro may also be reshaped during infection. We believe that an understanding of gene expression during growth in vivo is critical to validate in vitro studies and to define and prioritize the voids in our understanding.

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