Effect of alterations in non-coding regions in genomic expression reprogramming of two *Candida glabrata* clinical isolates

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Abstract

Up to now the majority of the genomic analysis undertaken in *C. glabrata* clinical isolates have focused modifications in coding regions ignoring what happens in the non-coding genome. In this work the non-coding genomes of the reference strain CBS138 and of a clinical isolate, named FFUL887, were compared. Among other phenotypic traits, the two strains are differently resistant to antifungals, the FFUL887 isolate being much more tolerant to fluconazole, voriconaozle and caspofungine than CBS138. Massive alterations had been identified in the non-coding genome of the two strains reflecting the great plasticity that characterizes these regions. Emphasis has been put in the examination of how these alterations may impact the governance of the overall regulation of genomic expression in the two strains by modulating the interaction of transcription factors with their binding sites. In particular, the regulatory network controlled by CgPdr1, a key regulator of resistance to antifungals in *C. glabrata*, was focused. 31 new putative CgPdr1 targets whose promoter in the FFUL887 harbored at least one binding sites for CgPdr1, which was absent in the CBS138 counter-partner, were identified. 7 of these putative CgPdr1 targets were confirmed to contribute for maximal *C. glabrata* tolerance to caspofungine and 2 were required for tolerance to fluconazole and voriconazole. The results obtained in this study are expected to contribute for the advance of the understanding of the mechanisms underlying the evolution of regulatory networks controlling the genomic expression of *C. glabrata* cells during colonization of the human host and during acquisition of resistance to antifungals.

Key-words: *C. glabrata*, resistance to antifungals, genomic analysis of non-coding regions, evolution of regulatory networks in *C. glabrata*

Introduction

The number of life-threatening infections caused by the members of the fungal genus *Candida*, generally known as invasive candidiasis, has risen significantly in the past decades mainly due to a significant increase in the size of the immunocompromised population as a result of the massification of aggressive therapeutic treatments that negatively affect the activity of the immune system[1–3]. . Other recognized risk factors for

the development of invasive candidiasis include the use of broad-spectrum antibiotics and the use of indwelling intravenous catheters[1,2,4]. Although *C. albicans* is the more common causative agent of invasive and superficial fungal infections, there has been a significant increase in the number of infections caused by non-*Candida albicans* species, generally known as NCAC[2]. The main factor attributed to the increase in the incidence of infections caused by *C. glabrata* is its reduced susceptibility to the main drug used in the treatment of fungal infections, fluconazole[5]. To overcome this problem of azole-resistance, drugs alternative to fluconazole had been developed including new azoles (e.g. voriconazole) and echinocandins[6].

The pleiotropic-drug resistance network (PDR) is a well-organized and complex regulatory network under the control of the transcription factor CgPdr1 and that has been found to play a fundamental role in the control of *C. glabrata* response to azoles [7,8]. Different transcriptomic analysis have unveiled the set of genes regulated by CgPdr1 during *C. glabrata* response to azoles these including several genes encoding ABC- and MFS MDR pumps, such as Cg*SNQ2*, Cg*CDR1*, *CgPDH1*, Cg*YOR1* and Cg*TPO3*, but also genes that are involved in the metabolism of lipids, fatty acids and sterol, stress response; transcription and adhesion. Although CgPdr1 is the main player in the control of the PDR network, several other transcription factors have also been found to play an important role in the regulation of PDR genes. In specific, it has been shown that Cg*PDR1* is activated by Cg*HST1*, Cg*RFM1* and Cg*SUM1*, being repressed by CgStb5. It has also been seen that Cg*PDR1* auto-regulates its own expression and that it represses Cg*RPN4*[8–17]. Yrr1 and Yrm1 were also found to play a critical role in the control of the PDR network in S. cerevisiae, however, in *C. glabrata* the relevance of these transcription factors has not yet been clarified. The regulation of the genes involved in the PDR network has also been demonstrated to differ in gain-of-function (GOF) CgPdr1 mutants that were recovered from azole resistant clinical isolates. in CgPdr1 GOF mutants the activity of the transcription factor becomes constitutively high thereby resulting in the constitutive expression of its target genes even in the absence of a xenobiotic stimulus[8–10,18]. The advances in genomic tools and computational methods have been playing a critical role in the identification of regulatory network components (such as transcription factors and their target genes) and to study their patterns of conservation and divergence across multiple species. Changes in regulatory mechanisms can be learned from patterns of evolutionary divergence in regulatory properties at multiple levels: gene expression (mRNA levels), characterization of *cis*-regulatory elements in orthologous promoter sequences, protein-DNA interactions measured across organisms (transcription factor binding), and duplication and divergence of regulators or comparing chromatin organization[19]. The emergence of drug resistance may be a result of this transcriptional rewiring that is resultant of the change of expression of several genes, such as Cg*PDR1* and the genes regulated by CgPdr1[7,8,10,20].

Within the last years a great number of studies has been published studying the molecular mechanisms underlying resistance of *C. glabrata* clinical isolates to azoles and echinocandins, with emphasis played by CgPdr1-regulated genes [21,22]. The majority of these studies have however focused on specific mechanisms and sets of regulated genes and little is known on the alterations occurring at a genome sequence level. Recently a clinical *C. glabrata* isolate recovered from the urinary tract, named FFUL887, was identified as being resistant to fluconazole and voriconazole, also exhibiting increased tolerance to caspofungin, in comparison with the reference strain CBS138 which was found to be susceptible to all these antifungals. One of the traits that was uncovered by the analysis of the genome sequence of the FFUL887 isolate was the fact that it encodes a novel gain-of-function CgPdr1 mutant, this being corroborated not only by the drug-resistance phenotype of the isolate but also by the observation that several genes regulated by CgPdr1 were found to be over-expressed in the FFUL887 strain during cultivation in drug-free growth medium.

Different from the approach that was previously performed, the work presented in this thesis is focused on the alterations that have occurred in the non-coding genome of the FFUL887 strain.

Materials and Methods

Strains and growth media

A cohort of 13 *C. glabrata* strains, FFUL887, CBS138, KUE100, its derived mutants ΔCgA, ΔB, ΔC, ΔD, ΔE, Δ*F*, Δ*G*, Δ*H*, Δ*I*, ΔJ, and the yeast *Saccharomyces cerevisiae* BY4741, acquired from the Euroscarf collection, were used. *C. glabrata* cells and *S. cerevisiae* BY4741 were batch-cultured at 30ºC, with orbital agitation (250rpm) in Yeast Peptone Dextrose (YPD) or in Minimal Medium (MM). YPD growth medium contains, per liter: 20 g glucose (Merck), 20 g yeast extract (Difco) and 10 g bactopeptone (Difco). MM growth medium contains, per liter, 20 g glucose (Merck), 1.7 g Yeast Nitrogen Base without amino acids and ammonium sulphate (Difco) and 2.65 g ammonium sulphate (Merck). To grow *S. cerevisiae* cells MM medium was further supplemented with 20 mg/L histidine, 60 mg/L leucine and 20 mg/L methionine to complement the auxotrophies of the BY4741 strain. Solid YPD or MM media were obtained by supplementing the corresponding liquid growth medium with 2% agar (Iberagar). The plasmid used in this work was pYEP354W.

Genomic DNA extraction

Gnomic DNA was extracted from FFUL887 and CBS138 cells cultivated in solid YPD. After growth, three loops of biomass were inserted in a 1 mL-Eppendorf tube with approximately 100µl of glass beads (0.5mm) and 200µl of lysis buffer (Tris 50 mM, EDTA 50 mM, NaCl 250 mM, SDS 0.3%). The tubes were vortexed for 2 minutes at maximum speed and then incubated at 65ºC for 1h, after which a second round of vortexing was performed. The obtained disrupted cell suspension was then centrifuged at 13000 rpm for 15 minutes at 4ºC and the supernatant transferred to a clean eppendorf. 20µl NaAC 3M (pH 4,8) and 400µl cold Ethanol were added to the suspensions to induce DNA precipitation. The samples were left at -20ºC for, at least, 30 minutes after which these were centrifuged at 13000 rpm, during 20min at 4ºC. The pellet obtained was washed with 500µl ethanol 70%, dried in the speed vacuum and finally resuspended in 50µl deionized water.

In vitro DNA manipulations

The PCR reactions were performed using Taqmed polymerase (Citomed) and a set of primers that were designed to specifically hybridize in the selected promoter regions of the genes *CDR1*, *PDH1*, and *MET10*. Beside the region that allows PCR amplification, the selected primers also contain the necessary regions of homology with the plasmid (pYEP354W), essential for homologous recombination (Table 1). Amplification of the fragments was confirmed by running the PCR products in a 0.8% agarose gel and purified from the agarose gel using the JETQUICK Gel Extraction Spin kit (Genomed).

Table 1 - Primers used in the amplification of the promoter fragments. Sequence includes specific promoter regions of each gene (red) and the homology region with the plasmid pYEP354W (black).

***CDR1*_Fw:** TCATTCCCGGAATTCCCGGGGATCCGTCGACCTGCAGCCA TAGCGCATGGAAATCCTTGG
***CDR1*_Rev:** TCCCAGTCACGACGTTGTAAAACGACGGCGGGAGCAAGCTT CTTGTCACTTGCAAGAGACA
***PDH1*_Fw:** TCATTCCCGGAATTCCCGGGGATCCGTCGACCTGCAGCCA TTGAACTATCCCAACACCAG
***PDH1*_Rev:** TCCCAGTCACGACGTTGTAAAACGACGGCGGGAGCAAGCT TAGAGTCATCGGGTGTGTTCA
***MET10*_Fw:** TCATTCCCGGAATTCCCGGGGATCCGTCGACCTGCAGCCA GTAAGTATGTCCCAGTATAC
***MET10*_Rev:** TCCCAGTCACGACGTTGTAAAACGACGGCGGGAGCAAGCT TCACAGACATTGCTTGGCTTT

Yeast transformation

The amplified promoter regions and the digested pYEP354W plasmid were transformed in *S. cerevisiae* BY4741 cells using the Alkali-Cation TM Yeast Transformation kit (MP Biomedicals) and plated on MMB plates. Confirmation of the integration of the different promoters in the pYEP354W plasmid in the clones that grew in MM plates was performed by colony PCR.

Drug resistance assays using C. glabrata FFUL887 derived mutants and C. glabrata KUE100 strain

Susceptibility of KUE100 and of the derived deletion mutants to fluconazole,

voriconazole and caspofungin was tested using an experimental setup based on 96-microwell plates. Three concentrations of each caspofungin, voriconazole and fluconazole were tested, the concentration that is known to correspond to the clinical resistance breakpoint (as defined by EUCAST, www.eucast.org); one below and one above that resistance breakpoint. The microplates were cultivated for 24h for fluconazole and 48h for caspofungin and voriconazole.

Testing the "white/dark brown" phenotype

Solid YPD was supplemented with 1mM sterile $CuSO_4$ after medium sterilization. FFUL887, CBS138 and KUE100 *C. glabrata* cells were cultivated in liquid YPD growth medium until mid-exponential phase (DO_{600} of 0.4).). After reaching the desired DO_{600} of 0.4, 1mL of each culture batch was used to performed dilution series to a final dilution of 10^{-6}. From these dilutions, only 10^{-4}, 10^{-5} and 10^{-6} cell suspensions were plated into solid YPD supplemented with 1mM of CuSO4. Cells were then incubated at 30ºC for 5 days and daily observations were performed to evaluate the phenotype switching progression between strains.

Spot assays

Solid MMB medium was prepared without glucose as a carbon source, instead three different concentrations of acetate, butyrate and propionate were used as carbon sources: 0.2%; 0.5% and 2%. FFUL887 and CBS138 strains were cultivated in liquid MMB until-mid-exponential phase (DO_{600} of 0.4). After reaching DO_{600} of 0.4, cell suspensions were prepared for an initial DO_{600} of 0.05 from the original suspension. Two more dilutions were performed for 1:5 and 1:25 from the initial DO_{600} of 0.05 cell suspension. Afterwards, 4µL of each cell dilution was used to plate in solid MMB medium with the different acetate, butyrate and propionate concentration. Cells were then incubated for 3 days at 30ºC.

Results

A total of 35438 SNP's were identified in the non-coding sequences of the FFUL887 strain, the vast majority of these being in promoter regions, from which 2 mutations were identified in one ncRNA , CaglfMr14. 73% of all genes predicted to be encoded by the genome of the FFUL887 strain harboured at least one SNP in their promoter, when compared with their CBS138 counter-partner (Figure 1). 26 genes were found to harbour more than 30 mutations in their promoter region, the more evident case being the C*gPWP4* gene whose promoter in the FFUL887 strain exhibited 136 different nucleotides comparing to the corresponding promoter encoded by the CBS138 genome (Figure 1 and Table 2)

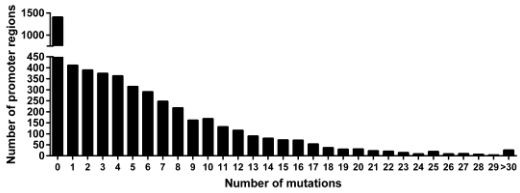

Figure 1 – Number of SNPs found in the promoter regions encoded by the FFUL887 strains.

Table 2 – Promoter regions found to harbour more SNPs in FFUL887 when comparing to CBS138 strain.

ORF	NAME	FUNCTION	NUMBER OF SNPS FOUND
CAGL0I10362G	*PWP4*	Lectin-like cell wall protein (flocculin) involved in flocculation	136
CAGL0C00231G	*FCY21*	Putative purine-cytosine permease	98
CAGL0J03058G	*ICL1*	Isocitrate lyase	53
CAGL0M01254G	*CgSTE12*	Putative transcription factor, required for filamentous growth induced by nitrogen starvation and for virulence	51
CAGL0C00253G		Putative cell wall adhesin	49
CAGL0L13288G	*NPR2*	Subunit of the Iml1p/SEACIT complex	42
CAGL0C00968G		Adhesin-like protein with a predicted role in cell adhesion	40
CAGL0L13299G	*EPA11*	Putative cell wall adhesin	40
CAGL0C03916G	*MNT3*	Role in protein glycosylation	38
CAGL0F01485G	*TIR2*	Putative GPI-linked cell wall mannoprotein of the Srp1p/Tip1p family	35

To take a hint into how the SNPs found were distributed throughout FFUL887 gene promoters' these were divided in 50bp windows and the number of mutations in each of these windows was computed (Figure 2).

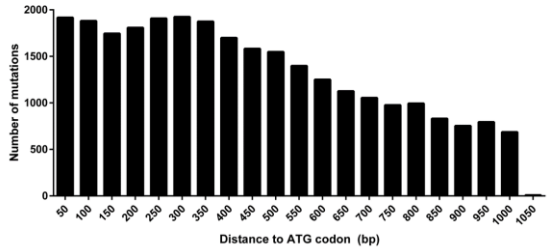

Figure 2 - Distribution of the SNPs throughout FFUL887 gene promoters. Each promoter region was divided in 50 bp segments and the number of SNPs (assessed upon comparing each promoter with its corresponding CBS138 counter-partner) was computed.

The results obtained showed that the mutations in the promoter regions are distributed all over the genes' promoters, however, a slight increase in the frequency of mutation was observed to occur closer to the ATG start codon (Figure 2). 1925 mutations identified in the promoters of FFUL887 strain are included in the 300 bp window, this number increasing to 13060 if we take into consideration the first 350 bp (Figure 2).

Correlation between gene expression and the SNPs found in promoters of FFUL887 genes.

To assess the relevance of the identified mutations in promoters of FFUL887 genes the results of a previous analysis in which the transcriptome of these cells was compared with the one of CBS138 cells during cultivation in RPMI growth medium were used[23]. In Figure 3 it is shown the correlation between the number of mutations found in each promoter with the difference in expression registered in the FFUL887 and in the CBS138 strains, according to the microarray analysis performed.

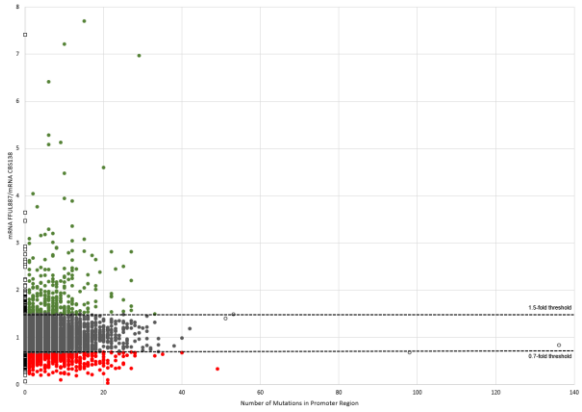

Figure 3- Correlation between the numbers of mutations found harbouring promoter regions in FFUL887 when compared to CBS138 genome and the expression of the corresponding genes in the two strains during cultivation in RPMI growth medium. Genes that have no mutations in their promoter regions, but are up- or down-regulated in the FFUL887 strain are highlighted as open squares. The genes whose promoters were found to be more strongly mutated (above 50 mutations) are highlighted as open circles. Genes that are above the 1.5-fold threshold are considered up-regulated and represented in green circles. Genes that are below the 0.7 threshold are considered down-regulated and are represented in red circles. Genes that were found to have no alterations in their expression are represented in grey circles.

82 genes over-expressed (above a 1.5-fold) and 173 repressed (below a 0.7-fold) genes in the FFUL887 strain were identified as not harbouring mutations in their promoter regions. The analysis undertaken allowed the identification of a set of genes that were differently expressed (above a threshold of 3-fold) in FFUL887 and CBS138 cells and whose promoters also differed in these two strains, (Figure 4).

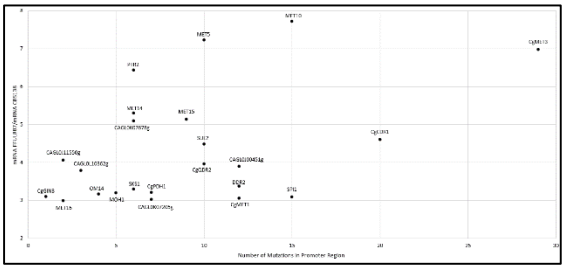

Figure 4 - Representation of the identified genes harbouring mutations in their promoter regions that were differently expressed (above a threshold of 3-fold) in FFUL887 and CBS138 cells.

Functional clustering of the genes up-regulated and whose promoters changed the most revealed a significant over-representation of genes involved in sulphate metabolism and, in particular, in biosynthesis of methionine: Cg*SUL1*, Cg*MET1*, Cg*MET3*, *MET5*, *MET10*, *MET14*, *MET15*, *MET16*. Three multidrug-resistance transporters, CgQDR2, CgPHD1 and CgCDR2 were also found in this dataset as well as Cg*PTR2*, involved in transport of peptides. Altogether, these observations lead to the hypothesis that the white-brown phenotypic switching, induced by *MET* genes, could be altered in the FFUL887 strain. To test this hypothesis FFUL887 and CBS138 cells were cultivated in YPD rich growth medium supplemented with 1 mM $CuSO_4$, a compound that is widely used to induce white to brown phenotype in *C. glabrata* (Figure 5)[24,25].

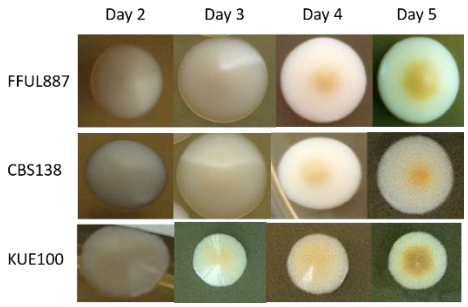

Figure 5 - Dark Brown phenotype assay with FFUL887, CBS138 and KUE100 strains. Cells were grown in YPD solid medium supplemented with 1mM $CuSO_4$ and incubated for 5 days at 30ºC.

The higher expression in FFUL887 cells of the genes shown in Figure 16 could result from the alteration(s) that were registered in their promoter or from the activity of the different factors involved in their transcriptional regulation, or even from the combination of these two things. In specific, a strategy was attempted to clone the promoter region of *MET10*, Cg*PHD1* and Cg*CDR1* genes obtained from the genome of the FFUL887 or of the CBS138 strains upstream of the *lacZ* gene present in the pYEP354w plasmid. The promoters of interest were at first amplified, by PCR, using as a template the genomic DNA of the FFUL887 strain. After recovery from the gel, and subsequent purification, this PCR product was transformed into *S. cerevisiae* cells together with the HindIII-digested pYEP354w plasmid. The experimental conditions used for the PCR reaction only allowed the amplification of the promoters of Cg*PDH1* and *MET10* and therefore it was decided to move forward only using these two promoters. After two days of incubation at 30ºC, the plates obtained from the transformations performed with *MET10* and Cg*PDH1* promoters had about 60 colonies each. The correct insertion of each promoter in the pYEP354w plasmid in these candidates was tested by colony PCR (Figure 6). The results obtained allowed the identification of three positive candidates for *MET10* (Figure 6A) and three also for Cg*PDH1* (Figure 6B).

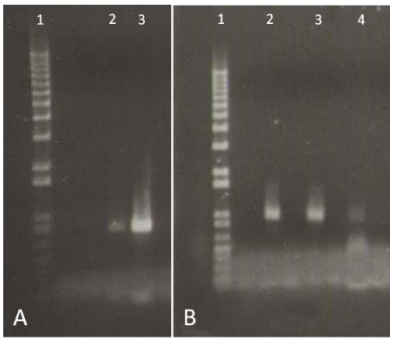

Figure 6- Agarose gel showing the result of the Colony PCR amplification for *MET10* (A) and Cg*PDH1* (B). A: 1 – 1kb DNA plus ladder, 2 and 3 – amplification of MET10 from the MET10-pYEP354w candidates; B: 1- 1kb DNA plus ladder, 2, 3 and 4 – amplification of CgPDH1 from the CgPDH1-pYEP354w candidates.

Effect of the SNPs found in gene promoters in the overall regulatory network controlling the genomic expression of the FFUL887 and CBS138 strains.

The observed modifications in the promoter region of FFUL887 genes, when compared to the gene promoters of the CBS138 strain, could contribute for the remodelling of the networks governing the control of gene expression in these strains for example by creating new DNA motifs that may serve as binding sites for transcription factors. To see how the alterations observed to occur in FFUL887 promoters could alter the regulatory associations that can be established between target genes and transcription factors in this strain, the promoters of this strain were searched for DNA motifs demonstrated (or hypothesized) to serve as binding sites for all predicted *C. glabrata* transcription factors. The exception to this were the transcription factors Cg*PDR1*, involved in the control of drug resistance in *C. glabrata*, and Cg*MSN4*, involved in control of response to environmental stress, whose binding sites were already characterized being HYCCRKGGRN and CCYYCCYYM, respectively[26,27]. Around 8042 new putative TF binding sites were found in FFUL887 gene promoters, these corresponding to motifs that were absent in the corresponding CBS138 promoters. On the other side, 3033 putative TF binding sites were lost in gene promoters of the FFUL887 strain. This difference in the number of putative TF binding sites corresponds to 2511 (48%) promoters being

identical in the two strains, 914 (17%) promoters that gained binding sites in the FFUL887 isolate, 1383 (26%) that lost binding sites in the FFUL887 isolate and 491 (9%) that suffered both types of modifications. In Figure 7 it is shown a representation of those binding sites that appeared/disappeared with higher frequencies in the genome of the FFUL887 isolate, in comparison with the genome of the reference strain.

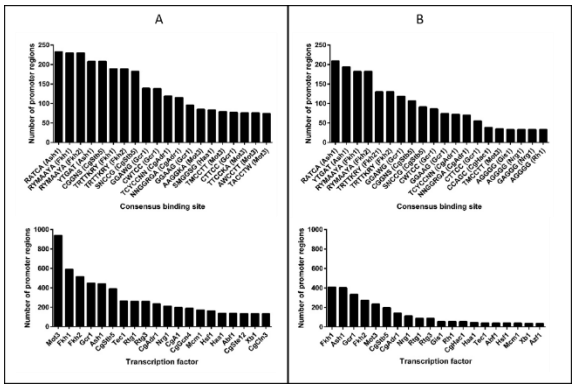

Figure 7- Representation of gained binding sites (A) and lost binding sites (B). Top graphic in box A refers to the consensus binding sites with more frequency of appearance in FFUL887 promoter regions, while bottom graphic in box A refers to transcription factors whose recognized consensus binding sites were more gained in FFUL887 promoter regions. Top graphic in box B refers to the consensus binding sites with more frequency of disappearance in FFUL887 promoter regions, while bottom graphic in box B refers to transcription factors whose recognized consensus binding sites were more lost in FFUL887 promoter regions.

Although it is clear that such modifications create a very strong alteration in what can be the potential transcriptome-wide regulatory network of the FFUL887 isolate, it is hard to understand the biological relevance of these modifications observed in the number of binding sites for the physiology of the FFUL887 isolate. This way, up- and down-regulated genes were clustered according to their gain or loss of binding sites harbouring their promoter region and the promoter regions of up- and down-regulated genes that suffered the most gains of binding sites were identified. This analysis lead to the identification of 139 up-regulated genes which did not lose or gain binding sites in their promoter regions in FFUL887 isolate; 30 up-regulated genes registered only losses of binding sites in their promoter region in this strain; 86 up-regulated registered only gains of binding sites and 92 genes were identified as lost and gained binding sites in their promoter region, is the case of genes of *MET* family.

CgPdr1 New Regulatory Network

81 of the genes found to be up-regulated in the FFUL887 strain were previously documented to be regulated by CgPdr1, this corresponding to approximately 22% of the overall set of genes over-expressed in this strain. Closer analysis of the promoter region of these documented CgPdr1 targets differently expressed in the FFUL887 and in CBS138 strains shows that only 16 of them harbour the DNA motif HYCCRKGGRN, the binding site recognized by CgPdr1[26]. Among these up-regulated genes that harbour the PDRE motif are the drug efflux pumps Cg*PDH1*, Cg*YOR1*, Cg*QDR2* and Cg*CDR1.* The Amt1 transcription factor, up-regulated in the FFUL887 isolate by 1.83-fold is a known documented target of CgPdr1[8,10,26] and 10 of the genes up-regulated in FFUL887 isolate are documented targets of CgAmt1. On the other direction, there are 39 documented CgPdr1 targets within the set of genes down-regulated in the FFUL887 isolate. In Figure 8 it was compiled a putative transcriptional regulatory network controlled by the CgPdr1 transcription factor encoded by the FFUL887 isolate.

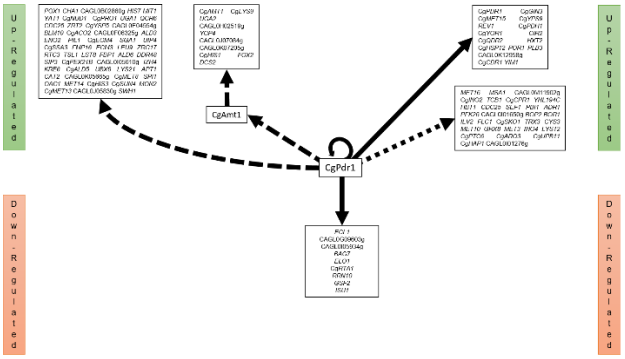

Figure 8 - Representation of CgPdr1 putative transcriptional regulatory network. Solid arrows represent documented target genes that harbour CgPdr1 consensus binding site; dashed-line arrows represent documented target genes that do not harbour CgPdr1 consensus binding site; dotted-line arrow represents undocumented targets that harbour CgPdr1 consensus binding site.

The regulatory network controlled by the CgPdr1 transcription factor mutant encoded by FFUL887 genome could also be altered as the result of modifications in the promoter region of target genes which can gain or lose PDRE motifs. To examine this

hypothesis all the promoter regions of FFUL887 genes were searched for the PDRE motif. The results obtained led to the identification of 19 genes that are candidates to be under CgPdr1 regulation by harbouring a PDRE motif in their promoter region, this being absent in the promoter region of the corresponding gene encoded by the genome of the CBS138 strain. Differently, 4 FFUL887 genes lost the PDRE motif from their promoter. The overall number of CgPdr1 binding sites harbouring in the FFUL887 strain reaches 420 while in the CBS138 only 405 promoter harbour this motif. To examine whether these genes that gained a PDRE motif in their promoter play a role in *C. glabrata* tolerance to azoles or echinocandins, the susceptibility of strains devoid of a selected set of these genes (Cg*A*, *B*, C, D, *E*, *F*, *G*, *H*, *I*, J) was compared with the one of the parental strain KUE100. In specific, three concentrations of voriconazole, fluconazole or caspofungin were tested: one corresponding to the considered clinical resistance breakpoint, one below that value and another one above it. Growth of the strains in the presence of the different concentrations of fluconazole and voriconazole was accompanied along 48h based on the increase of the OD600nm of the cultures. In the case of caspofungin an end-point assay was used in which the final OD600nm of the different cultures was compared after 48h of incubation in the presence or absence of the drugs (Figure 9).

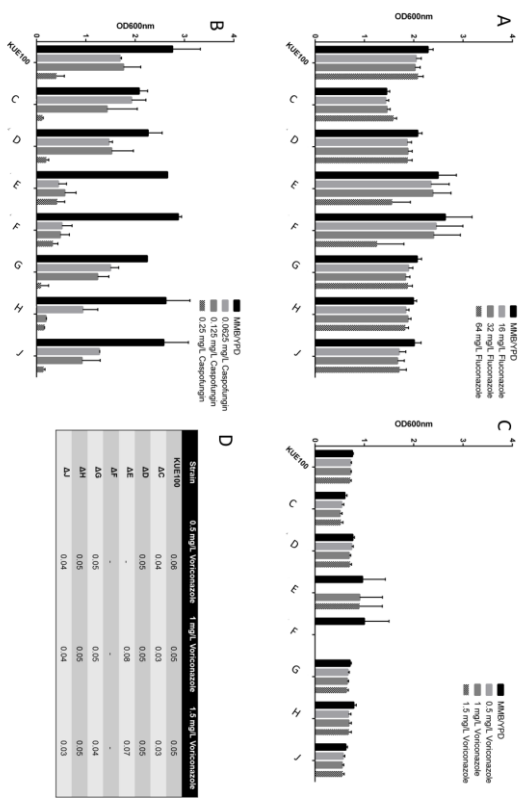

Figure 9 - Growth of the KUE100 strain and of the derived deletion mutants in MM growth medium supplemented with three concentrations of fluconazole (A), voriconazole (B) or caspofungin (C). In the case of voriconazole and fluconazole growth was compared after 24h of growth while for caspofungin this was extended to 48h due to the more inhibitory effect of the drug. Voriconazole growth values (C) and ratios (D). Derived mutants KUE100 ΔE and KUE100 ΔF were grown in rich YPD medium supplemented with the mentioned drug concentrations; derived mutants KUE100 ΔC, KUE100 ΔD, KUE100 ΔG, KUE100 ΔH and KUE100 ΔJ were grown in MMB medium supplemented with the indicated drug concentrations.

Because the deletion of the genes CgA, *B* and *I* was found to significantly reduce viability of *C. glabrata* cells it was not possible to use a true deletion strain and therefore in these cases the suppression of these genes was achieved by changing their promoter for a tetracycline-repressible promotor (Figure 10). In this case it was only compared the susceptibility of the wild-type cells with the mutant strains in the presence of caspofungine or fluconazole.

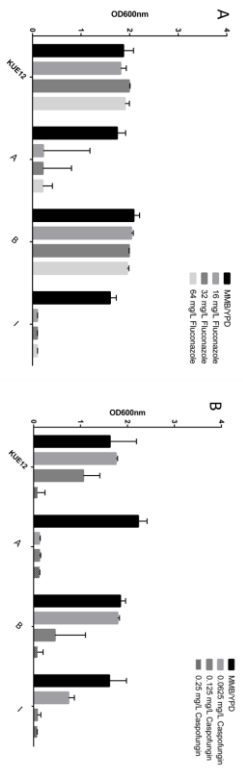

Figure 10 - Growth values for KUE100 and derived tetracycline-repressible promotor mutants under three different concentrations of fluconazole (A) after 24h of growth and caspofungin (B) after 48h of growth. Derived mutants B and I were grown in MMB medium supplemented with the indicated drug concentrations and tetracycline; derived mutant CgA was grown in rich YPD medium supplemented with the indicated drug concentrations and tetracycline.

Analysis of other CgPdr1 GOFs' regulatory network

Transcriptomic analysis of *C. glabrata* isolates expressing different gain-of-function Pdr1 mutants revealed a considerable dispersion in the different set of over-expressed genes leading to the hypothesis that the different mutations in the transcription factor impact the regulatory network in a different manner[10]. the set of genes up-regulated in the FFUL887 isolate was compared with the set of genes described to be regulated by two other Pdr1 gain-of-function mutants: SM3, harbouring the T2837C (L946A) mutation, and 6955 mutant, harbouring the G822T (K274N) mutation[10] (Figure 11).

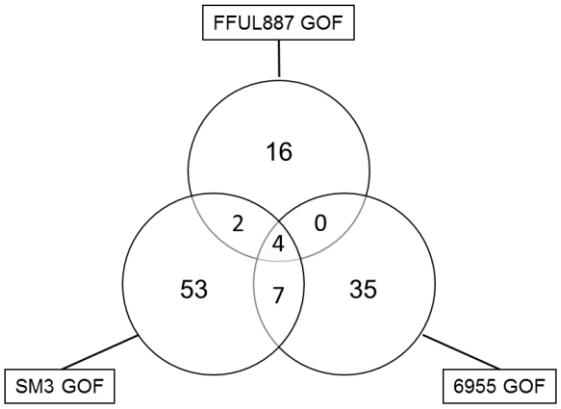

Figure 11- Representation of the comparison of the set of genes up-regulated and regulated by CgPdr1 in different clinical isolates harbouring different GOF CgPdr1 mutations[10].

Four genes were found to be in common in all the three datasets examined, out of which it stands out the Cg*PDR1* gene. The other 3 genes in the common dataset, CgY*PS9*, *REV1* and *HXT2* were previously documented to be regulated by CgPdr1.

Examination of the role of CgStb5, CgRpn4 and Yrr1 in the FFUL887 CgPdr1 regulated network

The control of the expression of the genes of the PDR network involves, besides CgPdr1, at least three other transcription factors, CgRpn4, CgYrr1 and CgStb5. It is the combined activity of these transcription factors that leads to the final expression of genes required for resistance to antifungals in *C. glabrata*[11,15,28]. In view of this, the FFUL887 gene promoters' were searched for binding sites for CgStb5, Yrr1 and CgRpn4, the results obtained showed that 390 new genes were found to harbouring new CgStb5-binding sites, 5 were found to harbour new CgYrr1-binding sites, while the total number of CgRpn4 binding sites was maintained in both FFUL887 and CBS138 strains. the total number of promoter regions harbouring CgStb5 and Yrr1 is similar in both FFUL887 and CBS138 strains, whereas CgStb5 potentially regulates 3870 genes in CBS138 and 4498 genes in FFUL887, while Yrr1 potentially regulates 715 genes in CBS138 and 761 genes in FFUL887 (Figure 12). Differently, CgPdr1 can potentially regulate 348 genes in CBS138 strain and 346 genes in FFUL887, as these were the genes found to harbour PDRE motif (Figure 12).

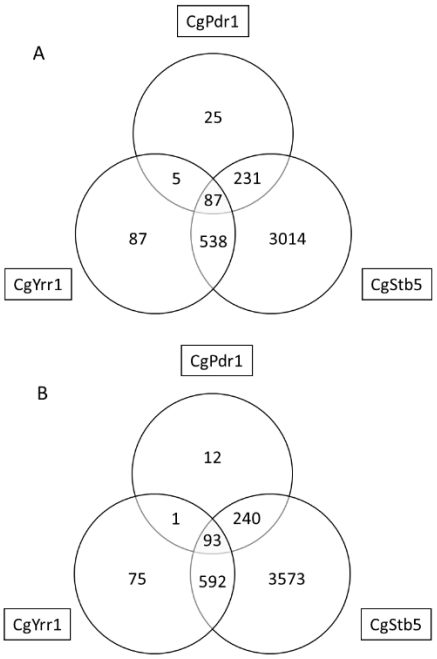

Figure 12- Potential regulatory network between CgPdr1, CgYrr1 and CgStb5. A – Interactions predicted in CBS138 strain. B – Interactions predicted in FFUL887 isolate.

With this results it was possible to identify a set of 83 genes that are co-regulated by these transcription factors in both CBS138 strain and FFUL887 isolate, whereas only 4 genes are predicted to be solely regulated by this network in CBS138 and 10 genes predicted to be regulated by this network in FFUL887 isolate. Interestingly, FFUL887 CgPdr1 predicted target genes include those already identified above, such as CgC, CgF and CgE, which is also predicted as a CgPdr1 in CBS138. Crossing the information with CgPdr1 documented target lead to the identification of 33 genes already documented as CgPdr1 targets in CBS138 strain and 34 genes identified as documented CgPdr1 target genes in FFUL887 isolate (Figure 13).

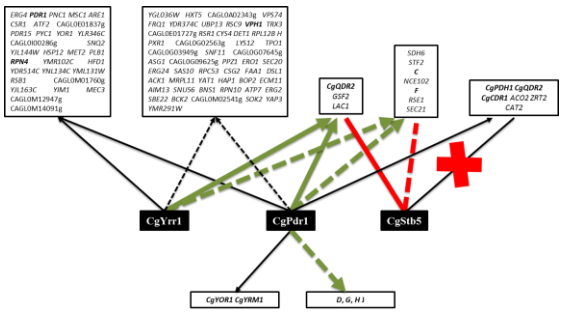

Figure 13 - Potential target genes of the CgPdr1, CgStb5 and CgYrr1 regulatory network in FFUL887. Target prediction was accomplished through the search of CgPdr1, CgStb5 and CgYrr1 binding sites in all CBS138 and FFUL887 promoter regions. Solid thicker arrows represent an interaction predicted by the CgPdr1 documented target genes; dashed thicker arrow represents an interaction not predicted by the CgPdr1 documented target genes; green arrows indicates an interaction that activates the expression of the target gene(s); red arrows indicates an interaction which represses the expression of the target gene(s); red arrow with a red cross (X) represents the loss of CgStb5 binding site in the target gene(s); black solid arrows indicate an interaction observed in both CBS138 and FFUL887 strains that is predicted by the documented target genes; black dashed arrows indicate an interaction observed in both CBS1338 and FFUL887 strain that is not predicted by the documented target genes, in these two cases the repressive action of CgStb5 is maintained; genes in bold represent genes which are described to be involved in drug resistance.

Unveiling other transcriptional regulators that could be involved in the control of genomic expression of the FFUL887 isolate: emphasis on the CgAdr1 transcription factor

To better understand the overall regulatory network governing the genomic expression of the FFUL887 isolate the documented regulatory network associations between transcription factors found to be up- or down-regulated in this strain, in comparison with the expression levels registered in the CBS138 strain, were searched. Besides CgPdr1, which regulated 23% of the genes up-regulated in the FFUL887 isolate, the transcription factor CgMsn4 was documented to regulate 2% of the up-regulated genes in this strain (Table 13). Those transcription factors whose expression decreased in the FFUL887 strain had no documented targets within the set of genes down-regulated in this strain. The transcription factor Adr1 (Table 15) emerges as a potential important transcription factor as a large number of the up-regulated genes was found to harbour a binding site for this transcription factor. It is important to stress that in *S. cerevisiae* Adr1 is involved in the control of the regulation of genes required for metabolization of alternative carbon sources, which as said before it is an hallmark of *C. glabrata* adaptation to the mammalian host[20]. The CgAdr1 transcription factor encoded by the FFUL887 strain has two point mutations (Ala650Gly and Gly627Ser) relative to its CBS138 counter-partners, however, it is not know the role of residues in the control of the activity of the transcription factor. These different insights prompted the examination of

whether the FFUL887 and CBS138 strains had a different capacity to grow in the presence of different carbon sources, the results obtained being shown in Figure 14.

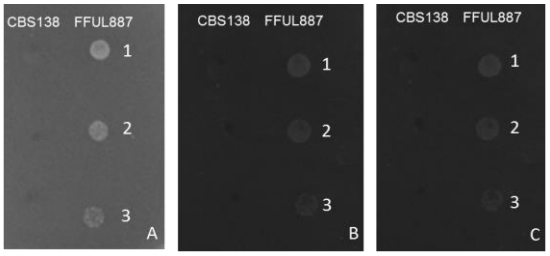

Figure 14 - Spot assays using MMB medium with 0.5% concentration of acetate (A), butyrate (B) and propionate (C) as the only carbon source for CBS138 and FFUL887 strains. Spots were prepared by plating a cellular suspension with initial OD600nm of 0.05 (1), followed by the dilution of 1:5 (2) and 1:25 (3) from the initial suspension (1).

Concluding Remarks

One of the clear observations that comes out of this study was the massive alteration in the non-coding genome of the FFUL887 strain, in comparison with the one of the reference strain CBS138. Although some of these modifications are likely to be the mere result of evolution and may not represent meaningful events affecting the physiology of *C. glabrata* cells, it is clear that many of these modifications will contribute to shape the evolution of the regulatory network of the strains thereby allowing a modification in the transcriptional landscape to fit better the needs of the microbe along colonization of the host. To better understand the true relevance of the herein reported alterations extensive transcription profiling of the FFUL887 and CBS138 strains under different experimental conditions would be required, along with a much better knowledge of the binding sites recognized by each *C. glabrata* transcription factor. In the case of CgPdr1 it was possible to identify a set of new putative target genes whose promoters gained at least one PDRE motif which was absent from the genome of the CBS138 strain. It remains to be confirmed if the expression of these genes is indeed regulated by CgPdr1, especially in the presence of antifungals to which CgPdr1 responds to. In case this hypothesis is confirmed, then it can be hypothesized that the expansion of regulatory networks through the acquisition of novel TF-DNA interactions could represent an important mechanism by which *C. glabrata* cells acquire resistance to antifungals. It will also be of interest to further confirm if the loss of binding sites for CgStb5 may contribute to enhance the expression of the drug-efflux pumps CgQDR2, CgPHD1 and CgCDR1 in the FFUL887 isolate. If this is confirmed, then it will be an important demonstration of the evolution of the PDR network occurring by alteration in the promoter region of target genes.

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