Phenotypic Switching in *Candida glabrata* Accompanied by Changes in Expression of Genes with Deduced Functions in Copper Detoxification and Stress

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Received 1 April 2005/Accepted 31 May 2005

Most strains of *Candida glabrata* switch spontaneously between a number of phenotypes distinguishable by graded brown coloration on agar containing 1 mM CuSO₄, a phenomenon referred to as “core switching.” *C. glabrata* also switches spontaneously and reversibly from core phenotypes to an irregular wrinkle (IWr) phenotype, a phenomenon referred to as “irregular wrinkle switching.” To identify genes differentially expressed in the core phenotypes white (Wh) and dark brown (DB), a cDNA subtraction strategy was employed. Twenty-three genes were identified as up-regulated in DB, four in Wh, and six in IWr. Up-regulation was verified in two unrelated strains, one a and one α strain. The functions of these genes were deduced from the functions of their *Saccharomyces cerevisiae* orthologs. The majority of genes up-regulated in DB (78%) played deduced roles in copper assimilation, sulfur assimilation, and stress responses. These genes were differentially up-regulated in DB even though the conditions of growth for Wh and DB, including CuSO₄ concentration, were identical. Hence, the regulation of these genes, normally regulated by environmental cues, has been usurped by switching, presumably as an adaptation to the challenging host environment. These results are consistent with the suggestion that switching provides colonizing populations with a minority of cells expressing a phenotype that allows them to enrich in response to an environmental challenge, a form of rapid adaptation. However, DB is the most commonly expressed phenotype at sites of host colonization, in the apparent absence of elevated copper levels. Hence, up-regulation of these genes by switching suggests that in some cases they may play roles in colonization and virulence not immediately obvious from the roles played by their orthologs in *S. cerevisiae*.

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switching. Rather, spontaneous switching directly regulated expression of these genes, regardless of extracellular CuSO₄ levels, presumably by phase-specific trans-acting factors. Lachke et al. (29) demonstrated that genes other than those involved in copper assimilation, such as HLP1, were also regulated by core switching. Furthermore, Srikantha et al. (59) demonstrated that MTLα1 was regulated by core switching, but in reverse order, with expression highest in Wh and lowest in DB.

Here we have performed a cDNA subtraction screen of phase-specific libraries in order to identify additional genes regulated by core switching. Genes enriched in cDNA pools of Wh or DB cells subtracted with excess driver cDNA from the alternative phenotype were then tested by Northern blot hybridization for levels of expression in Wh, DB, and 1W cells derived from either an a or an α strain. To determine if the differential expression of phase-regulated genes was mediated by upstream promoter regions, the promoters of select genes were fused with the reporter gene expressing Renilla reniformis luciferase (RLUC), and reporter activity was assessed in Wh and DB cells. The results indicate that more genes are up-regulated in DB than in Wh, that the majority of these genes are related to copper detoxification and stress responses, and that these genes are regulated by core switching at the level of the promoter. However, because DB is a common colonizing phenotype, and because copper toxicity does not appear to be a limiting factor contributing to colonization, we propose that some of these differentially expressed genes may play roles other than those deduced from their S. cerevisiae orthologs.

### MATERIALS AND METHODS

**Strains and culture conditions.** The origins of the strains used in this study are provided in Table 1. All strains were stored in 20% glycerol at −80°C. In the course of an experiment, cells of different switch phenotypes were isolated and plated onto YPD agar (2% [wt/vol] Bacto peptone, 2% [wt/vol] glucose, 1% [wt/vol] yeast extract, 1 mM CuSO₄, 1.5% [wt/vol] agar). For experiments, cells were obtained from colonies grown at 25°C for 4 to 6 days. The ura3Δ strains were maintained on defined synthetic dextrose agar (2 g of a complete amino acid mixture, 20 g dextrose, 5 g ammonium sulfate, 1.45 g yeast nitrogen base, and 50 mg uracil per liter [54]). Only colonies exhibiting a homogenous switch phenotype were used for experiments.

**cDNA subtraction.** The generation of cDNA libraries enriched for mRNA species of the switch phenotypes Wh and DB was based on the subtraction strategy of Hubank and Schatz (18). The Oligotex mRNA kit (QIAGEN, Valencia, CA) was used to isolate poly(A)+ mRNA individually from the Wh and DB phenotypes of strains 35B11 (MTLaα) and 1480.49 (MTLaα). This RNA was used to synthesize double-stranded cDNA, employing the PCR-Select cDNA subtraction kit (BD Sciences, Palo Alto, CA) with minor modifications. Subtractions were performed reciprocally between Wh and DB for both the a and α strains. The cDNA sample from each cell phenotype was digested with Rsal to create blunt ends and then split into three aliquots, one to be used as a driver in subtraction and the other two, ligated at both ends to adaptors 1 and 2, respectively, to be used as “targets” (Table 2). Two sequential subtraction hybridizations were performed, the first for the duplicate Wh targets versus the DB driver and the second for the duplicate DB targets versus the Wh driver, for both the a and α strains. The eight subtracted cDNA pools, two from Wh and two from DB of each of the two strains, were then selectively amplified, first by using a PCR strategy to eliminate unsubtracted cDNAs and second by a PCR enrichment strategy employing nested primers 1 and 2 (Table 2). The secondary PCR-amplified cDNA pools were purified with the Wizard-PCR Clean kit and ligated to the pGEM-T-Easy plasmid and to the tandem repetitive elements of CLONTECSwitch1435 (57) and the S. glabrata genome database (http://www.duke.edu/web/microlabs/mccusker/). Northern blot analyses were performed by methods previously described (58, 59).

| Strain | Parent strain | Genotype | Mating type | Reference |
|--------|--------------|----------|-------------|-----------|
| 35B11  | —            | —        | MTLα        | 59        |
| PB921  | —            | —        | MTLα        | 59        |
| 1480.49| —            | —        | MTLα        | 59        |
| 1480.47| —            | —        | MTLα        | 59        |
| 1480.50| —            | —        | MTLα        | 59        |
| 40F1   | 35B11        | ura3Δ(−85 + 932)::Trn903NeoR | MTLα        | This study |
| 12F1   | PB921        | ura3Δ(−85 + 932)::Trn903NeoR | MTLα        | This study |

a —, strain is natural and therefore has no identifiable parent.
b —, wild type.
| Primer or adaptor | Gene or purpose | Sequence |
|-------------------|----------------|----------|
| Adaptor 1 cDNA subtraction | 5′-CTA ATG CTA CGG CGG CTG CTG GAG GAG GAG CAG GTG-3′ | |
| Adaptor 2R cDNA subtraction | 5′-CTA ATG CTA CGG CGG CTG CTG GAG GAG GAG CAG GTG-3′ | |
| PCR primer 1 cDNA subtraction | 5′-CTA ATG CTA CGG CTG CTG GAG GAG GAG CAG GTG-3′ | |
| Nested primer 1 cDNA subtraction | 5′-TCG ACC GCG CCG CCC GCG ACC GGC AGG-3′ | |
| Nested primer 2R cDNA subtraction | 5′-AGC TGT TGT GGC AGG-3′ | |
| REFV RLuc ORF | 5′-TAA CTC GAG TCG CGG CCG CTG ATG ACT TCG AAA GGT TAT GAT-3′ | |
| REV RLuc ORF | 5′-TAA GAG CTC TTA TCG TIC ATT TTG GAG ACG-3′ | |
| ADFV 3′ end ADE2 | 5′-TAA GAG CTC TAA TAG AGC ACC ATC TAT AAA ACCA-3′ | |
| ADRF 3′ end ADE2 | 5′-TTA ACG TAT TGG CGT TGA TAT TGG CTC GT-3′ | |
| TCMF1 SICTCMI | 5′-TCG CCC GGG CAG CTG CTA AGC CGG GAA GTT-3′ | |
| TCMR1 | 5′-TCG CCC GGG ATA TGT ACA TGT ACG-3′ | |
| TCIn-R1 | 5′-TCG TCG CAG GTC GAC GAA GTT TTG TTA GAA AAT AAATC-3′ | |
| TCIn-F1 | 5′-TCG TCG CAG GTC GAC GAA GTT TTG TTA GAA AAT AAATC-3′ | |
| NATSacF1 | 5′-ATT GAG CTC ATG ACC ACT CTT GAC GAC ACC-3′ | |
| NATSacR1 | 5′-ATT GAG CTC ATG ACC ACT CTT GAC GAC ACC-3′ | |
| cghor1 | 5′-TAA CTC GAG TCT AAT CTC TAG ATC CGC ATG TTT CTC-3′ | |
| cghor1 | 5′-ATT GTG GTC GCG GCC GAG GGA-3′ | |
| PMPF1 | 5′-TGG CTG CAG TTA GTA GGA GTA CTA GAC TAC-3′ | |
| PMFR1 | 5′-TGG CTG CAG TTA GTA GGA GTA CTA GAC TAC-3′ | |
| TARF2 | 5′-TGG CAG TAT CAG TAT TGG TGA TAT TGG GCC ACC TTA GAT-3′ | |
| TARR1 | 5′-TGG CAG TAT CAG TAT TGG TGA TAT TGG GCC ACC TTA GAT-3′ | |
| ECFM17 F1 | 5′-TAA CTC GAG CTG TAA AAA AAG TGG TGC ATC TAT GAT GC-3′ | |
| ECFM17 R1 | 5′-TAA CTC GAG CTG TAA AAA AAG TGG TGC ATC TAT GAT GC-3′ | |
| ACPF1 | 5′-ATT GAG CTC ATG ACC ACT CTT GAC GAC ACC-3′ | |
| APCR1 | 5′-ATT GAG CTC ATG ACC ACT CTT GAC GAC ACC-3′ | |
| HSPF1 HSP104 | 5′-TGG CTG CAG TTA GTA GGA GTA CTA GAC TAC-3′ | |
| HSPR1 | 5′-TGG CTG CAG TTA GTA GGA GTA CTA GAC TAC-3′ | |
| PGKF1 PGK1 | 5′-TGG CTG CAG TTA GTA GGA GTA CTA GAC TAC-3′ | |
| PGKR1 | 5′-TGG CTG CAG TTA GTA GGA GTA CTA GAC TAC-3′ | |
| TEFF1 TEF4 | 5′-TGG CTG CAG TTA GTA GGA GTA CTA GAC TAC-3′ | |
| TEFR1 | 5′-TGG CTG CAG TTA GTA GGA GTA CTA GAC TAC-3′ | |
| SUTF1 | 5′-TGG CTG CAG GTC GAC GAA GTT TTG TTA GAA AAT AAATC-3′ | |
| SUTR1 | 5′-TGG CTG CAG GTC GAC GAA GTT TTG TTA GAA AAT AAATC-3′ | |
| M14F1 | 5′-GAT AG AAG CTA CCA CCA CTG TAC-3′ | |
| M14R1 | 5′-GAT AG AAG CTA CCA CCA CTG TAC-3′ | |
| M25F1 | 5′-GAT AGA CCG ACC ATG TCG TCA GTG ACC-3′ | |
| M25R1 | 5′-GAT AGA CCG ACC ATG TCG TCA GTG ACC-3′ | |
| R8F1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| R8R1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| ZF1F ZF1R | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| ZF2F ZF2R | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| PI0F1 HSP104 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| PI0R1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| AXF1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| AXR1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| OXX1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| OXR1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| GOF1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| GOR1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| LLP1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| LLP1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| PCF1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| PCR1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| CTV2F1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| CTV2R1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| CCC2F1 CCC2R1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| Zrtf1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| Zrf1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| Ctrf1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| Ctr1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| fre6f1 FRE6 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| fre6r1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| AQYf1 AQY1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| AQYr1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| chtf1 CB1F | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |
| cht1 | 5′-TGG ATT GTT GAT GTT GTA CTC-3′ | |

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pair NATSacF1–NATSacR1 (Table 2), followed by digestion with SacI and end repair using T4 DNA polymerase (New England Biolabs, Beverly, MA). This was inserted at the NrI site of the inverse PCR fragment to derive the NAT
dominant marker module. The module was amplified from the derivative plasmid, end repaired, phosphorylated using T4 polynucleotide kinase, and subcloned into pT2.1 at the PstI site. The plasmid derivative containing the intact PstI site abutting the ATG start codon of the
promoters was verified by DNA sequencing.

To replace wild-type C. glabrata gene harboring the neomycin resistance cassette. The dominant marker module. The module was amplified from the derivative plasmid to derive the
inserted at the NruI site of the inverse PCR fragment to derive the

TABLE 2—Continued

| Primer or adaptor | Gene or purpose | Sequence |
|-------------------|----------------|----------|
| EcM17f1           | ECM17          | 5′-ACC AAC ATG TGG TCT AGC-3′ |
| EcM17r1           |                | 5′-GCA TCT TCA GAG ATC TCC-3′ |
| Met3f1            | MET3           | 5′-ATG TCT ACG GGA GAT GATC-3′ |
| Met3r1            |                | 5′-GGC TTC ATT TAT TAG GAC TCC-3′ |
| McsF1             | MSC1           | 5′-CAC TGA CGA CTG TGT TACG-3′ |
| Mcsr1             | RFX1           | 5′-GTA CGA CAT CAC ATG ATA CC-3′ |
| Mcsr1             | RFXr1          | 5′-TGC ACA GTA TTT CGG AGC-3′ |
| YapF1             | YAP1           | 5′-AAA CGA CTA CTG TAC AAC-3′ |
| Yapr1             | MET4           | 5′-ACA GAG CTC AGA CGA CAC-3′ |
| met4f1            | TSA1           | 5′-ATG TCA AAA GGC TTC GTC-3′ |
| met4r1            |               | 5′-GTA TTC ATT TAT GAG TTC-3′ |
| TSAR1             |               | 5′-GTA CTC CCT GGA GTC TTC-3′ |

* ORF, open reading frame.
cDNA subtraction. To identify genes regulated by the core switching system, a cDNA subtraction strategy (18) was applied. In the first step of this strategy, two different cDNA pools were generated for Wh and two for DB of both the a strain 35B11 and the α strain 1480.49. The first of each pair contained primer sequences for cloning into the pGEM-T-Easy plasmid and represented the target cDNA pool. The second of each pair lacked these sequences and represented the driver pool. Each target cDNA pool was hybridized with an excess of the driver cDNA pool of the alternative phenotype of that strain (i.e., target DB cDNA and driver Wh cDNA, or target Wh cDNA and driver DB cDNA). After two successive hybridizations, those target cDNAs that had not hybridized with excess driver were cloned into the pGEM-T-Easy plasmid to generate phase-enriched subpools.

Approximately 1,500 clones of each of the four subpools (35B11-Wh, 35B11-DB, 1480.49-Wh, 1480.49-DB) were screened for hybridization to MT-II and MT-I, which were presumed to be expressed at high levels in DB cells (29). Approximately 80% of the clones from the DB cDNA subpools of both the a and α strains hybridized with the MT-II and MT-I probes, while only 5% of the clones from the Wh cDNA subpools of both strains hybridized to these probes, indicating that the subtractions resulted in phenotype-specific enrichment. The identified MT-II and MT-I clones were excluded from further analysis. Clones from the a strain 1480.49 were also screened with MTLα1, which was presumed to be overexpressed in α cells (59), and the clones thus identified were excluded from further analysis. The total number of putative recombinant clones from the Wh pools was approximately 6,000 and that from the Db pools 12,000. Restriction enzyme analysis and sequencing of 85 clones from the former and 165 from the latter subpools revealed that 87% (218) contained recombinant sequences and that 40% of these were represented once or twice, while 60% were represented three or more times. Forty-five unique clones were subsequently selected for Northern blot analysis, 13 from the Wh subpools and 32 from the DB subpools, which represented the approximate proportions of putative unique sequences from the respective core phenotypes. Each of the 45 clones was used to probe Northern blots containing total-cell RNA from Wh, DB, and IWr cells from each of the a (35B11) and α (1480.49) strains. Comparisons of the intensities of hybridization of each gene probe (Table 2) with Wh, DB, and IWr RNAs were made within each strain, either a or α, not between strains (i.e., not between a and α strains). The patterns of relative expression between the switch phenotypes held true within both strains for all genes tested (Tables 3 and 4).

Elevated expression in DB. Of the 32 putative DB-enriched genes analyzed, 22 (69%) proved by Northern blot analysis to be expressed at higher levels in DB than in Wh (Table 3; Fig. 2). In deducing the putative functions of these up-regulated genes from the functions of their S. cerevisiae orthologs, it became apparent that 18 of them, representing the majority (77%), were involved in either sulfur assimilation, copper assimilation, or stress responses (Table 3).

Sulfur assimilation. Studies with both S. cerevisiae and M. rouxii have demonstrated that copper-induced brown coloration results from the reduction of CuSO4 to CuS (2, 62, 66). In S. cerevisiae, this reaction is catalyzed primarily by sulfite reductase, which is a heterotetramer of the gene products of ECM17 and MET10 (62, 66). Both of these genes were identified in the subtracted DB cDNA pool and demonstrated by Northern blot analysis to be up-regulated in DB cells (Table 3; Fig. 2). In S. cerevisiae, the promoters of both ECM17 and MET10 contain the binding site CACGTG for the trans-activator Cbf1p, which has been demonstrated to be involved in the regulation of both centromere function and sulfur assimilation in S. cerevisiae (9). In C. glabrata, the promoter of MET10 contained two Cbf1p binding sites and the promoter of ECM17 contained one site (http://cbi.labri.fr/Genolevures/elt/CAGL). CBF1 was also identified in the subtracted DB cDNA pool and demonstrated by Northern blot analysis to be up-regulated in DB cells (Table 3). These results suggest that up-regulation of ECM17 and MET10 in DB cells may be mediated by Cbf1p. The ECM17 promoter also contained a binding site for Amt1p, a transactivator of genes in the copper-sequestering pathway of S. cerevisiae (15, 45). Although we did not identify AMT1 in the subtraction screen, we tested its expression by Northern blot analysis and found it to be up-regulated in DB cells of both the a and α strains, like CBF1 (Table 3). Hence, it is possible that Amt1p may also play a role in up-regulating ECM17 in DB cells.

In S. cerevisiae, Cbf1p is a member of the transcription factor family of transcription factors that are involved in the regulation of gene expression in response to copper deficiency. Cbf1p is known to bind to the CACGTG motif, which is found in the promoter regions of many copper-responsive genes in S. cerevisiae. The presence of Cbf1p binding sites in the promoters of ECM17 and MET10 suggests that these genes are likely to be regulated by Cbf1p in response to copper deficiency. The elevation of expression of these genes in DB cells suggests that copper deficiency may be a significant factor in the differential expression of these genes in the two strains. This is consistent with the observation that copper deficiency has been shown to be a significant factor in the switching of the core phenotypes.
TABLE 3. Genes up-regulated in DB

| General category         | Gene     | Identification in screen | Relative transcript levela | Relative transcript abundanceb | Deduced functionc |
|-------------------------|----------|--------------------------|---------------------------|-------------------------------|-------------------|
|                         |          |                          | Strain 35B11 (a) | Strain 1480.49 (α) | Wh | DB | IWr | Wh | DB | IWr |          |
| Sulfur metabolism       | ECM17    | √                         | + + + + + + + + | – – + + + + | Low | Sulfite reductase subunit (β subunit) |
|                         | CBF1     | √                         | + + + + + + + + | + + + + + + + + | Low | Transcription factor, methionine biosynthesis |
|                         | MET31    | √                         | + + + + + + + + | + + + + + + + + | Low | Transcription factor, methionine biosynthesis |
|                         | MET10    | √                         | + + + + + + + + | + + + + + + + + | Low | Sulfite reductase subunit (α subunit) |
| Copper metabolism       | FRE6     | √                         | + + + + + + + + | + + + + + + + + | Low | Copper-iron reductase |
|                         | CTR1     | √                         | + + + + + + + + | – + + + + + + + + | Low | Copper transporter, high affinity |
|                         | MAC1     | √                         | + + + + + + + + | + + + + + + + + | Low | Copper-sensing transcription factor |
|                         | CCC2     | √                         | + + + + + + + + | + + + + + + + + | Low | Copper transport, P-type ATPase |
|                         | MT-H     | √                         | + + + + + + + + | + + + + + + + + | Very high | Metallothionein Ia and Ib |
|                         | FET3     | √                         | + + + + + + + + | + + + + + + + + | Medium | Multicopper oxidoreductase |
|                         | FTR1     | √                         | + + + + + + + + | + + + + + + + + | Low | High-affinity copper and iron permease |
|                         | AMT1     | X                         | + + + + + + + + | + + + + + + + + | High | Transcriptional activator of MT-II, MT-I |
|                         | MT-1     | √                         | + + + + + + + + | + + + + + + + + | High | Metallothionein I |
| Stress response         | ROX1     | √                         | + + + + + + + + | + + + + + + + + | Low | Repressor of hypoxic genes |
|                         | TSA1     | √                         | + + + + + + + + | + + + + + + + + | Medium | Thioredoxin peroxidase, redox homeostasis |
|                         | PMP3     | √                         | + + + + + + + + | + + + + + + + + | Low | Salt tolerance |
|                         | HSP104   | √                         | + + + + + + + + | + + + + + + + + | Medium | Chaperone, stress response |
|                         | RB12     | √                         | + + + + + + + + | + + + + + + + + | High | Vacuole fusion, endopeptidase inhibitor |
| Other                   | ECM14    | √                         | + + + + + + + + | + + + + + + + + | Medium | Zinc carboxypeptidase |
|                         | ECM25    | √                         | + + + + + + + + | + + + + + + + + | Low | Cell wall organization |
|                         | TAR1     | √                         | + + + + + + + + | + + + + + + + + | Medium | Mitochondrial RNA, Pol-associated |
|                         | MSC1     | √                         | + + + + + + + + | + + + + + + + + | Low (a)/High (α) | Meiotic recombination |
|                         | SGO1     | √                         | + + + + + + + + | + + + + + + + + | Low (a)/High (α) | Chromosome segregation |

a Checks indicate genes identified in screen. X indicates gene not identified in screen but analyzed by Northern blot hybridization.
b Symbols: +++, maximum; +++, slightly reduced; +, very reduced; +, extremely reduced; –, not detectable. Luminescence measurements of bands in Northern blot hybridization patterns indicate that the difference between – and + + + + + is between 6- and 35-fold, the difference between + or + + and + + + + + is at least 4-fold, and the difference between + + + and + + + + is approximately 2-fold. These fold differences are underestimates because of pixel saturation artifacts at the high end of band intensity.
c Level of maximum expression relative to levels of expression of other messages.
d Deduced from the demonstrated functions of S. cerevisiae orthologs.

complexes Cbf1p/Met4p/Met28p (24) and Met31p/Met32p (4). MET31 was also identified in the subtracted DB cDNA pool and demonstrated by Northern blot analysis to be up-regulated in DB (Table 3). Hence, the two genes encoding the subunits of sulfite reductase and three genes encoding trans-acting factors that regulate their expression in S. cerevisiae were up-regulated in DB cells.

**Copper assimilation.** *C. glabrata,* like other microorganisms, has evolved intricate molecular mechanisms to deal with both limiting and toxic concentrations of copper (15, 48, 65). Several genes that either were previously demonstrated to be involved in copper assimilation or were orthologs of *S. cerevisiae* genes involved in copper assimilation were identified in the subtracted DB cDNA pool and demonstrated by Northern blot analysis to be up-regulated in DB cells (Table 3; Fig. 2). They included the two metallothionein genes MT-II and MT-I, originally observed by Lachke et al. (29) to be up-regulated by core switching in DB cells; a copper and iron reductase gene, FRE6 (50); two copper transporter genes, CTR1 (27) and CCC2 (69); a copper-sensing transcription factor gene, MAC1 (49); a multicopper oxidoreductase gene, FET3 (50); and a high-affinity copper/iron permease gene, FTR1 (50) (Table 3). As previously noted, Northern blot analysis revealed that AMT1, which regulates MT-I and MT-II expression in response to extracel-
lular copper levels (72), was also up-regulated in DB cells (Table 3). While the promoters of *MT-II* and *MT-I* contained putative Amt1p binding sites, the promoters of *FRE6, FET3*, and *FTR1*, which were similarly up-regulated in DB cells, did not contain binding sites for Amt1p (http://cbl.labri.fr/Genolevures/elit/CAGL).

**Stress response pathways.** A surprisingly high proportion of the remaining nine genes identified in the screen as up-regulated in DB had deduced functions in stress response pathways. *ROXI*, which encodes a transcription factor that represses hypoxic genes in response to oxidative stress in *S. cerevisiae* (52, 61), was identified in our screen and demonstrated by Northern blot analysis to be up-regulated in DB cells (Table 3). *CCC2*, a copper chaperone and a Rox1p target gene in *S. cerevisiae* (61, 69), was also identified in the subtraction screen and demonstrated by Northern blot analysis to be up-regulated in DB (Table 3). However, Northern blot analyses revealed that orthologs of four additional *S. cerevisiae* target genes of *ROXI (CYT1, SOD2, TIR1, and SUT1)* (61) were not similarly up-regulated in DB (data not shown). *TSAI*, which encodes

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### Table 4. Genes up-regulated in Wh, up-regulated or down-regulated in IWr, or constitutively expressed

| General category | Gene | Identification in screen | Relative transcript level<sup>a</sup> Strain 35B11 (a) | Relative transcript abundance<sup>b</sup> Strain 1480.47 (a) | Deduced function<sup>c</sup> |
|------------------|------|--------------------------|----------------|----------------|-----------------|
| Up-regulated in Wh | *APC9* | √ | +++ + +++++ ++++++++ ++++++ ++++++++ | Medium | Ubiquitin protein ligase, cell cycle |
|                  | *FAR8* | √ | +++ + ++++ ++++++++ ++++++ ++++++++ | Low | Cell cycle, pheromone response |
|                  | *CTR2* | √ | +++ ++++++++ ++++++ ++++++++ | Low | Copper transporter, low affinity |
|                  | *YAP1* | √ | +++ ++++++++ ++++++ ++++++++ | Low | Transcription factor, oxidative response |
| Down-regulated in IWr | *TEF4* | √ | + + ++++++++ ++++++++ ++++++++ | Low | Elongation factor, translation |
|                  | *RPS23A* | √ | ++ ++ ++++ ++++ ++++++++ | High | 40S ribosome subunit, translation |
|                  | *SIL1* | √ | ++ ++ ++++ ++++ ++++++++ | Medium | Protein translocation into ER |
|                  | *SUT1* | √ | ++ ++ ++++ ++++ ++++++++ | Medium | RNA Pol II regulator, sterol transport |
|                  | *PUT1* | √ | + + ++++ ++++ ++++++++ | Low | Proline dehydrogenase, mitochondria |
|                  | *HEM4* | √ | ++ ++ ++++ ++++ ++++++++ | Low | Uroporphyrin III synthase |
| Constitutive | *EFT1* | √ | +++ +++ +++ +++ +++ +++ +++ +++ +++ | Medium | Elongation factor, translation |
|                  | *MET4* | √ | +++ +++ +++ +++ +++ +++ +++ +++ | Low | Transcription factor, methionine biosynthesis |
|                  | *S32* | √ | +++ +++ +++ +++ +++ +++ +++ +++ | Low | Regulator of sulfur aa biosynthesis |
|                  | *SPC98* | √ | +++ +++ +++ +++ +++ +++ +++ +++ | High | Microtubule nucleation |
|                  | *RAX2* | √ | +++ +++ +++ +++ +++ +++ +++ +++ | High | Bud site selection |
|                  | *RFX1* | √ | +++ +++ +++ +++ +++ +++ +++ +++ | High | DNA replication checkpoint |
|                  | *CYT1* | √ | +++ +++ +++ +++ +++ +++ +++ +++ | High | Cytochrome electron transport |
|                  | *PST2* | √ | +++ +++ +++ +++ +++ +++ +++ +++ | High | Flavodoxin-like protein biosynthesis |
|                  | *ZRT1* | √ | +++ +++ +++ +++ +++ +++ +++ +++ | High | Zinc transporter, high affinity |
|                  | *TIR1* | √ | +++ +++ +++ +++ +++ +++ +++ +++ | High | Cold shock induced, cell wall |
|                  | *ENO2* | √ | +++ +++ +++ +++ +++ +++ +++ +++ | High | Enolase |
|                  | *SOD2* | √ | +++ +++ +++ +++ +++ +++ +++ +++ | High | Mn<sup>2+</sup>-containing superoxide dismutase |

<sup>a</sup> Checks indicate genes identified in screen, X indicates gene not identified in screen but analyzed by Northern blot hybridization.  
<sup>b</sup> Symbols: +++, maximum; ++, slightly reduced; +, very reduced; -, extremely reduced; -, not detectable. Luminescence measurements of bands in Northern blot hybridization patterns indicate that the difference between – and ++ is between 6- and 35-fold, the difference between + or ++ and +++ is at least 4-fold, and the difference between +++ and ++++ is approximately 2-fold. These fold differences are underestimates because of pixel saturation artifacts at the high end of band intensity.  
<sup>c</sup> Level of maximum expression relative to levels of expression of other messages.  
<sup>d</sup> Deduced from the demonstrated functions of *S. cerevisiae* orthologs. ER, endoplasmic reticulum; aa, amino acid.
Of the 13 putative Wh enriched genes analyzed, only 4 (31%) proved by Northern blot analysis to be expressed at higher levels in Wh than in DB (Table 4; Fig. 2). One of these genes, CTR2 (47), functions as a low-affinity copper transporter, and another, FAR8 (22), as a key regulator of cell cycle arrest in the pheromone response in *S. cerevisiae*. The third gene, YAPI (42), functions as a b-zip transcription factor involved in the oxidative response, and the fourth gene, APC9 (70), is a ubiquitin protein ligase involved in the cell cycle of *S. cerevisiae*. It seems no coincidence that three of these four genes are involved in copper assimilation, a stress response, or a pheromone response.

**Elevated expression in IWr.** In this study, genes enriched in subtracted Wh or DB cDNA pools were tested for relative expression by Northern blot analyses not only in Wh and DB cells but also in IWr cells. Northern blot analysis revealed five patterns that involved differential gene expression in IWr: (i) DB > Wh ≡ IWr, (ii) DB ≡ IWr > Wh, (iii) Wh ≡ IWr > DB, (iv) Wh ≡ DB > IWr, and (v) IWr > Wh ≡ DB (Tables 3 and 4). IWr exhibits the coloration of Wh, independently of the core phenotype of origin (28). Hence, similarities between the gene expression patterns of IWr and Wh (Table 3) may reflect this commonality. The patterns of expression of genes involved in sulfur and copper assimilation appeared to reflect this. Three out of the four genes involved in sulfur assimilation and all nine genes involved in copper assimilation that were expressed at higher levels in DB were also expressed at lower levels in Wh (Table 3). In addition, all of the genes expressed at higher levels in Wh than in DB were up-regulated in IWr as well (Table 4). However, IWr has a propensity to switch back to the core phenotype from which it emerged, suggesting that even though the coloration is that of Wh, IWr maintains, or “remembers,” its original core phenotype (28). The patterns of expression of genes involved in stress responses appeared to reflect this. Three out of the five stress response-related genes that were expressed at higher levels in DB were also expressed at higher levels in IWr (Table 3).

Six genes were selectively up-regulated and three down-regulated in IWr, but not in either Wh or DB (Table 4). Interestingly, the deduced functions of several of these genes (*TEF4, RPS23A, SIL1, SUT1, EFT1, and MET4*) involved RNA synthesis, protein synthesis, or protein translocation in *S. cerevisiae* (5, 21, 35, 41, 60, 64). None of the genes selectively up-regulated in IWr were related to copper detoxification or stress. In addition, none were involved in pseudohypha formation, an involvement one might have expected given the high proportion of cells in IWr colonies that express this phenotype (28). However, both Wh and DB colonies also contain pseudohyphae, although at lower proportions, and furthermore, the screens were not designed for the enrichment of IWr-specific transcripts.

**Constitutively expressed genes.** In our screen, we serendipitously identified nine genes that were enriched in either Wh or DB cDNA pools but proved to be constitutively expressed by Northern blot analysis (Fig. 2; Table 4). All of these genes exhibited high transcript abundance in the three switch phenotypes tested, while only 17% of these genes in the regulated
categories (6 of 36) exhibited high abundance, which may explain why the former may have slipped through the cDNA screening protocol. It is also noteworthy that none of these genes were involved in copper detoxification or stress responses. Verification of regulation of select genes in additional strains. To verify regulation patterns, we analyzed the expression of PMP3, TARI, CBF1, ECM17, CTR1, and CTR2 in Wh and DB in two additional, unrelated strains, the a strain 1480.50 and the a strain 1480.47. As was the case for the a strain 35B11 and the a strain 1480.49 (Tables 3 and 4), expression of the first five of these genes was up-regulated in DB cells, while that of CTR2 was up-regulated in Wh cells (data not shown).

Promoter activity reflects phenotype-specific expression. To test whether the patterns of differential gene expression among the switch phenotypes reflected promoter activity, the promoter regions of select genes from different categories were fused to the cDNA screening region of the RLUC reporter gene (57). The plasmid constructs were then targeted to the HO locus of C. glabrata (7), which plays a specific role only in mating type switching and hence was considered a neutral, nonessential gene for growth, core switching, and IWr switching. Two unrelated strains, one MTLa (35B11) and one MTLa (PB921) strain, were transformed with each of eight genes representing different categories of gene regulation. Two independent transformants were selected for each promoter and strain combination. Cells of each transformant were then plated, and three Wh colonies and three DB colonies were separately pooled in a growth medium containing 1 mM CuSO4. Cells were assayed at late-exponential phase. Luciferase activities in Table 5 are presented as the means (± standard deviations) of six measurements, which included three from each of the two independent transformants. For every targeted gene, the results of the promoter comparison were similar to the results of the Northern blot comparison (Table 5), indicating that the regulation of gene expression by switching occurs at the level of the promoter.

To test whether growth phase affected phenotype-specific promoter activity, we compared luciferase activities between exponential- and saturation-phase cells grown in liquid culture for a third strain, 35B1, which is MTLa. For every tested category of gene expression, regulation between Wh and DB was similar in the two growth phases (Table 6). Hence, regulation of promoter activity by switching was independent of growth phase. It should also be noted that the reproducibility of Wh and DB regulation in the three unrelated test strains was remarkably high (compare the PMP3, TARI, HSP104, APC9, and SUT1 genes in Tables 5 and 6).

DISCUSSION

A majority of C. glabrata strains switch spontaneously between core phenotypes and to the irregular wrinkle phenotype (6, 28, 29). It has been suggested that switching in the infectious fungi provides variants in natural colonizing populations

### Table 5. Phenotypic regulation of gene expression assessed by Northern blot analysis reflects promoter activity assessed in cells transformed with promoter-luciferase fusions

| Gene | Northern blotting result | RLUC sp act (\(10^4\) RLUC units/30 s/µg protein) | RLUC result | Fold difference of RLUC comparison |
|------|--------------------------|-----------------------------------------------|-------------|-----------------------------------|
|      |                          | Wh phase                                      | Saturation phase |                          |
|      |                          | Log phase                                     | Saturation phase |                          |
|      |                          | Wh                                            | DB           |                       |
|      |                          | 2.8 ± 1.6                                     | 1.1 ± 0.5     | 4.8 ± 1.8             | DB > Wh | DB/Wh | 7.5 | 4.3 |
| PMP3 | DB > Wh                  | 1.6 ± 0.8                                     | 1.2 ± 0.6     | 6.5 ± 2.8             | DB > Wh | DB/Wh | 11.6 | 9.5 |
| TARI | DB > Wh                  | 1.2 ± 0.6                                     | 1.2 ± 0.7     | 7.3 ± 4.6             | DB > Wh | DB/Wh | 8.9  | 6.1 |
| ECM17| DB > Wh                  | 1.9 ± 0.9                                     | 14.0 ± 6.3    | 2.3 ± 1.5             | Wh > DB | Wh/DB | 5.9  | 6.1 |
| HSP104| Wh > DB                 | 13.1 ± 5.1                                    | 2.6 ± 1.7     | 2.9 ± 1.8             | Wh ≈ DB | Wh/DB | 1.2  | 0.9 |
| APC9 | Wh ≈ DB                  | 4.3 ± 2.4                                     | 1.5 ± 0.5     | 1.9 ± 0.9             | Wh ≈ DB | Wh/DB | 1.3  | 0.8 |
| SUT1 | Wh ≈ DB                  | 1.9 ± 1.1                                     | 1.5 ± 0.8     | 1.5 ± 0.5             | Wh ≈ DB | Wh/DB | 1.2  | 1.2 |
| TEF4 | Wh ≈ DB                  | 2.8 ± 16.9                                    | 1.1 ± 0.5     | 4.8 ± 1.8             | DB > Wh | DB/Wh | 7.5  | 4.3 |

### Table 6. Phenotypic regulation of promoter activity is independent of the growth phase in liquid culture

| Gene | Northern blotting result | RLUC sp act (\(10^4\) RLUC units/30 s/µg protein) | RLUC result | Fold difference of comparison |
|------|--------------------------|-----------------------------------------------|-------------|--------------------------------|
|      |                          | Wh phase                                      | Saturation phase |                          |
|      |                          | Log phase                                     | Saturation phase |                          |
|      |                          | Wh                                            | DB           |                       |
|      |                          | 3.5 ± 2.1                                     | 4.9 ± 2.7     | 24.7 ± 10.6           | DB > Wh | DB/Wh | 6.6  | 5.0 |
| PMP3 | DB > Wh                  | 1.8 ± 0.9                                     | 2.7 ± 1.5     | 24.6 ± 8.8            | DB > Wh | DB/Wh | 11.9 | 9.1 |
| TARI | DB > Wh                  | 1.9 ± 0.9                                     | 2.9 ± 1.8     | 21.9 ± 9.7            | DB > Wh | DB/Wh | 9.7  | 7.6 |
| HSP104| DB > Wh                 | 17.4 ± 7.2                                    | 23.0 ± 10.4   | 2.7 ± 1.6             | Wh > DB | Wh/DB | 7.6  | 8.5 |
| APC9 | Wh > DB                  | 4.1 ± 2.2                                     | 4.9 ± 2.6     | 4.5 ± 2.4             | Wh ≈ DB | Wh/DB | 1.1  | 1.1 |
| SUT1 | IWr > Wh ≈ DB           | 1.7 ± 0.6                                     | 2.7 ± 1.3     | 2.3 ± 1.2             | Wh ≈ DB | Wh/DB | 1.1  | 1.0 |

### Discussion

A majority of C. glabrata strains switch spontaneously between core phenotypes and to the irregular wrinkle phenotype (6, 28, 29). It has been suggested that switching in the infectious fungi provides variants in natural colonizing populations.

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*a* The basal RLUC activity of strains harboring the promoterless vector pH12.7 targeted to the HO gene ranged between \(5.9 \times 10^3\) and \(6.2 \times 10^3\) units. This was subtracted from all RLUC activities obtained with promoter-containing constructs. Each measurement shown is the mean (± standard deviation) of six measurements of relative luminescence, three from each of two independent transformants.
which may be enriched in response to rapid environmental challenges, a mechanism for rapid adaptation (55, 56). White-opaque switching in C. albicans has been demonstrated to facilitate skin colonization (26) and to be essential for mating (32, 39). One way of deducing the roles these complex phenotypic transitions play in host colonization and pathogenesis is to identify the genes regulated by them (30). Here we have used a subtraction strategy to identify genes differentially regulated by the core switching system. This screen identified 35 such genes, the majority of which were up-regulated in DB. Regulation of these genes was verified in two unrelated strains, one a and one α, and select genes were verified in two additional strains. In the white-opaque transition in C. albicans, more genes appear to be up-regulated in the white-to-opaque direction than in the opaque-to-white direction (30), suggesting a more specialized role for the opaque-phase phenotype and, by inference, a more specialized role for the DB phenotype in C. glabrata.

The S. cerevisiae orthologs of 17 of the 22 genes (77%) identified in the screen as up-regulated in DB function in S. cerevisiae in sulfur assimilation, copper assimilation, and stress responses. They included reductases, transporters, and permeases, as well as trans-acting factors that regulate these genes. In several organisms including S. cerevisiae, the genes encoding proteins involved in copper homeostasis and detoxification, as well as in stress responses, are up-regulated in response to environmental cues such as toxic levels of CuSO₄, increases in temperature, or changes in oxygen tension (1, 19, 31, 53). In C. glabrata, the orthologs of some of the same genes have also been demonstrated to be regulated by environmental cues. MT-I and MT-II in C. glabrata have been demonstrated to be up-regulated by high levels of extracellular CuSO₄ (71, 72). However, in our comparison of gene expression, both Wh and DB cells were grown in media containing the same concentration of CuSO₄. They were also grown in the same nutrient medium at the same temperature and were harvested for comparison at the same growth phase. Therefore, in C. glabrata these genes are also regulated by spontaneous phenotypic switching and, as we have demonstrated here, at the level of promoter activation. We found that the differences in promoter activity between Wh and DB were similar in mid-log-and saturation-phase cells. Lachke et al. (29) further demonstrated that at least in the case of MT-II, graded expression (i.e., Wh < LB < DB) was similar in cells grown in the presence and in the absence of 1 mM CuSO₄. They also demonstrated this to be the case for the hemolysin-like protein gene HLP1, which is expressed in a similar graded fashion (i.e., Wh < LB < DB) (29). Therefore, core switching in C. glabrata regulates a number of genes normally under the regulation of environmental cues in other organisms.

Our results would appear to be consistent with the hypothesis that switching provides populations with a minority of cells expressing variant phenotypes that can be rapidly enriched in response to an environmental challenge (44, 55, 56). However, this explanation does not appear to be sufficient in the case of C. glabrata, since DB may represent the common core phenotype expressed at sites of colonization (6; S. Lachke and D. R. Soll, unpublished observations). The up-regulation of genes associated with sulfur homeostasis, copper homeostasis, and stress responses, therefore, may not be associated with a rare phenotype, but possibly with the most common colonizing phenotype of C. glabrata. However, Brockert et al. (6) also observed that for one patient, while DB was the predominant phenotype in cheek and tongue samples, Wh of the same strain was the predominant phenotype in the vaginal canal, indicating a specialization that may be based on the differences in gene expression patterns demonstrated here.

While only four genes were identified as up-regulated in Wh, three were orthologs of S. cerevisiae genes regulated by environmental cues. These three included a low-affinity copper transporter, a protein involved in the pheromone response, and a transcription factor involved in the oxidative response. In marked contrast, only one of eight constitutively expressed genes that were picked up in the screen due to their high abundance had a deduced function in copper or sulfur assimilation or a stress response. Hence, the functional bias of genes up-regulated in DB or Wh toward copper detoxification and stress responses cannot be due to chance. This conclusion is further supported by the deduced functions of the six genes identified in the screen as up-regulated in IWr. None of them had a deduced role in copper assimilation, sulfur assimilation, or a stress response.

In addition to the 6 genes identified as up-regulated in IWr, 5 of the 18 genes up-regulated in DB were also up-regulated in IWr, which may reflect the DB origin of the IWr isolates analyzed here. More interestingly, all four of the genes up-regulated in Wh were also up-regulated in IWr. This is consistent with our original assessment (28) that IWr seemed to exhibit Wh characteristics, regardless of the core phenotype from which it arose. These characteristics included white color on agar containing 1 mM CuSO₄, red color on agar containing phloxine B, a high switching frequency, and low levels of MT-II transcript (28). Our results further suggest that although the core and IWr switching systems appear to be distinct, there may be overlap in the genes that are regulated by the two programs.

Our results, therefore, indicate that core switching in C. glabrata regulates a subset of genes that have been implicated in copper detoxification and stress responses in S. cerevisiae. The majority of these genes are up-regulated in the DB phenotype, which may represent the prevalent phenotype at sites of infection. Although we have suggested that up-regulation of these genes in vitro in the transition from Wh to DB is a result of switching and not environmental cues, it may be that for this pathogen, the regulation of such genes has been usurped by the spontaneous core-switching system in adaptation to the challenging host environment. As such, switching may represent a supervirulence factor regulating a number of genes, the combined expression of which facilitates pathogenesis. Because the host environment does not include high levels of CuSO₄, we further suggest that the functions of these and perhaps other genes up-regulated in DB may not be the same as the functions in S. cerevisiae, which has not similarly evolved as a pathogen.

ACKNOWLEDGMENTS

This research was supported by National Institutes of Health grant DE014219.
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