

# **Investigation of the role of Mediator tail subunits in antifungal drug resistance and cellular aggregation in *Candida* species**

Wen Jun Lim  
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Microbiology Research Unit  
Division of Oral Biosciences  
Dublin Dental University Hospital  
Trinity College Dublin



## **Declaration**

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## Summary

*Candida albicans* is a commensal fungal pathogen that resides on various human mucosal surfaces (e.g. oral cavity) and can cause severe infection in immunocompromised individuals. Azole antifungal resistance in *C. albicans* is increasing, making candidemia difficult to treat due to the lack of alternative antifungal drugs. Studies have revealed that azole resistant *C. albicans* isolates possess hyperactive Tac1, a transcription factor which activates expression of the efflux pump Cdr1 and ultimately confers azole resistance. Previous studies also revealed that tail components of the Mediator complex were required for Tac1-activated azole resistance in *C. albicans*. In this study we investigate whether the Mediator tail component Med2, encoded by the massively expanded (n=14) *TLO* gene family of *C. albicans* was required for Tac1 activity. Based on these findings, we also aimed to investigate the function of specific *TLO* genes, namely *TLO $\alpha$ 1* and *TLO $\beta$ 2*, in the hyperactive Tac1-activated resistance to the antifungals fluconazole and terbinafine. To carry out this specific study, CRISPR-Cas9 was used to introduce a homozygous *TAC1-5* gain-of-function point mutation into wild-type (WT), *tlo $\Delta$*  and *med3 $\Delta$*  strains of *C. albicans* which enables them to express hyperactive Tac1. qRT-PCR analysis revealed that *tlo $\Delta$ -TAC1-5* has reduced *CDR1* expression compared to WT *TAC1-5* and antifungal susceptibility testing showed that the minimal inhibitory concentration (MIC) values of fluconazole and terbinafine were decreased 2-fold. Individual copies of *TLO $\alpha$ 1* and *TLO $\beta$ 2* were introduced into *tlo $\Delta$ -TAC1-5* to investigate their role in Tac1-activated fluconazole and terbinafine resistance. qRT-PCR revealed that *TLO $\alpha$ 1* is highly expressed but *TLO $\beta$ 2* is weakly expressed in *tlo $\Delta$  TAC1-5*. *TLO $\alpha$ 1* could restore the Tac1-activated fluconazole and terbinafine resistance of *tlo $\Delta$  TAC1-5* whereas *TLO $\beta$ 2* could only partially restore the Tac1-activated fluconazole resistance of *tlo $\Delta$  TAC1-5*. Surprisingly, *TLO $\beta$ 2* restores the terbinafine resistance of *tlo $\Delta$*  but reduces the terbinafine resistance of *tlo $\Delta$ -TAC1-5*, suggesting *TAC1-5* antagonized *TLO $\beta$ 2* expression. ChIP-qPCR was used to investigate the interaction of *Tlo $\alpha$ 1*

and the *CDR1* promoter. These experiments revealed that Tlo $\alpha$ 1 co-localised to the drug response element (DRE) which is the site of Tac1 binding in the *CDR1* promoter, whereas the interaction of Tlo $\beta$ 2 was much weaker.

The second part of this study aimed to investigate the function of *MED2* and *MED15* in another multidrug resistant pathogen *C. auris*. CRISPR-Cas9 was used to delete *MED2* and *MED15* in a weakly aggregative strain 8985 and highly aggregative strain 8977. The deletion of *MED2* and *MED15* did not reduce the antifungal resistance of *C. auris* strains. However, the deletion of *MED2* and *MED15* increased the resistance of 8985 towards calcofluor white and congo red, suggesting cell wall changes in these mutants. The deletion of *MED2* and *MED15* reduced the aggregation capability of 8977 in PBS and the echinocandin induced aggregation phenotype of 8985. The deletion of *MED2* and *MED15* also significantly reduced the biofilm formation capability of 8977 on hydrophobic surface and in particularly on fetal bovine serum precoated surfaces. The white-opaque switching (environmental adaptation) of 8977 might also be affected by *MED2* and *MED15* deletion. Lastly, RNA sequencing revealed that an important aggregation-contributing adhesin, *SCF1*, was downregulated in the Mediator tail mutants of 8977 along with other putative adhesins and important genes. Surprisingly, the strong downregulation of a putative Als3p encoding gene B9J08\_004498 might contribute to the low aggregation capability of Mediator tail mutants of 8985 despite the upregulation of *SCF1*.

In summary, *C. albicans* Mediator tail protein Tlo $\alpha$ 1 is required for Tac1-activated fluconazole and terbinafine resistance whereas the corresponding Mediator tail component of *C. auris*, Med2 and its interacting partner Med15, regulate *SCF1* and other putative adhesins required for cellular aggregation in this species.

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## Abbreviations

|                |                                                           |
|----------------|-----------------------------------------------------------|
| AAD            | <b>Acidic activation domain</b>                           |
| ABC            | Adenosine triphosphate binding cassette                   |
| ALS            | Agglutinin-like sequence                                  |
| ANOVA          | Analysis of Variance                                      |
| ATP            | Adenosine triphosphate                                    |
| ATPase         | Adenosine triphosphatase                                  |
| bp             | Base pair(s)                                              |
| CAGE           | Cap analysis gene expression                              |
| cDNA           | Complementary DNA                                         |
| CFW            | Calcofluor white                                          |
| ChIP           | Chromatin Immunoprecipitation                             |
| cm             | Centimetre                                                |
| Co-IP          | Co-immunoprecipitation                                    |
| CRISPR         | Clustered regularly interspaced short palindromic repeats |
| crRNA          | CRISPR derived RNA                                        |
| C <sub>T</sub> | Threshold cycle                                           |
| DBD            | DNA binding domain                                        |
| DDUH           | Dublin Dental University Hospital                         |
| DNA            | Deoxyribonucleic acid                                     |
| dNTP           | Deoxyribonucleotide triphosphate                          |
| DRE            | Drug response element                                     |
| DSB            | Double stranded break                                     |
| DTT            | Dithiothreitol                                            |
| e.g.           | <i>Exempli gratia</i> ; for example                       |
| ECL            | enhanced chemiluminescence                                |
| ECM            | Extracellular matrix                                      |
| EDTA           | Ethylenediaminetetraacetic acid                           |
| EPC            | esophageal candidiasis                                    |

|               |                                                    |
|---------------|----------------------------------------------------|
| <i>et al.</i> | <i>Et alia</i> ; and others                        |
| etc.          | <i>Et cetera</i> ; and the rest                    |
| FBS           | Fetal bovine serum                                 |
| FGR           | Fungal gene regulator                              |
| g             | Gram                                               |
| GI            | Gastrointestinal                                   |
| GO            | Gene Ontology                                      |
| GOF           | Gain of function                                   |
| GPI           | Glycosylphosphatidylinositol                       |
| gRNA          | Guide RNA                                          |
| GSEA          | Gene set enrichment analysis                       |
| h             | Hour(s)                                            |
| HA            | Hemagglutinin                                      |
| HCAI          | Healthcare associated infection                    |
| HDR           | Homology-directed repair                           |
| HEPES         | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HF            | High fidelity                                      |
| HIV           | Human immunodeficiency virus                       |
| HNH           | His-Asn-His                                        |
| HSP           | Heat shock protein                                 |
| HYG           | Hygromycin                                         |
| HYG400        | 500 µg/ml hygromycin                               |
| i.e.          | <i>Id est</i> ; that is                            |
| IP            | Immunoprecipitation                                |
| ITS           | Internal transcribed spacer                        |
| KEGG          | Kyoto Encyclopedia of Genes and Genome             |
| kV            | Kilovolts                                          |
| L             | Litre                                              |
| LEU           | Leucine                                            |

|        |                                      |
|--------|--------------------------------------|
| M      | Molar                                |
| MDR    | Multidrug-resistant                  |
| MF     | Major facilitator                    |
| mg     | Milligram                            |
| MHR    | Middle homology region               |
| MIC    | Minimum inhibitory concentration     |
| min    | Minute(s)                            |
| ml     | Millilitre                           |
| mM     | Millimolar                           |
| mRNA   | Messenger RNA                        |
| NAC    | Non-albicans Candida                 |
| NAT    | Nourseothricin                       |
| NAT200 | 200 µg/ml nourseothricin             |
| NEB    | New England Biolabs                  |
| ng     | Nanogram                             |
| NGS    | Next generation sequencing           |
| NHEJ   | Non homologous end joining           |
| nm     | Nanometre                            |
| NP-40  | Ethoxylated nonylphenol              |
| OC     | Oral candidiasis                     |
| OD     | Optical density                      |
| ODU    | Optical density per unit             |
| ORF    | Open reading frame                   |
| ORF    | Open reading frame                   |
| PAM    | Protospacer adjacent motifs          |
| PB     | Phloxine B                           |
| PBS    | Phosphate buffer saline              |
| PBS-T  | Phosphate buffer saline triton x-100 |
| PC     | Personal computer                    |

|             |                                                           |
|-------------|-----------------------------------------------------------|
| PCA         | Principal component analysis                              |
| PCR         | Polymerase chain reaction                                 |
| pH          | Potential hydrogen                                        |
| PI          | Protease inhibitor                                        |
| PIC         | Pre-initiation complex                                    |
| PMSF        | Phenylmethanesulfonyl fluoride                            |
| PVDF        | Polyvinylidene fluoride                                   |
| qPCR        | Quantitative polymerase chain reaction                    |
| qRT-PCR     | Quantitative real time polymerase chain reaction          |
| RAM         | Regulation of <i>ACE2</i> and the Morphogenesis           |
| RNA         | Ribonucleic acid                                          |
| RNase       | Ribonuclease                                              |
| RPMI        | Roswell Park Memorial Institute                           |
| RVVC        | Recurrent vulvovaginal candidiasis                        |
| SAGE        | Serial analysis of gene expression                        |
| SDS         | Sodium dodecyl sulfate                                    |
| SDS-PAGE    | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| sgRNA       | Single guide RNA                                          |
| SNP         | Single nucleotide polymorphism                            |
| <i>spp.</i> | Species                                                   |
| TAE         | Tris-acetate-EDTA                                         |
| TBP         | TATA-binding protein                                      |
| TE          | Tris-EDTA                                                 |
| <i>TLO</i>  | Telomere associated                                       |
| tracrRNA    | Tracer CRISPR derived RNA                                 |
| UK          | United Kingdom                                            |
| USA         | United States of America                                  |
| UV          | Ultraviolet                                               |
| V           | Volts                                                     |

|      |                                |
|------|--------------------------------|
| v/v  | Volume per volume              |
| VVC  | vulvovaginal candidiasis       |
| w/v  | Weight per volume              |
| WT   | Wild-type                      |
| YEPD | Yeast extract peptone dextrose |
| YNB  | Yeast nitrogen base            |
| YPG  | Yeast extract peptone glycerol |
| β-ME | β-Mercaptoethanol              |
| μg   | Microgram(s)                   |
| μl   | Microlitre(s)                  |
| μM   | Micromolar                     |
| %    | Percentage                     |
| '    | Prime                          |
| <    | Less than                      |
| >    | More than                      |
| °C   | Degree(s) Celcius              |

# **Chapter 1**

## General Introduction

## 1.1 Introduction: *Candida albicans* and disease

Fungi are one of the largest eukaryotic kingdoms that exhibit a huge variety of life-cycle patterns, metabolic adaptations and morphogenetic processes that enable them to adapt to ever-changing ecosystems (Choi and Kim, 2017). It is estimated that there are approximately 2 to 3.8 million fungal species and only a few hundred of those have been identified to cause clinical disease in humans (Hawksworth and Lücking, 2017). Even though there are only a few hundred human fungal pathogens, they infect billions and cause more than 1.5 million human deaths annually, a mortality rate similar to tuberculosis and >3 fold more than malaria (Brown *et al.*, 2012; Fisher *et al.*, 2018). A recent epidemiological study estimated the global mortality rate of fungal infection to be in the region of 2.5 million human deaths per year (Denning, 2024). Fungal diseases have various infection sites, including human skin, oral cavity and nails with severities ranging from asymptomatic or mild mucocutaneous infections to potentially life-threatening systemic infections like candidemia (Reddy *et al.*, 2022). Despite having such a detrimental impact on human health, fungal infections have often been neglected by the public. Over the past few decades and despite the epidemiological changes in fungal diseases, *Candida* species remain the main fungal pathogen of humans responsible for most serious fungal infections (Bongomin *et al.*, 2017).

*Candida* species are responsible for most human fungal infections and among these, *Candida albicans* is the main cause of opportunistic human infections (Guinea, 2014; Macias-Paz *et al.*, 2023). *C. albicans* is a commensal pathogen that inhabits various human mucosal surfaces such as the gastrointestinal (GI) tract, reproductive tract, and the oral cavity (Nobile and Johnson, 2015). Due to *C. albicans* robust adherence properties and a high level of pathogenicity (Millsop and Fazel, 2016), it is responsible for most mucosal fungal infections and systemic infections (Bongomin *et al.*, 2017). It normally resides as a harmless commensal in healthy

individuals but can cause various superficial infections as well as severe life-threatening infections in critically ill or immunocompromised patients (Nobile and Johnson, 2015). The alteration in host microbiota (e.g. due to antibiotics), changes in host immune response (e.g. due to stress, other microbial infection), or variations in the local environment (e.g. pH or temperature) contribute to the overgrowth of *C. albicans* and can cause infection (Nobile and Johnson, 2015).

Infections caused by *Candida spp.* can be classified into two major subtypes based on site of infection: superficial and systemic (Lopes and Lionakis, 2022). The primary superficial sites affected by *C. albicans* are the vaginal mucosa (vulvovaginal candidiasis [VVC]), the oral mucosa (oral candidiasis [OC]), the oesophageal mucosa (oesophageal candidiasis [EPC]), and the least common site, the nails (onychomycosis) (Lopes and Lionakis, 2022). Systemic candidiasis mainly affects sterile body sites like the human bloodstream, central nervous system, liver, and heart (Pappas *et al.*, 2018). Unlike mucosal candidiasis, systemic candidiasis is associated with high mortality despite the administration of antifungal therapy (Pappas *et al.*, 2018).

#### 1.1.1 Vulvovaginal candidiasis

Vulvovaginal candidiasis is a common disease among pregnant women, diabetic women, and women undergoing antibiotic treatment (Martins *et al.*, 2014). It is estimated that at least 75% of women will experience VVC once in their lifetime and 40-50% will suffer from a recurrence (Gonçalves *et al.*, 2016). Recurrent vulvovaginal candidiasis (RVVC) is defined as three or four symptomatic episodes of vulvovaginal candidiasis per year, with asymptomatic periods in

between (Sobel, 2016). RVVC is common among women between ages 25 – 34 and is estimated to affect 5% - 9% of the female population (Sobel, 2016).

#### 1.1.2 Oral candidiasis

Oral candidiasis predominantly affects immunocompromised adults and neonates such as HIV-infected patients or individuals taking immunosuppressive drugs (Millsop and Fazel, 2016). *Candida* spp. are present in the normal oral flora of 45% - 65% of healthy infants and 30% - 55% of healthy adults (Millsop and Fazel, 2016). Patients using steroid inhalers, dentures, or having xerostomia are high-risk groups for oral candidiasis (Millsop and Fazel, 2016). Oral candidiasis commonly presents as a white lesion on the tongue and *C. albicans* is the major cause as it was found in more than 80% of oral lesions (Millsop and Fazel, 2016). This classic presentation is known as pseudomembranous candidiasis, also known as “thrush”, and presents as confluent white plaques on tongue, buccal mucosa, oral pharynx, and hard and soft palate (Millsop and Fazel, 2016). These white plaques can be easily scraped off to reveal an erythematous surface and patients might sometimes experience other symptoms like the feeling of dysgeusia or a burning sensation in the mouth (Millsop and Fazel, 2016). It is important to identify atypical presentations of OC like “denture stomatitis”, which commonly presents with edematous, erythematous gums in contact with the dentures, and “hyperplastic candidiasis”, which forms white lesions that cannot be easily wiped off and makes it hard to distinguish it from leukoplakia (Millsop and Fazel, 2016). *C. albicans* is also involved in the development of angular cheilitis, presenting with painful fissures at the corners of the mouth, and central papillary atrophy of the tongue (Millsop and Fazel, 2016).

### 1.1.3 Invasive candidiasis

*Candida* spp. can also enter the bloodstream to cause a form of invasive candidiasis known as candidemia. *C. albicans*, *Candida glabrata* [recently renamed *Nakaseomyces glabrata* (Borman and Johnson, 2021)] and *Candida parapsilosis* are responsible for the majority of cases of candidemia, which are estimated at 8.05 cases per 100,000 inhabitants annually (Denning, 2024). In certain parts of the world, an increasingly common species known as *Candida auris*, has emerged as a cause of invasive candidiasis (Chowdhary *et al.*, 2017; Lockhart *et al.*, 2017). Among all four *Candida* species, *C. albicans* is the leading cause of all healthcare-associated bloodstream infections in the United States with a crude mortality rate of around 40% despite antifungal intervention (Lee *et al.*, 2021). The incidence of candidemia in Europe has also increased from 2.2 cases/100,000 inhabitants per year to 3.2 cases/100,000 inhabitants per year between 2000 and 2019 (Koehler *et al.*, 2019). The increase of serious disease caused by fungi in recent decades is due to the increasing number of immunocompromised patients such as cancer patients, organ transplant recipients, individuals infected by HIV, and the increasing elderly population (Pfaller and Diekema, 2010). Furthermore, the rise of antimicrobial resistance in fungal pathogens is particularly concerning due to the lack of antifungal drugs for treatment and the emergence of multidrug resistant fungal pathogens, particularly certain *Candida* species (Lee *et al.*, 2021). The high consumption of first line antifungals and their inappropriate use are the other two main factors that contribute to the uprise of antifungal resistance fungal pathogens (Galia *et al.*, 2022).

### 1.1.4 Epidemiology of antifungal susceptibility in *Candida albicans* and non-*albicans Candida* species

In Europe, *C. albicans* is mainly responsible for invasive candidiasis, accounting for 70-90% of cases (Bongomin *et al.*, 2017). Antifungal susceptibility reports of *Candida* species isolated

from patients with candidemia showed a wide range of geographical variabilities. For example, the incidence of fluconazole resistant strains ranges from 2% in Ireland (Ryan *et al.*, 2019) to a 10% incidence in Spain (Pemán *et al.*, 2005). A recent surveillance report of blood culture *Candida* isolates from six Southern European tertiary hospitals showed a significant increase of azole resistant *C. albicans* isolates from 0.7% to 4.5% (Boattini *et al.*, 2023). Echinocandin resistance seems negligible as currently there are very few cases of echinocandin resistant strains being reported (1.4% of strains for caspofungin, 2.9% for anidulafungin, and 1.3% for micafungin) (Kritikos *et al.*, 2018). Even though there are several antifungal susceptibility reports of *Candida* species in Europe (Bongomin *et al.*, 2017; Pemán *et al.*, 2005; Ryan *et al.*, 2019), the overall incidence of antifungal resistance in *C. albicans* is poorly documented (Galia *et al.*, 2022).

The other worrying concern is the increased incidence of non-*albicans Candida* (NAC) strains. Over the past decade, there was a significant increase in the proportion of NAC strains among all candidemia cases (Guinea, 2014; Kajihara *et al.*, 2022) and was most likely due to the high usage of fluconazole and other antifungal drugs for therapy, resulting in the selection of NAC species with reduced susceptibility to antifungal drugs (Taei *et al.*, 2019). Among all NAC, *Candida parapsilosis* and *Candida glabrata* are the most predominant NAC species that causes invasive candidiasis, whose distribution varies at different countries (Guinea, 2014). Between the two NAC species, *C. glabrata* is more prevalent in Northern Europe and USA whereas *C. parapsilosis* is more frequent in Spain and Brazil (Guinea, 2014). Multiple European studies have shown an increase in azole resistance among invasive NAC species with different percentages and variability. For instance, the fluconazole resistance of *C. glabrata* ranges from 2.8% in Iceland (Asmundsdottir *et al.*, 2013) to 88.6% in Denmark (Arendrup *et*

*al.*, 2013), whereas 4% of *C. tropicalis* isolates were found to have fluconazole resistance across six European countries (Arendrup *et al.*, 2023).

## **1.2 A newly emerged multidrug resistant NAC species: *Candida auris***

*Candida auris*, also known as *Candidozyma auris* (Liu *et al.*, 2024), is a recently identified multidrug-resistant (MDR) NAC yeast pathogen that was first isolated in Japan in 2009 from a female patient (Satoh *et al.*, 2009). *C. auris* belongs to the *Candida* group and was discovered to be phylogenetically closely related to *Candida ruelliae* and *Candida haemulonii* based on analyses of the 26S rDNA D1/D2 domain, nuclear ribosomal DNA internal transcribed spacer (ITS) region sequences, and chemotaxonomic studies (Satoh *et al.*, 2009). Currently, *C. auris* isolates are grouped into six geographically restricted genetic groups called clades: clade I (South Asia), clade II (East Asia), clade III (South Africa), clade IV (South America) (Bravo Ruiz and Lorenz, 2021; Du *et al.*, 2020; Spivak and Hanson, 2018), clade V from Iran (Spruijtenburg *et al.*, 2022) and a Clade VI from Singapore (Suphavilai *et al.*, 2024). *C. auris* is well known for causing significant healthcare-associated invasive fungal infections and evidence suggests that it can persistently colonize the hospital environment and patients, leading to high transmissibility and prolonged outbreaks (Schelenz *et al.*, 2016; Vallabhaneni *et al.*, 2017). *C. auris* was later found to have a unique tropism for skin which allows it to persist on human skin for a long period of time (Huang *et al.*, 2021) and act as a commensal of human skin (Proctor *et al.*, 2021) unlike *C. albicans* which is a commensal of human mucosal surface and the gastrointestinal tract (Lopes and Lionakis, 2022). A recent surveillance study revealed that there was an increase in *C. auris* cases and outbreaks in the European Union and European Economic Area between 2020 and 2021 (Kohlenberg *et al.*, 2022) and up till December 2023, *C. auris* has been identified in 61 countries across the world (Figure 1.1). Furthermore, *C. auris* can cause candidemia (systemic bloodstream infection) with a high crude mortality rate ranging

from 30 to 72% (Cortegiani *et al.*, 2018) and causes outbreaks in hospitals across the globe (Biswal *et al.*, 2017; Ruiz-Gaitán *et al.*, 2018; Ruiz-Gaitán *et al.*, 2019; Schelenz *et al.*, 2016). Most *C. auris* clinical isolates are resistant to one or more commonly used antifungal drugs to treat *Candida* infections, making it harder to be treated (Chowdhary *et al.*, 2014; Lockhart *et al.*, 2017). Overall, the emergence of this problematic fungus urges the need to develop new strategies to control the global outbreak of *C. auris*. Despite the large number of publications addressing *C. auris* infection published since its discovery in 2009, both the pathogenicity and virulence mechanism of this newly emerged human pathogen are still poorly understood (Rossato and Colombo, 2018).

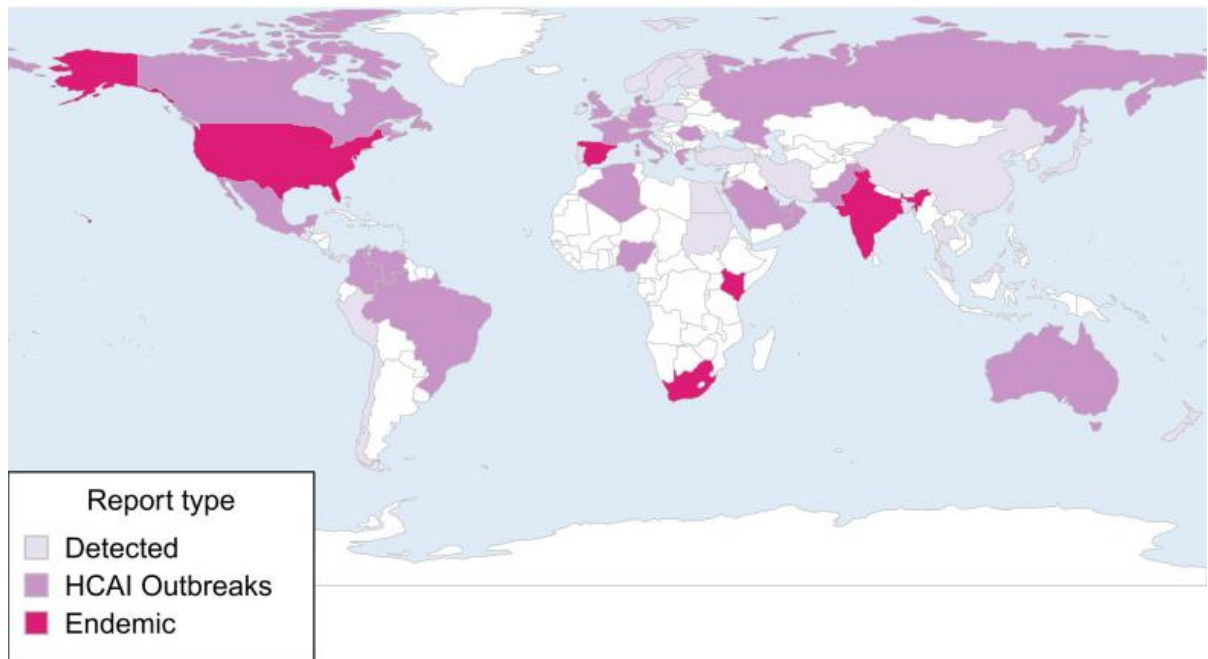


**Figure 1.1. Global epidemiology of *C. auris*.**

Countries where *C. auris* has been reported were categorised into three different groups with different colours with pale lilac representing reports on the isolation of *C. auris*, lilac representing reports about HCAI (healthcare associated infection) outbreaks of *C. auris*, and dark pink representing areas where reports indicate endemic cases of *C. auris*. Figure adapted from (Jones *et al.*, 2024).

### 1.3 Antifungal treatment for candidiasis

The treatment for most cases of oral and vulvovaginal candidiasis are similar as *C. albicans* is the main causative agent of mucosal candidiasis (McCort, 2021). Most *C. albicans* related infections can be treated with many available antifungal drugs (azoles, polyenes and echinocandins) as they are generally susceptible to most antifungals except in some rare cases (McCort, 2021).

#### 1.3.1 Oral candidiasis

The treatment for oral candidiasis involves trying to contain the overgrowth of yeast as well as removing possible underlying risk factors. For mild oral candidiasis, topical antifungal agents like clotrimazole troches or nystatin suspension for seven days is sufficient to eliminate mild oral candidiasis (Pappas *et al.*, 2016). Nystatin is a widely used polyene antifungal drug available in various topical forms that disrupts the fungal cell membrane via ergosterol binding (Millsop and Fazel, 2016). For more severe oropharyngeal candidiasis, oral fluconazole tablets (200 – 400mg daily) will be prescribed to patients for one to two weeks depending on clinical improvements (Millsop and Fazel, 2016). Patients with denture stomatitis are recommended to have their dentures washed regularly in an antifungal solution to reduce recurrences (Pappas *et al.*, 2016). For patients with chronic recurrent oral candidiasis, prophylaxis with 150mg fluconazole three times weekly is the safest and most effective approach (Pappas *et al.*, 2016).

#### 1.3.2 Vulvovaginal candidiasis

For uncomplicated vulvovaginal candidiasis, the first line treatment will be topical antifungal agents (like clotrimazole or nystatin cream) or a single dose of 150mg oral fluconazole tablet. Topical agents are effective in 80-85% of uncomplicated cases (Pappas *et al.*, 2016). Three

doses of 150mg fluconazole is recommended to be taken every 72 hours by patients with severe acute vulvovaginal candidiasis (Pappas *et al.*, 2016). Oral azole therapy is often preferred due to convenience and ease of use despite topical agents have fewer risk of systemic side effects (Vazquez and Sobel, 2002). Pregnant women are the only group exempted from oral azole therapy due to the risk of fetal toxicity and they can only be treated with topical agents (Pappas *et al.*, 2016). For recurrent vulvovaginal candidiasis patients, treatment includes an induction course of one to two weeks of 150mg oral fluconazole every 72 hours followed by a continuous maintenance treatment of fluconazole 150mg weekly for six months (Pappas *et al.*, 2016).

### 1.3.3 Invasive candidiasis

For invasive candidiasis caused by both *C. albicans* and *C. auris*, echinocandins are currently chosen as the first line treatment drug as they are equally as effective as fluconazole in the initial treatment of approximately 70-75% of cases of invasive candidiasis, according to randomized clinical trials (Bretagne *et al.*, 2021; Pappas *et al.*, 2018). An analysis of seven randomized clinical trials in around 2,000 patients with invasive candidiasis revealed that patients having echinocandin as their initial treatment have a significantly lower 30-day mortality rate compared to others having azole or amphotericin B (Andes *et al.*, 2012). Another double-blinded study based on 245 patients with systemic candidiasis showed similar results as the overall response rates were significantly higher with anidulafungin (76%) compared to fluconazole (60%) with significantly fewer persistent infections (2.7% vs 13.1%,  $p < 0.05$ ) (Reboli *et al.*, 2011).

After the initial treatment with echinocandins, a step-down therapy changing from echinocandins to azoles will be applied to treat invasive candidiasis caused by *C. albicans* as it

provides favourable results in invasive candidiasis with different organ involvement (Barantsevich and Barantsevich, 2022). It is normally started after 3 – 7 days of initial treatment depending on the results of susceptibility testing (Barantsevich and Barantsevich, 2022). Fluconazole is often the first choice as it is effective against most *Candida* species and has oral formulations with high absorption rates (>90%) from the gastrointestinal tract (Barantsevich and Barantsevich, 2022). Several clinical trials revealed that there are no differences in the 30-day survival or mycology cure rates of patients who received echinocandins throughout the whole therapy and patients who received a step-down therapy with oral azoles, based on susceptibility testing after 5 days of treatment (Kullberg *et al.*, 2017; Vazquez *et al.*, 2014).

In the case of invasive candidiasis caused by *C. auris*, lipid formulation of amphotericin B would be the main choice for step-down therapy as most *C. auris* are multidrug resistant to both azoles and echinocandins (Barantsevich and Barantsevich, 2022). However, multiple reports in recent years revealed the uprise of pan-drug-resistant *C. auris* strains that are resistant against 3 to 4 classes of antifungal agents including amphotericin B worldwide, especially in the United States (Geremia *et al.*, 2023; Jacobs *et al.*, n.d.; Ostrowsky *et al.*, 2020).

#### **1.4 Antifungal mechanisms of action**

Candidiasis caused by *Candida* spp. can be treated by different classes of drugs that target different cellular processes, leading to either inhibition of growth (fungistatic) or death (fungicidal) of these pathogenic yeasts (Bhattacharya *et al.*, 2020). Antifungal drugs mainly target the biosynthesis of the cell wall, cell membrane, and biosynthesis of RNA (Bhattacharya *et al.*, 2020). For example, terbinafine (Lamisil), which is an allylamine commonly used to treat dermatophyte infections, targets squalene epoxidase (Erg1p) in the ergosterol biosynthetic

pathway and inhibits fungal growth (Bhattacharya *et al.*, 2020). Currently, polyenes, azoles and echinocandins are the three major classes of antifungal drugs available to treat *Candida* infections, each with varying efficacy depending on the type and site of infection and the sensitivity of *Candida* species (Whaley *et al.*, 2017).

#### 1.4.1 Polyenes

Among the 3 classes of antifungals, polyenes were the first to be discovered. They are natural, amphipathic molecules that have specific antifungal properties and were introduced into the market to treat systemic fungal infections. In the 1950s, the most widely used polyene was amphotericin B which exhibits potent activity against a broad spectrum of clinically relevant fungal species, including species of *Candida*, *Aspergillus*, and *Cryptococcus* (Robbins *et al.*, 2016). In the past, polyenes were thought to directly bind to ergosterol and form drug-lipid complexes that intercalates into fungal cell membranes, thus causing leakage of intracellular components and eventually, leading to cell death (Ostrosky-Zeichner *et al.*, 2010). However, this model was being challenged by recent structural and biophysical studies, revealing that amphotericin B could form extramembranous aggregates that act like a fungicidal “sterol sponge” by extracting ergosterol from fungal cell membranes, as shown in Figure 1.2 (Anderson *et al.*, 2014). Despite its broad-spectrum antifungal activity, amphotericin B was not favoured in clinical use in the past as it has poor oral bioavailability and dose-dependent toxic effects toward the host due to the similarities between ergosterol and cholesterol (Anderson *et al.*, 2014). Fortunately, this problem was solved in the 1990s when the lipid formulations of amphotericin B were introduced with much lower nephrotoxicity (Robbins *et al.*, 2016) and thus, making these formulations an alternative treatment option in cases where antifungal availability is restricted or resistance to other more suitable antifungals precludes their utility (Pappas *et al.*, 2016). Fortunately, resistance to amphotericin B in *C. albicans* is extremely rare

due to the severe fitness costs associated with the evolution of polyene resistance (Vincent *et al.*, 2013). However, clinical isolates of *C. auris* were found to have resistance to amphotericin B ranging from 0% to 30% with preliminary evidence suggesting that there is transient and inducible amphotericin B resistance in this species (Lockhart, 2019).

#### 1.4.2 Azoles

Fluconazole, a member of the azoles, is the most prescribed antifungal used for most *Candida* infections (Whaley *et al.*, 2017). Exposure to azoles causes the accumulation of toxic sterol intermediates in *C. albicans* by inhibiting the cytochrome P450 enzyme lanosterol 14- $\alpha$ -demethylase, an enzyme involved in the biosynthesis of the fungal-specific membrane sterol ergosterol encoded by the *ERG11* gene (Whaley *et al.*, 2017) (Figure 1.2). Compared to other antifungals, many azole drugs have high levels of oral bioavailability and are available in both oral and intravenous formulations (Allen *et al.*, 2015). However, azoles have two major drawbacks including a high potential to interact with other drugs as they inhibit mammalian cytochrome P450 (Roemer and Krysan, 2014) and their fungistatic mechanism which inhibits the growth of *Candida* without killing them which promotes the development of antifungal drug resistance (Lee *et al.*, 2021).

Currently, *C. albicans* isolates are generally susceptible to fluconazole, with one large study showing only 0.3% fluconazole-resistant isolates identified in a total of 7179 isolates investigated (Pfaller *et al.*, 2019). However, significant increases in the number of azole-resistant *C. albicans* recovered in Southern Europe have been reported recently (Boattini *et al.*, 2023). For *C. auris*, most of its clinical isolates are resistant against fluconazole; for example,

a study that collected 350 clinical isolates from 10 hospitals in India showed that 90% of the isolates exhibit a certain degree of fluconazole resistance (Chowdhary *et al.*, 2018).

#### 1.4.3 Echinocandins

The most recently introduced antifungal drug class are the echinocandins, which are natural product derivatives composed of a cyclic hexapeptide core with an N-linked fatty-acyl side chain (Lee *et al.*, 2021). Echinocandin exhibits antifungal properties by disrupting the fungal cell wall via binding non-competitively to the catalytic subunit of (1,3)- $\beta$ -D-glucan synthase, which is encoded by *FKS1* in both *C. albicans* and *C. auris* (Letscher-Bru and Herbrecht, 2003) (Figure 1.2). The inhibition in the biosynthesis of (1,3)- $\beta$ -D-glucan would cause a disruption of cell wall integrity and an imbalance in osmotic pressure, resulting in a fungicidal effect (Letscher-Bru and Herbrecht, 2003). Currently, echinocandins are being used as a first-line treatment for invasive candidiasis due to their potent activity against *Candida* species (Pappas *et al.*, 2016). Even though echinocandins have higher fungal selectivity and low potential for drug-drug interactions, they exhibit poor oral absorption and being limited to intravenous administration in clinical use (Pappas *et al.*, 2016). As echinocandins were widely used, resistance to echinocandins in *C. auris* has emerged recently. The most utmost concern is the emergence of multidrug-resistant isolates of *C. auris* that are resistant to all the 3 main drug classes, meaning that these pathogens are untreatable (Chowdhary *et al.*, 2018). Fortunately, *C. albicans* are still highly susceptible to Amphotericin B (Vincent *et al.*, 2013) and echinocandins (Pfaller *et al.*, 2019).

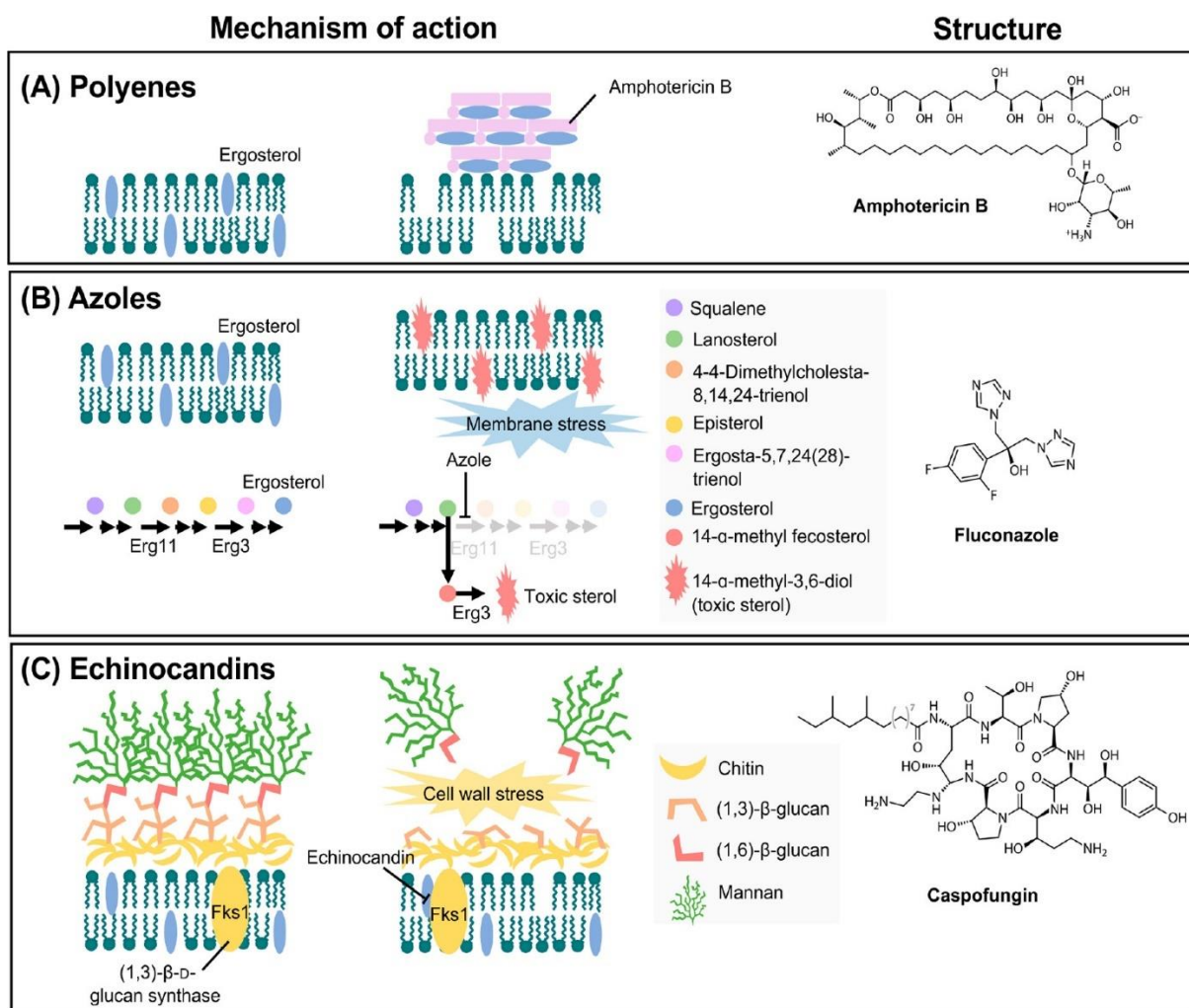


**Figure 1.2. Antifungal mechanisms of action and structures.**

(A) Polyenes like amphotericin B act as a fungicidal “sterol sponge” by forming extra-membranous aggregates that extract ergosterol from lipid bilayers. (B) Azoles exert fungistatic activity by inhibiting lanosterol 14- $\alpha$ -demethylase (encoded by *ERG11*), which inhibits ergosterol synthesis and causing the accumulation of toxic sterol intermediates, including 14- $\alpha$ -methyl-3,6-diol produced by Erg3. (C) Fungal cell walls are composed of (1,3)- $\beta$ -D-glucan covalently linked to (1,6)- $\beta$ -D-glucan, as well as chitin and mannan. Echinocandins prevent the synthesis of (1,3)- $\beta$ -D-glucan by inhibiting the (1,3)- $\beta$ -D-glucan synthase (encoded by *FKS1* in *C. albicans* and *C. auris*), resulting in a loss of cell wall integrity and severe cell wall stress. The structure of a representative antifungal from each class is depicted to the right of each mechanism. Figure adapted from Lee *et al.*, 2021.

## 1.5 Mechanism of antifungal resistance

Due to the high usage of antifungals since their discovery, many *Candida* species have developed several ways to gain resistance against these antifungals. The term resistance can be defined as a strain having a higher minimum inhibitory concentration (MIC) for a specific antifungal than the control/reference strain or above a specific clinical breakpoint that indicates the dosage of antifungal required to treat the patient (Berman and Krysan, 2020). This is opposed to the term tolerance, which refers to the ability of a drug-susceptible fungal strain to adapt and grow in the presence of a specific antifungal above the MIC (Berman and Krysan, 2020).

### 1.5.1 Resistance against polyenes

For polyenes, *C. albicans* can develop amphotericin B resistance by acquiring mutations in several biosynthesis enzymes such as *ERG2* (Jensen *et al.*, 2015), *ERG3* (Martel *et al.*, 2010), and *ERG11* (Sanglard *et al.*, 2003) as the alteration of these ergosterol biosynthetic enzymes could reduce drug-binding affinity or deplete ergosterol from the membrane (Figure 1.3). The whole genome sequencing of *C. auris* revealed that amphotericin B resistant strains have mutations in *ERG11* that might contribute to their high resistance to amphotericin B (Muñoz *et al.*, 2018).

### 1.5.1 Resistance against echinocandins

The most common antifungal resistance mechanism that *Candida* species acquires against echinocandins is the alteration of drug target (Figure 1.3) via acquisition of mutations in the *FKS* genes. Both *C. albicans* and *C. auris* acquire their echinocandin resistance through point mutations in *FKS1* (Chowdhary *et al.*, 2018; Desnos-Ollivier *et al.*, 2008), a major subunit of

glucan synthase that is the presumed target of echinocandins. Mutations in two hot spot regions in the *FKS1* gene of *C. albicans* that correspond to amino acids 641-649 (hot-spot 1) and amino acids 1357-1364 (hot-spot 2) were shown to reduce susceptibility to caspofungin, in particular serine 645 (S645) which falls within hot-spot region 1 (Perlin, 2007). In *C. auris*, a phenylalanine substitution at serine 639 (S369F) (equivalent to S645 in *C. albicans*) in the Fks1 enzyme was identified in 38 *C. auris* clinical isolates that exhibited echinocandin resistance (Chowdhary *et al.*, 2018). This mutation was further investigated and proven to be clinically relevant using an *in vivo* murine model of invasive candidiasis, showing that mice infected by *C. auris* S639F mutant did not respond to caspofungin treatment compared to its susceptible wild-type counterpart (Kordalewska *et al.*, 2018).

#### 1.5.2 Resistance against azoles

For azoles, the 3 major azole resistance mechanisms of *C. albicans* are the alteration of drug target (Erg11), the overexpression of Erg11, and the upregulation of plasma membrane efflux pumps (Figure 1.3) (Lee *et al.*, 2021). The most described acquired azole resistance mechanism in *Candida* species is the overexpression of efflux pumps. In *C. albicans*, the two main classes of efflux pumps that are highly involved in azole drug resistance are the ATP-binding cassette (ABC) superfamily and the major facilitator (MF) superfamily (Lee *et al.*, 2021). The two homologous ABC transporters, Cdr1 and Cdr2, can be overexpressed and confer azole resistance in *C. albicans* clinical strains. Among the two ABC transporters, Cdr1 is the major determinant of azole resistance as the deletion of *CDR1* in a clinical isolate reduces its azole resistance by 4- to 8- fold whereas the deletion of *CDR2* only reduces its azole resistance by 1.5- fold or less (Tsao *et al.*, 2009). The transcription factor Tac1(transcriptional activator of *CDRs*) regulates the expression of *CDR1* and *CDR2* via binding to cis-acting drug response elements (DREs) located in their promoters (Lee *et al.*, 2021). A single amino acid substitution

in the activation domain of Tac1 can also result in hyperactive alleles and lead to the overexpression of both *CDR1* and *CDR2*, ultimately contributing to azole resistance in clinical isolates of *C. albicans* (Coste *et al.*, 2006). For example, two alleles of *TAC1*, termed Tac1-5 and Tac1-7, were found to encode hyperactive Tac1 proteins that conferred fluconazole resistance, terbinafine resistance, and caused a *C. albicans* wild type strain to express high levels of Cdr1p and Cdr2p, in particular Cdr1p (Coste *et al.*, 2006). A single point mutation (N977D) known as *TAC1-5* was identified in both *TAC1* alleles of an azole resistant clinical *C. albicans* isolate. Introduction of this mutation into wild-type *C. albicans* strains confers fluconazole and terbinafine resistance due to hyperactive Tac1 activity and high Cdr1p expression (Coste *et al.*, 2006).

Among the 95 MF transporters in *C. albicans* (Gaur *et al.*, 2008), only Mdr1 (multidrug resistance 1) is implicated in fluconazole resistance (Figure 1.2) (White, 1997). The expression of *MDR1* is regulated by transcription factor Mrr1 (multidrug resistance regulator 1) as the deletion of *MRR1* eliminates *MDR1* expression and increases susceptibility to fluconazole (Morschhäuser *et al.*, 2007) whereas certain point mutations in *MRR1* also increase azole resistance (Dunkel *et al.*, 2008). The deletion of *MRR1* reduces fluconazole resistance to a greater extent than deleting *MDR1*, suggesting that *MRR1* might regulate other pathways that contributes to azole resistance (Morschhäuser *et al.*, 2007). Recently, the *C. albicans* Swi/Snf chromatin remodelling complex was implicated as a major coactivator of Mrr1 as the deletion of *SNF2* (catalytic subunit of the Swi/Snf complex) in *MRR1* gain-of-function strains drastically reduced the activation of Mdr1 and fluconazole resistance (Liu and Myers, 2017a).

Phylogenetic analysis revealed that *C. auris* can encode for many transporters orthologous to those in *C. albicans* that belongs to the ABC and MF families like *MDR1*, *CDR1/2* and *CDR4* (Muñoz *et al.*, 2018). A study by Rybak in 2019 showed that both *CDR1* and *MDR1* were overexpressed in azole-resistant *C. auris* isolates but only the deletion of *CDR1* led to significant reductions in azole MIC values, indicating that this efflux pump is important for azole resistance in *C. auris* (Rybak *et al.*, 2019). The importance of efflux pumps was further confirmed in a separate study where the deletion of *CDR1* conferred an 8-fold increase in fluconazole sensitivity (Kim *et al.*, 2019). Apart from drug transporters, *C. auris* has numerous transcription factors including 2 copies each of *TAC1* and *MRR1* (Muñoz *et al.*, 2018), suggesting that *C. auris* may have a different transcriptional regulatory machinery that enables efflux pumps to be transcribed at a higher constitutive level.

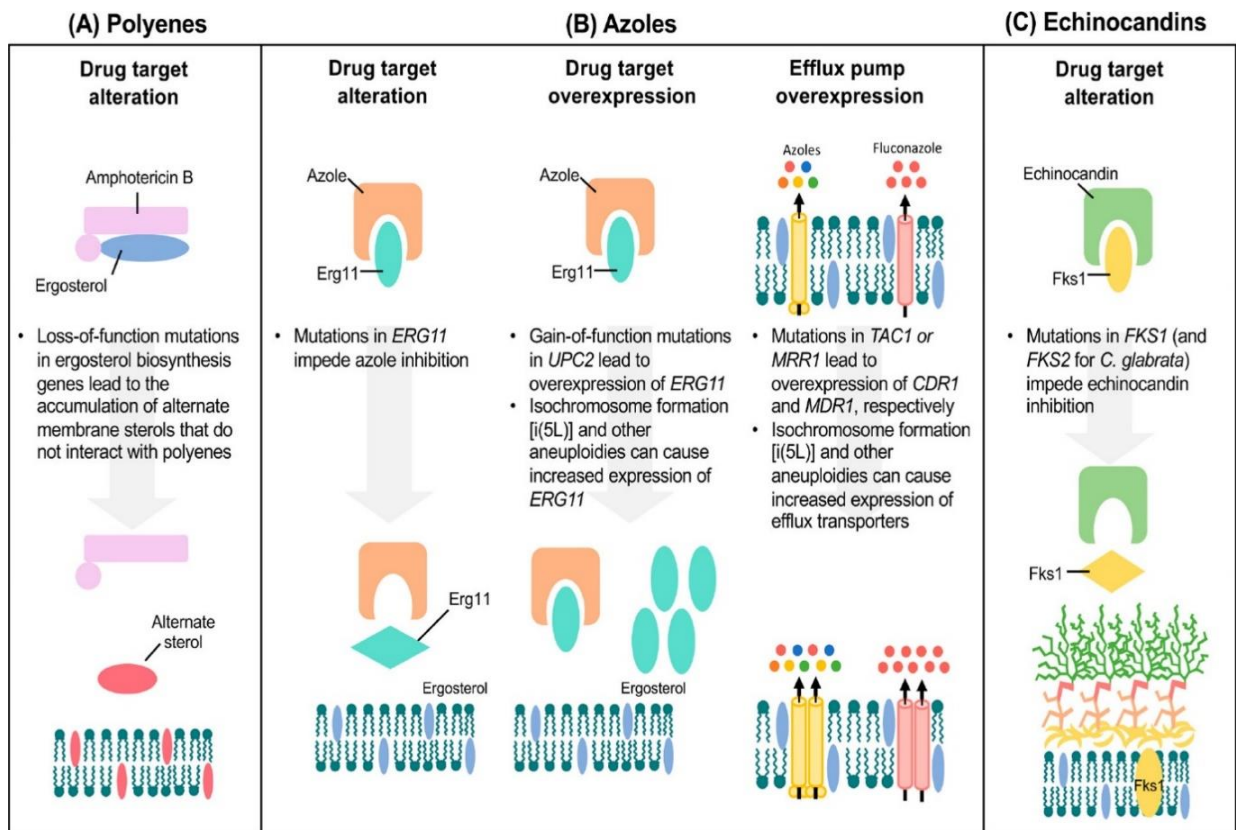


**Figure 1.3. Molecular mechanisms of antifungal drug resistance.**

(A) Resistance to polyenes is primarily mediated by the alteration of ergosterol through loss-of-function mutations in ergosterol biosynthesis genes. This leads to the production of sterols with alternative structures that could not be recognized by polyenes and therefore are not extracted from the fungal cell membrane. (B) Resistance to azoles can occur through substitutions to the azole target, Erg11, which lowers the drug-binding affinity for the lanosterol demethylase enzyme (left panel). Overexpression of the drug target can occur through gain-of-function mutations in the transcriptional activator, *UPC2*, or through the formation of aneuploidies, such as [i(5L)], which directly increase the copy number of *ERG11* (middle panel). Most azole resistance is acquired through the upregulation of ABC transporters (yellow), in particularly Cdr1 and Cdr2, by activating mutations in specific transcription factors (*TAC1* in *C. albicans* and *C. auris*). Furthermore, overexpression of the MF transporter (pink), Mdr1, through activating mutations in the transcriptional factor, *MRR1*, confers azole resistance (right panel). Efflux pumps can also be overexpressed through aneuploidy formation. (C) Resistance to echinocandins primarily involves mutations in *FKS* genes that encode the catalytic subunit of the drug target (1,3)- $\beta$ -D-glucan synthase. For *C. albicans* and *C. auris*, mutations conferring echinocandin resistance occur in *FKS1*, while for *C. glabrata*, mutations occur in both *FKS1* and its paralogue *FKS2*. Adapted from Lee *et al.*, 2021.

## 1.6 Distinct fungal virulence of *C. albicans* and *C. auris*

*C. albicans* is a highly adapted commensal and fungal pathogen that can switch morphology from yeast to pseudo-hyphal or true hyphal forms *in vitro* and *in vivo*, depending on the surrounding environmental conditions (Chen *et al.*, 2020). The plasticity of the mycelial form is a major factor that contributes to the virulence of *Candida* species (Thomson *et al.*, 2015). However, in the case of *C. auris*, it does not form filaments when grown *in vitro* under similar conditions that induce hyphal growth in *C. albicans* (Wang *et al.*, 2018) and can only form pseudohyphae under specific growth conditions such as the presence of high salt concentrations (10% NaCl) (Wang *et al.*, 2018) and following the inhibition of *HSP90* (Kim *et al.*, 2019). Recent studies on *C. auris* revealed that it can undergo a tristable phenotypic switch between typical yeast, filamentation-competent yeast, and filamentous cells after passaging through murine models of systemic candidiasis (Yue *et al.*, 2018).

The other unique switching morphological phenotype of *C. auris* is its ability to form aggregates. Certain strains/clades of *C. auris* can switch between the single-cell (non-aggregated) form and the aggregated form. Aggregates are generally more tolerant to antifungal drugs than their non-aggregated form, although they exhibit lower virulence *in vivo* in both *Galleria mellonella* and *Caenorhabditis elegans* models (Hernando-Ortiz *et al.*, 2021). However, aggregates did show higher capacity to form biofilms in those two invertebrate infection models (Hernando-Ortiz *et al.*, 2021). A recent study showed that aggregation could be induced by media or the presence of antifungal drugs and aggregation seems to benefit long-term persistence of fungal infection in the host, as macrophages could not effectively clear out aggregates (Pelletier *et al.*, 2024). Until now, three distinct mechanisms of aggregation have been unravelled which are (1) adhesin-mediated aggregation; (2) cell-separation defects; and (3) extracellular matrix aggregation (Santana *et al.*, 2024). The secretion of cell surface proteins

allows cell-cell adhesion that drives the formation of aggregation in *C. auris*. Some *C. auris* strains have cell separation defects which caused them to aggregate and others might form extracellular matrix that attached cells together to form aggregates.

Biofilm growth is an important characteristic in pathogenic yeast as it allows colonisation of abiotic surfaces and facilitates infections associated with medical devices and intravenous catheters. Although both *C. albicans* and *C. auris* can form adherent biofilms, their cellular morphology in biofilms differs to each other. The biofilms of *C. albicans* are normally densely packed with hyphal cells whereas the biofilms of *C. auris* consists of mainly yeast cells both *in vivo* and *in vitro* (Bing *et al.*, 2023; Gulati and Nobile, 2016; Horton *et al.*, 2020; Sherry *et al.*, 2017). Interestingly, even though *C. albicans* can form a higher biofilm density than *C. auris* on most abiotic surfaces (Sherry *et al.*, 2017), *C. auris* can form biofilms with 10-fold greater burden than *C. albicans* in synthetic sweat medium on abiotic surfaces (Horton *et al.*, 2020). *C. auris* was also shown to form biofilms with high density on porcine skin *ex vivo* compared to *C. albicans*, which was higher again in the presence of synthetic sweat medium (Horton *et al.*, 2020). The differences between *C. albicans* and *C. auris* biofilm formation suggest that *C. auris* might express its adhesins under specific conditions or produce adhesins with functions that differ to *C. albicans*. Currently, several adhesins in *C. auris* (e.g. Scf1, Als4) have been found to be required for both its aggregation and biofilm formation on both abiotic and skin surfaces (Bing *et al.*, 2023; Louvet *et al.*, 2024; Santana *et al.*, 2023).

## 1.7 The role of Mediator in yeast pathogenesis

Like all other eukaryotes, pathogenic fungi like *C. albicans* have evolved a complex process to initiate the transcription of genes (Moran *et al.*, 2019). Mediator acts as a conserved central co-regulator of RNA polymerase II (Pol II)-mediated transcription across eukaryotes and is crucial for the expression of protein coding and non-coding RNA genes (Soutourina, 2018). It is a multi-subunit protein that controls transcription through various mechanisms such as acting as a scaffold for the assembly of the pre-initiation complex (PIC), facilitating phosphorylation of the C-terminal domain of Pol II, and the remodelling of chromatin (Jeronimo and Robert, 2017). Since Mediator is highly involved in various crucial cellular functions, Mediator subunits of fungal pathogens have been suggested as potential therapeutic targets to treat antifungal resistance of *C. albicans* (Moran *et al.*, 2019).

In *Candida spp.*, Mediator is divided into structurally distinct modules like in *Saccharomyces cerevisiae*, referred to as tail, middle, head, and Cdk8 (Figure 1.4) (Moran *et al.*, 2019). The combination of tail, middle and head is usually referred as the “core” of Mediator (Tsai *et al.*, 2014). Even though the general function of Mediator is conserved among eukaryotes, Mediator composition differs between and within species (Allen and Taatjes, 2015). These different Mediator subunits (especially the tail and kinase modules) acquired gene specific functions by having a unique composition and therefore, can mediate the expression of specific subsets of genes in different cells and/or tissues (Allen and Taatjes, 2015).

As the most clinically relevant fungus in humans, *C. albicans* thrives in the human body due to its ability to form adherent biofilms and invasive hyphae and to resist various environmental and nutrient stresses (Moran *et al.*, 2019). In 2012, Uwamahoro *et al.* deleted the genes encoding *CaMED31* (from the middle module), *CaMED20* (from the head module), and

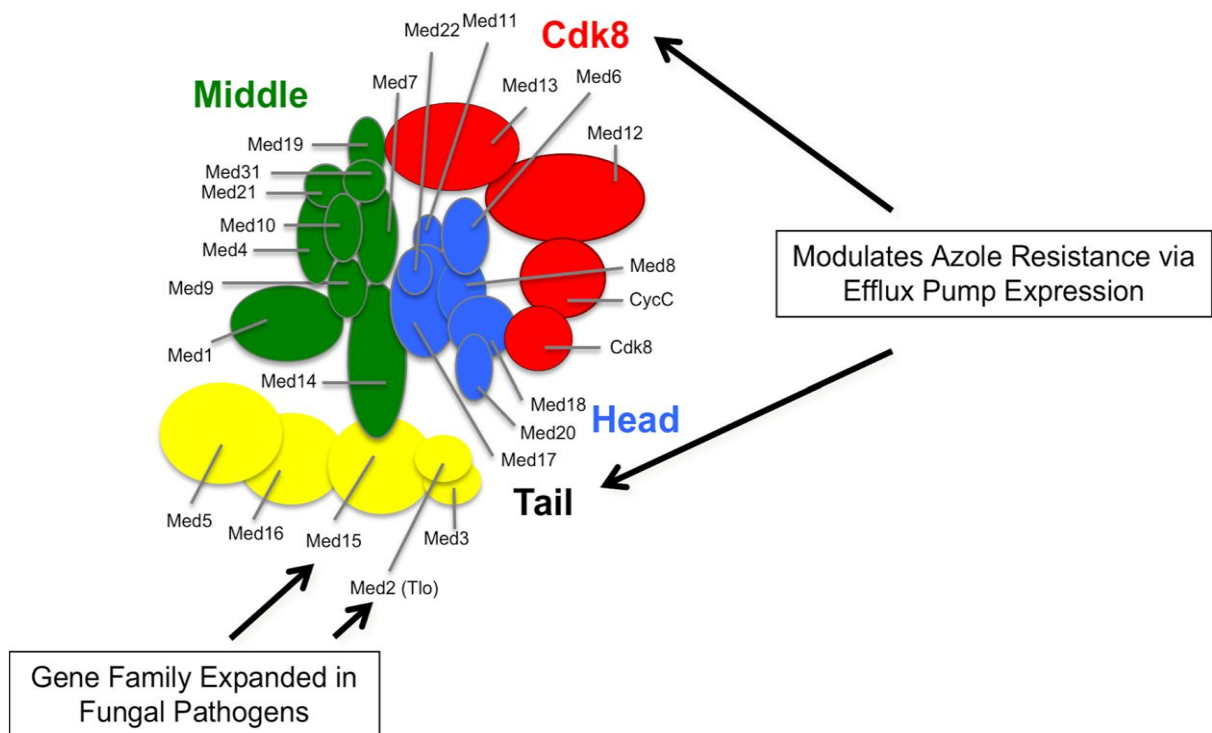


**Figure 1.4. Diagram illustrating the domain structure of mediator in fungal pathogens.**

Individual subunits are colour coded to indicate their location in the Head (blue), Middle (green), Tail (yellow) or Cdk8/kinase (Red) module (Moran *et al.*, 2019).

*CaMED13* (from the kinase module) in *C. albicans*. The loss of these three genes largely affected a variety of phenotypes such as hypha and biofilm formation which are both important for fungal virulence (Uwamahoro *et al.*, 2012). The Med31 and Med13 subunits of *S. cerevisiae* repress genes required for adhesion (the *FLO* family) whereas *C. albicans* requires these orthologous proteins for transcriptional activation of the most potent adhesins (the ALS family) expressed by this species (Uwamahoro *et al.*, 2012). This discovery supports the evolution of species- and gene-specific functions within mediator (Uwamahoro *et al.*, 2012). Similar research focusing on Mediator components carried out by Zhang *et al.* in 2012 revealed the biochemical structure of *C. albicans* Mediator and showed that deletion of *C. albicans* *CaMED3* caused defective assembly of Mediator tail and thus, greatly reduced its filamentous growth. A later *in vivo* study confirmed that a *Δcamed3* deletion mutant was avirulent in a murine model of systemic candidiasis (Liu *et al.*, 2016). A comprehensive study also identified defects in the white-opaque phenotypic switch that is responsible for the virulence and environmental adaptation of *C. albicans* by deleting eight Mediator subunit-encoding genes (i.e. *CaMED3*, *CaMED5*, *CaMED15* and *CaMED16* [tail]; *CaMED1* and *CaMED9* [middle]; *CaMED20* [head] and *CaMED12* [kinase]) further supporting the role of Mediator in virulence and mating conferred by the opaque cell state (Zhang *et al.*, 2013).

Deletion of Med7 (essential for viability in *S. cerevisiae*) in *C. albicans* impairs biofilm formation, hypha formation, and the ability to colonize the GI tract of mice (Tebbj *et al.*, 2014). Further chromatin immunoprecipitation (ChIP) analysis revealed that Med7 is involved in regulating the expression of glycolytic genes (Tebbj *et al.*, 2014). Med7 was shown to regulate the fungal gene regulator (*FGR*) family associated with hyphal growth and was further shown to have higher binding levels in hyphal cells, correlating with increased transcriptional activity (Tebbj *et al.*, 2014). Med7 was shown to bind the *WOR1* and *WOR2* genes, suggesting a

possible role in white-opaque switching as well (Tebbj *et al.*, 2014). Deletion of other Mediator complex subunits in *C. albicans* such as *MED3*, *MED15* and *MED12* also negatively impairs the phenotypic switching from white to opaque (Zhang *et al.*, 2013). Furthermore, the deletion of *MED3* in *C. albicans* impairs its filamentous growth and the ability to cause systemic candidiasis in mice (Fletcher *et al.*, 2023).

Deletion of Mediator tail component *MED15* in *Candida lusitanae* (another opportunistic *Candida* pathogen) resulted in defective cell separation, hyphal growth and mating (Sabra *et al.*, 2023). Proteomic analysis showed that the deletion of *C. lusitanae MED15* led to the upregulation of stress-response genes and genes involved in alternative carbon source utilisation (Sabra *et al.*, 2023). The same study also concluded that *C. lusitanae MED15* might contribute to its wild-type level of virulence (Sabra *et al.*, 2023). In *Aspergillus fumigatus*, the downregulation of *MED15* impairs the conidiation and virulence of *A. fumigatus* in the *Galleria mellonella* wax moth infection model (Guan *et al.*, 2020). Interestingly, the overexpression of *MED15* was lethal for this fungus, possibly due to the dysregulation of genes involved in calcium and iron uptake (Guan *et al.*, 2020).

Conclusively, all these studies showed that Mediator is responsible for regulating various phenotypes in *C. albicans*, and in many studies is shown to be directly required for its virulence. For *C. auris*, since it has only been discovered quite recently in 2009, the role of Mediator tail subunits in its antifungal and virulence properties remain unknown and require more studies to determine its function.

## 1.8 The role of Mediator in the response of yeast to antifungal drugs

The Mediator of yeast (in particularly *Candida* spp.) was discovered to have a major role in regulating various antifungal resistance related mechanisms. One of the earliest involvements of Mediator in antifungal resistance mechanisms was discovered in *S. cerevisiae*. These studies revealed that the ScPdr1/3-dependant recruitment of Mediator to the DRE (Drug Response Element) in the promoter region of *ScPDR5* (encoding an ABC transporter) can be activated in response to gain of function mutations in Pdr1, induction by azole drugs and loss of the mitochondrial genome (Gao *et al.*, 2004; Simonicova and Moye-Rowley, 2020). The upregulation of the ScPdr5 efflux pump was shown to be dependent on the Mediator tail component ScMed15 (Thakur *et al.*, 2008). The exact same mechanism also exists in another pathogenic yeast, *C. glabrata*. CgMed15A, a ScMed15 ortholog, along with its transcription factor CgPdr1, directly regulated the expression of the ABC transporter encoding genes *CgCDR1* and *CgCDR2* (orthologs of *ScPDR5*) (Thakur *et al.*, 2008). The upregulation of these transporters, in particularly CgCdr1, increase azole resistance in *C. glabrata* (Paul *et al.*, 2011). However, the other *MED15* ortholog in *C. glabrata*, *CgMED15B*, was not involved in either ABC transporter expression or azole resistance (Thakur *et al.*, 2008; Paul *et al.*, 2011). These findings in *C. glabrata* were supported by a later study showing that the inhibition of the interaction between CgMed15A and CgPdr1 using a small molecule inhibitor *iKIX1* could result in a reduction of fluconazole induced *CgCDR1* and *CgCDR2* expression, and azole resistance (Nishikawa *et al.*, 2016). The other Mediator tail module subunit CgMed2 was also shown to affect CgPdr1-activated *CgCDR1* and *CgCDR2* expression, but to a weaker extent compared with CgMed15A (Borah *et al.*, 2014). A more recent study showed that CgPdr1-dependent transactivation was mediated by a complex network of transcriptional coactivators, including cgMed15A, interacting with the extreme C-terminal part of CgPdr1 (Simonicova and Moye-Rowley, 2020).

A study on *C. albicans* revealed that 2 Mediator tail subunits, Med3 and Med15, are both required for Tac1-activated azole resistance. Both Med3 and Med15 were equally required for the induction of *CaCDR1* expression under the control of Tac1 in response to xenobiotics or in the presence of hyperactive Tac1 protein and thus azole resistance in *C. albicans* (Liu and Myers, 2017b). Another subunit of the Mediator kinase module CaSSN3 was also shown to be involved in azole resistance (Liu and Myers, 2017a).

The Mediator of *C. albicans* was also found to interact with the transcription factor CaAce2 and the *CaERG* genes responsible for sterol biosynthesis in *C. albicans*. The alteration of sterol biosynthesis gene expression, such as *ERG251* and *ERG6*, is often linked with azole resistance in *Candida* species (Elias *et al.*, 2022; Lu *et al.*, 2023). CaAce2 is a transcription factor that regulates expression of genes with roles in cellular processes such as hyphal development, nutrient metabolism, and cellular respiration (Mulhern *et al.*, 2006). Depending on the cellular morphology of *C. albicans*, the deletion of CaAce2 affects *CaERG* gene expression differently. Hyphal cells without CaAce2 showed decrease in *Erg11* and *Erg251* whereas yeast cells without CaAce2 showed increase in *Erg2*, *Erg9*, *Erg10* and *Erg24* (Mulhern *et al.*, 2006). Gene set enrichment analysis (GSEA) revealed similarities in gene expression between *med31ΔΔ* and *ace2ΔΔ* mutants, suggesting that Med31 might interact with Ace2 to mediate transcriptional control in a subset of genes, especially cytokinesis genes. A *med31ΔΔ* mutant also showed downregulation of genes required for cell wall stability and altered cell wall composition, resulting in increased sensitivity to the ergosterol targeting polyene drug nystatin (Uwamahoro *et al.*, 2012). CaMed7, which is responsible for the virulence of *C. albicans*, was shown to interact with CaAce2, *CaErg6* and *CaErg25* (Tebbj *et al.*, 2014). This finding suggests that CaMed7 might have a role in Ace2-regulated control of ergosterol biosynthesis genes. Further evidence for the role of Mediator in regulating ergosterol biosynthesis was presented by

(O'Connor-Moneley *et al.*, 2024) who showed that the *tlo* mutant had altered expression of *ERG* genes which might contribute to azole tolerance in the mutant.

Several studies in *C. glabrata* further support the role of Mediator in ergosterol biosynthesis. The deletion of CgMed3 (encode by CgMEDA and CgMEDB) in *C. glabrata* resulted in increased expression of *HMG1*, *ERG10*, *ERG11*, *ERG3*, and *ERG4* (Lin *et al.*, 2017). The changes in expression of these sterol biosynthetic and sterol pathway related genes lead to accumulation of squalene and lanosterol and a decrease in zymosterol, fecosterol and ergosterol in the plasma membrane compared to the wild-type strain (Lin *et al.*, 2017). CgMED15B, which was not involved in ABC transporter expression, was later found to have a role in maintaining lipid metabolism and ergosterol biosynthesis homeostasis (Qi *et al.*, 2017). The deletion of CgMED15B leads to high expression of multiple ergosterol biosynthetic genes such as lanosterol 14- $\alpha$ -demethylase (*ERG11*), methylsterol monooxygenase (*ERG2*), and C-5 sterol desaturase (*ERG3*). All these changes resulted in an increase in zymosterol in the membrane and reduced synthesis of fecosterol and ergosterol, indicating major changes in membrane sterol components (Qi *et al.*, 2017). Transcriptomics revealed that the deletion of CgMED15B led to changes in lipid metabolism, suggesting changes in the structure and architecture of plasma membrane due to altered membrane lipid composition. These changes were found to ultimately influence membrane integrity, fluidity, and H<sup>+</sup>-ATPase activity (Qi *et al.*, 2017).

## 1.9 The expanded *TLO* gene family in *C. albicans*

*C. albicans* has a family of genes known as the *TLO* (Telomere-associated) genes situated close to the telomeres of each chromosome (Zhang *et al.*, 2012). Since their discovery, it has been established that the *TLO* genes encode multiple *MED2* (mediator tail subunit) paralogs. Comparative genomics revealed that most *C. albicans* strains have between 10 to 15 distinct *TLO* paralogs whereas other *Candida* species typically have a single *MED2* gene. The closely related, low-pathogenicity species *C. dubliniensis* is slightly unusual as it has two *MED2* genes (*CdTLO1* and *CdTLO2*). The two *C. dubliniensis* *MED2* paralogs only share 58% identity and the expression of *TLO1* is much higher than *TLO2* (Haran *et al.*, 2014). Despite all *Tlos* having a conserved Med-2 like domain, the 14 *TLO* genes in the *C. albicans* reference strain SC5314 can be separated into three distinct clades, which are the  $\alpha$ -,  $\beta$ - and  $\gamma$ -clades, largely based on the presence of long terminal repeat (LTR) insertions in the C-terminal domain of the genes (Figure 1.5A) (Anderson *et al.*, 2012). In SC5314,  $\gamma$  clade (n=7) is the largest *TLO* clade having the shortest *TLO* genes at around 525bp and is the only *TLO* clade that has undergone alternative splicing (Figure 1.5B). The  $\alpha$  clade of *TLOs* (n=5) have a length between 675 and 750bp whereas the single  $\beta$  clade gene has a length of 822bp (Figure 3B). *TLO* genes are highly similar to each other as *TLO* genes within a clade can share up to 97% amino acid sequence similarity and exhibit approximately 82% identity between clades. It was suggested that all *Tlo* proteins mainly localized to the nucleus except for  $\gamma$  clade *Tlos* which sometimes produce unspliced gene products that localize within the mitochondria as well as the nuclei. The expression of the *TLO* $\alpha$  clade mRNA is the highest whereas *TLO* $\gamma$  has a much lower expression level compared to the other two clades. The tertiary structure predicted by the AlphaFold structure prediction tool suggests that all *Tlos* are largely  $\alpha$  helical in structure with a less structured C-terminal domain (absent in *TLO* $\gamma$ ) (Liu and Myers, 2015; Varadi *et al.*, 2021; O'Connor-Moneley *et al.*, 2023).

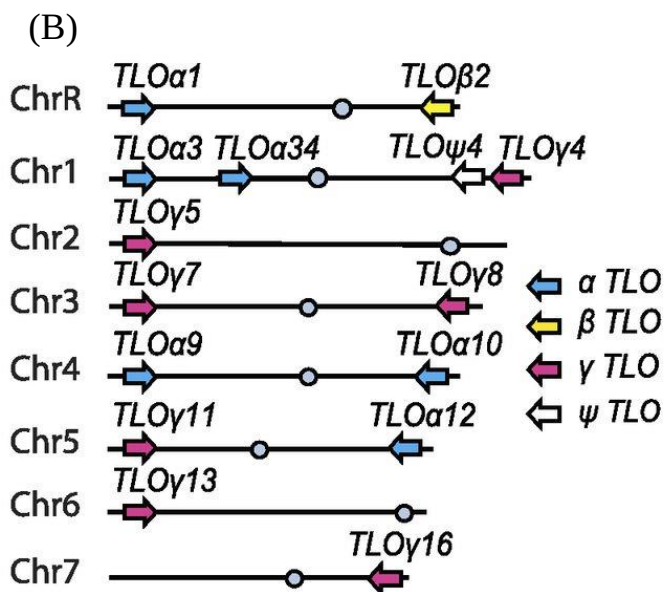
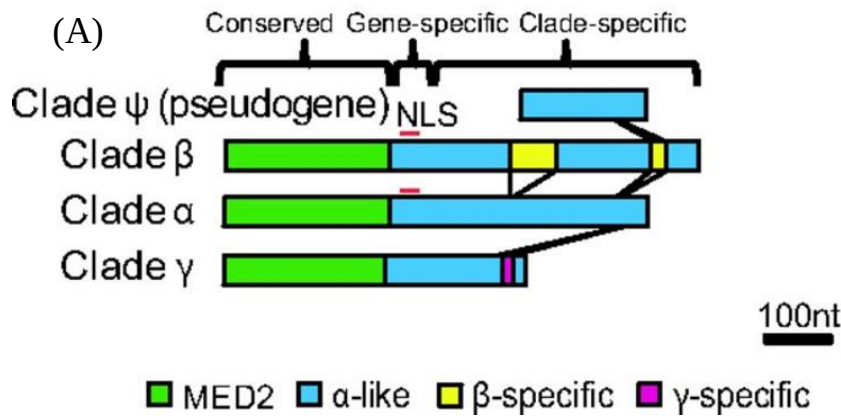


**Figure 1.5. Structures and locations of the *TLO* genes in *C. albicans***

A) *TLO* genes are divided into three clades ( $\alpha$ ,  $\beta$  and  $\gamma$ ) based on the presence of long terminal repeat (LTR) insertions in the C-terminal domains. All *TLO*s (except for a pseudogene) consist of a *MED2*-like domain (green) at the N-terminus. Family specific domains are indicated by colour ( $\alpha$ - blue,  $\beta$ - green,  $\gamma$ -pink). The pseudogene *TLO $\psi$ 4* has no *MED2*-like domain but has a C-terminus homology to *TLO*  $\alpha$ -like domain. The predicted nuclear localisation sequence located in  $\alpha$  and  $\beta$  *TLO* clade was written as NLS and indicated by a red line. B) The complete *TLO* genetic mapping across the *C. albicans* SC5314 genome. Each *TLO* clade is represented by coloured arrows ( $\alpha$  *TLO* – blue,  $\beta$  *TLO* – yellow,  $\gamma$  *TLO* – red,  $\psi$  *TLO* - white) and grey circles represents centromeres. Adapted from (Anderson *et al.*, 2012).

Given the highly expanded nature of the *TLO* gene family in *C. albicans*, studies have shown that there is a high level of Tlo protein produced, resulting in an excess ‘Mediator-free’ population of Tlo proteins in addition to the Mediator-associated population in *C. albicans* (Zhang *et al.*, 2012). However, a recent study showed that *C. albicans* reference strain SC5314 contains a non-functioning RNAi system, which when repaired results in a significant decrease in *TLO* gene expression, suggesting that the amount of free Tlo proteins present in SC5314 could be an artefact of this defective RNAi regulation (Iracane *et al.*, 2024). Further studies are required to determine whether Mediator-free Tlo is truly a phenomenon in *C. albicans* or just an unusual feature of strain SC5314.

Comparative genomic studies have suggested that the *C. albicans* *TLO* gene family may have expanded from one ancestral *TLO* gene (possibly *TLO $\beta$ 2*) through sub-telomeric recombination events (Jackson *et al.*, 2009). A microbial evolutionary-based study revealed that sub-telomeric recombination and loss of heterozygosity can affect the copy number and clade representation of *TLOs* in *C. albicans* (Anderson *et al.*, 2015). A subsequent *C. albicans* *TLO* genomic sequencing study confirmed that *TLO* gene copy number can vary between *C. albicans* strains providing further evidence suggesting that the *TLO* gene family is continuing to rapidly evolve (Hirakawa *et al.*, 2015).

The expansion of the large *TLO* gene family in *C. albicans* has been suggested to be linked with its success as a highly adapted human pathogen. The widely expanded Mediator complexes are hypothesized to interact with multiple virulence-associated genes and regulate them to adapt, colonize and cause diseases in humans. Tlo was first shown to have a role in pathogenicity in the closely related species *C. dubliniensis* (Cd*TLO1* and Cd*TLO2*) as the deletion of both genes

resulted in a mutant that was defective in various virulence-associated traits, including the capacity to produce hyphae and to tolerate oxidative stress (Haran *et al.*, 2014). In support of this, the expansion of the *TLO* gene family in *C. dubliniensis* by ectopic introduction of the *C. albicans* *TLO* $\beta$ 2, *TLO* $\gamma$ 11 and *TLO* $\alpha$ 12 genes led to an increase in virulence in the wax moth (*G. mellonella*) infection model, suggesting that the expansion of *TLO*s conferred increased virulence (Flanagan *et al.*, 2018). Recent studies in *C. albicans* revealed that the deletion of the entire *TLO* gene family produced a mutant with defective virulence traits such as reduced growth rates, the inability to form hyphae, and reduced virulence in the wax moth infection model (Fletcher *et al.*, 2023). The complementation of the mutant with individual *TLO* $\alpha$ 1, *TLO* $\beta$ 2 and *TLO* $\gamma$ 11 constructs resulted in different restoration levels in various virulence phenotypes except for *TLO* $\gamma$ 11, having no restoration effect (Fletcher *et al.*, 2023).

### **1.10 Mediator and Drug Resistance of *C. albicans***

As previously mentioned, one of the major antifungal drug resistance mechanism of *C. albicans* is the upregulation of efflux pump genes to reduce the level of intracellular drug accumulation. Mediator has been shown to play an important role in this mechanism (Moran *et al.*, 2019). The hyperactivation of transcription factors, which is the major cause of the increase of efflux pump gene expression, may increase recruitment of Mediator and associated co-activators to the promoters of the genes encoding efflux pumps (Moran *et al.*, 2019). The transcriptional activator of the *CDR1* and *CDR2* genes, Tac1, may become hyperactivated due to the presence of certain xenobiotics (e.g. fluphenazine) or due to gain-of-function mutations in its transcriptional activating domain (Liu and Myers, 2017b). Mediator tail region, in particularly the Med15 subunit, is required for azole resistance and the activation of *CDR1* by several different *TAC1* gain of function mutations (Moran *et al.*, 2019). *CDR1* expression is equally dependent on the Med3 tail module subunit in *C. albicans* and Med3 is required for the

recruitment of Mediator to the *CDR1* promoter by the Tac1 transcriptional activation domain (Liu and Myers, 2017b) (Figure 1.6). The tail module is also required for the activation of *CDR1* by fluphenazine, although this xenobiotic activation is weaker than the gain of function mutations (Liu and Myers, 2017b).

Studies on Mediator structure in *C. albicans* all suggest that *TLO* genes in *C. albicans* are responsible for the production of the Mediator tail subunit similar to Med2 in other yeasts, thus suggesting a role similar to other Mediator tail subunits (Med3 and Med15) in regulating fungal pathogenesis and antifungal drug resistance (Nishikawa *et al.*, 2016). This could possibly make them a potential drug target for Mediator targeted drugs to modulate fungal virulence and drug susceptibility (Nishikawa *et al.*, 2016). Even though various studies mentioned above indicate that Tlo and Mediator subunits are crucial for various phenotypic and virulence traits of *C. albicans*, further in-depth studies are required to reveal how Tlo protein and Mediator subunits work together to regulate genes that are responsible for antifungal resistance of *C. albicans*. Hence, this study will focus on investigating the role of *C. albicans* *TLO* genes in *TAC1*-mediated fluconazole resistance. For *C. auris*, since the function of Mediator tail subunits remains unknown, this study will also investigate the role of individual Mediator tail subunits in the virulence and antifungal properties of multidrug-resistant *C. auris* isolates by optimizing a novel CRISPR method developed by Ennis *et al.* (2021).

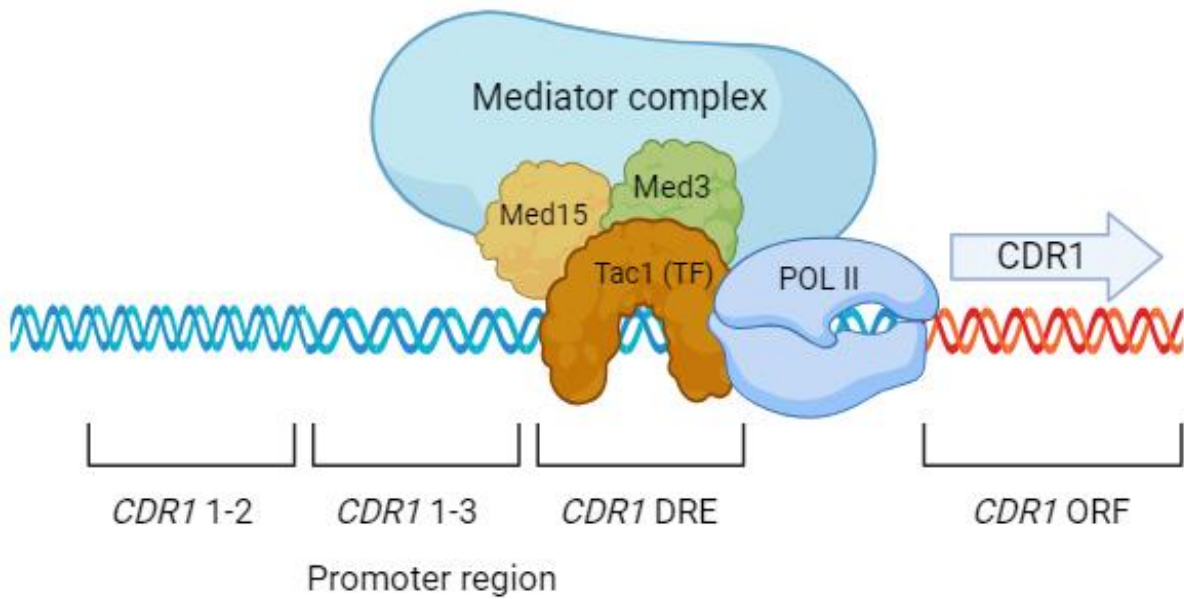


**Figure 1.6. A model of the interaction of Mediator tail with Tac1 and other components at the *CDR1* promoter.**

Three *CDR1* promoter regions described by Liu and Myers (2017b) are labelled upstream of the *CDR1* ORF. Protein components shown to interact with the promoter using ChIP are indicated including Med3 and Med15 which mediate the interaction of hyperactive Tac1 at the *CDR1* DRE site with RNA polymerase II (Pol II) to initiate *CDR1* expression.

### 1.11 Aims of this study

This study aimed to investigate the role of Mediator tail in *C. albicans* and *C. auris* in drug resistance and in the case of *C. auris*, to also carry out the first investigations of Mediator tail and virulence.

Specifically, I aimed to:

- Investigate the role of the *C. albicans* *TLO* gene family in *TAC1*-activated fluconazole resistance.
- Investigate if individual *TLO* genes from 2 major different clades ( $\alpha$  and  $\beta$ ) differ in their ability to mediate fluconazole and terbinafine resistance.
- Optimise a CRISPR-Cas9 method (Ennis *et al.*, 2021) for use in *C. auris* clinical strains.
- Delete Mediator tail subunits in *C. auris* and assess their contribution to drug resistance and virulence.

## **Chapter 2**

### General Materials and Methods

## **2.1 General Materials and Methods**

### **2.1.1 Buffers and solutions**

All buffers and solutions used in this study were molecular biology grade. Sterile ultrapure Milli-Q Biocel purified water (Millipore, Carrigtohill, Cork, Ireland) was used to make up all solutions and buffers. DNA and oligonucleotides were diluted in molecular biology grade water (Sigma-Aldrich, Arklow, Co. Wicklow, Ireland) and used in all molecular reactions (e.g. polymerase chain reaction, CRISPR-Cas9 editing, etc.). Phosphate Buffer Saline (PBS) 1X stock was made from Oxoid tablets (Oxoid, Basingstoke, Hampshire, UK) and Tris acetate EDTA (TAE) 10X buffer was made from Tris-HCL base (Sigma-Aldrich), EDTA powder (Sigma-Aldrich), and acetic acid solution (Sigma-Aldrich). 10X TAE was further diluted into 1X TAE buffer for running gel electrophoresis and dissolving agarose (Sigma-Aldrich).

### **2.1.2 Chemicals, enzymes, antibiotics and antifungals**

Chemicals were obtained from Roche (Roche Products [Ireland] Limited, Citywest, Dublin 24, Ireland), Promega (Promega Corporation, Madison, Wisconsin, USA), Sigma-Aldrich (Arklow, Co. Wicklow, Ireland), Bioline (London, United Kingdom) and Life Technologies (Dun Laoghaire, Dublin, Ireland). Enzymes, including restriction-endonucleases, were obtained from Promega and New England Biolabs (Ipswich, Massachusetts, USA). All antibiotics and antifungals were obtained from Sigma-Aldrich except for Nourseothricin (Werner Bioagents [Jena, Germany]). All items listed above were stored and used following the given manufacturer's guidelines.

### 2.1.3 Oligonucleotides

All oligonucleotides were obtained from Sigma-Aldrich, unless otherwise stated. The sequence of all oligonucleotides used in this study was listed in Table 2.1. Prior to use, 100 $\mu$ M stocks were diluted to 10 $\mu$ M in Molecular Biology grade water, unless otherwise stated.

**Table 2.1.** Sequences of oligonucleotides used in this study.

| <b>Name</b>                      | <b>Sequence (5'-3')</b>                                                         |
|----------------------------------|---------------------------------------------------------------------------------|
| <b>qRT-PCR</b>                   |                                                                                 |
| ACT1 F                           | AGCTCCAGAAGCTTTGTTTCAGACCAG                                                     |
| ACT1 R                           | TGCATACGTTTCAGCAATACCTGGG                                                       |
| RT CDR1 F                        | TGTGCTGAACGTGAATATGTTT                                                          |
| RT CDR1 R                        | TTGGTGGAACCTCATTTGACAG                                                          |
| CaTLO1 qPCR F                    | ACTAGCCCCAACAACGAACT                                                            |
| CaTLO1 qPCR R                    | CATAACGCCGAGACACCACT                                                            |
| CaTLO2 qPCR F                    | TCAACGACATGCAGAACGAC                                                            |
| CaTLO2 qPCR R                    | TCATGTCCAAGTCGCTGTCT                                                            |
|                                  |                                                                                 |
| <b><i>C. albicans</i> CRISPR</b> |                                                                                 |
| AHO1096                          | GACGGCACGGCCACGCGTTTAAACCGCC                                                    |
| AHO1098                          | CAAATTA AAAAATAGTTTACGCAAG                                                      |
| AHO1097                          | CCCGCCAGGCGCTGGGGTTTAAACACCG                                                    |
| TAC1-5 gRNA                      | CGTAACTATTTTTAATTTG <b>ACAATAATTTGGGGA</b><br><b>TTTAAG</b> TTTTAGAGCTAGAAATAGC |
| TAC1-5 Repair F                  | TTTAGTCAATTTAATAATTTACCCAATTTTTTCT<br>TTGACGATAATTTGGGGATTAAAGT                 |
| TAC1-5 Repair R                  | TATACATCGCTTTCACCAATTACAACCTCTTTTTT<br>AACACTTTAAATCCCCAAATTATCGT               |
|                                  |                                                                                 |
| <b>TAC1-5 Sanger-sequencing</b>  |                                                                                 |
| TAC1 ampF                        | AACTCAACTGGAGAGCCTCAAG                                                          |
| TAC1-5R                          | AAAGTCATCTTCTTGCTTAC                                                            |

|                                    |                                                                  |
|------------------------------------|------------------------------------------------------------------|
| <b>TLO insert checks</b>           |                                                                  |
| Pan TLO                            | GACCAGATATTGAAATCATC                                             |
| TLO1 R                             | ACTTTTGTGGGACGGTCTCT                                             |
| TLO2 R                             | GTCGTTCTGCATGTCGTTGA                                             |
| Tlo tag ( <i>Bgl</i> III) R        | ATGCAGATCTACCTAAGCGTAATCTGGAAC                                   |
|                                    |                                                                  |
| <b><i>C. auris</i> CRISPR-Cas9</b> |                                                                  |
| AHO1096                            | GACGGCACGGCCACGCGTTTAAACCGCC                                     |
| CJNO3235                           | TGTTTTCTGCTGAGGGAGTC                                             |
| AHO1097                            | CCCGCCAGGCGCTGGGGTTTAAACACCG                                     |
| AHO1237                            | AGGTGATGCTGAAGCTATTGAAG                                          |
| CJNO3755                           | TACTGTATAAGTCGAAGAGCACAAG                                        |
| CJNO3080                           | TTATTTCTGCAAAAGCTTCTTTAC                                         |
| MED2 repair F1                     | TCCATTTCTTCTTTCATTTGCCA                                          |
| MED2 repair R1                     | <u>CCTCATGTCTGAGCACTCGTCTCGAGCCGCTGGT</u><br>AGTAATGGG           |
| MED2 repair F2                     | <u>CGAGACGAGTGCTCGACATGAGGGGGCTGCCA</u><br>ATGGTTAGAC            |
| MED2 repair R2                     | GGAGAGCCACAATTGCAAGT                                             |
| MED15 repair F1                    | CCCACTCTCTCACGATCCTT                                             |
| MED15 repair R1                    | <u>CCTCATGTCTGAGCACTCGTCTCGGATGGTGGAG</u><br>GCGAAAAG            |
| MED15 repair F2                    | <u>CGAGACGAGTGCTCGACATGAGGTGAGATATT</u><br>TACTAGAAGACGCA        |
| MED15 repair R2                    | AAAGATAGCGCACGTGACAC                                             |
| MED15com repair F                  | AAGCTTCCATTTTCGGTCCC                                             |
| MED15com repair R                  | AAAGATAGCGCACGTGACAC                                             |
| MED2 gRNA                          | GACTCCCTCAGCAGAAAACATGTTCAAGCCAA<br>GATCCATAGTTTTAGAGCTAGAAATAGC |
| MED15 gRNA                         | GACTCCCTCAGCAGAAAACAACAAGGGTTGA<br>CTTCAACGGTTTTAGAGCTAGAAATAGC  |

|                                                    |                                                                                   |
|----------------------------------------------------|-----------------------------------------------------------------------------------|
| MED15com gRNA                                      | GACTCCCTCAGCAGAAAACAC <b>CGAGACGAGTGC</b><br><b>TCGACATG</b> GTTTTAGAGCTAGAAATAGC |
|                                                    |                                                                                   |
| <b><i>C. auris</i> MED2 deletion confirmation</b>  |                                                                                   |
| M2 MD1 F1 (Sanger)                                 | TTTCAGTCATGGCCAGGGTT                                                              |
| M2 MD1 R1 (Sanger)                                 | GTGAAGTCGTGTGAACCTTTGA                                                            |
| Med2 out F1                                        | TTTCAGTCATGGCCAGGGTT                                                              |
| Med2 out R1                                        | GTGAAGTCGTGTGAACCTTTGA                                                            |
|                                                    |                                                                                   |
| <b><i>C. auris</i> MED15 deletion confirmation</b> |                                                                                   |
| Med15 F2 (Sanger)                                  | GTTGCCCATGTGTCACCTTT                                                              |
| Med15 R2 (Sanger)                                  | AAGATAGCGCACGTGACAC                                                               |
| Med15 up2 F1                                       | GTTGCCCATGTGTCACCTTT                                                              |
| Med15 up2 R1                                       | TATATCCTTGAGCGTGGCGA                                                              |
| Med15 dn1 F1                                       | AAGGGTGGTAGTGCTGGTAC                                                              |
| Med15 dn1 R1                                       | GCTGACAGAGAGAATGCACG                                                              |
| Med15 out F1                                       | CTTTGCTGGAAATCTCGGGG                                                              |
| Med15 out R1                                       | GGCGAGCGACAACCTATAGGT                                                             |
|                                                    |                                                                                   |
| <b>ChIP-qPCR</b>                                   |                                                                                   |
| <b>CDR1 primers</b>                                |                                                                                   |
| 1-upF                                              | ATTGACGACGATGATGAC                                                                |
| 1-upR                                              | ATAAGAAGTTGAGCGGAAG                                                               |
| 1 F                                                | ATCCTCGTTACTCAATAAG                                                               |
| 1 R                                                | AGACACTAACTCCGTATT                                                                |
| 2 F                                                | TGCGTGACCCAAACATAATCT                                                             |
| 2 R                                                | TGTGACGAGGTGGCTGAT                                                                |
| 3 F                                                | GTAAGAGGAGTAGCAAGTG                                                               |
| 3 R                                                | TTAAGCGATTCTGGTAGTTAT                                                             |

|                       |                               |
|-----------------------|-------------------------------|
| 1-DREF                | TTTCAACATATTAGAATCGAATCATTACG |
| 1-DRER                | GCGGCTGTGTGTTTGTGTG           |
|                       |                               |
| <b>Pol II primers</b> |                               |
| <i>NEG1</i> F         | CTTCAAGGTCATTCCGAA            |
| <i>NEG1</i> R         | GAAGCTGAAACTGAACTAAA          |
| <i>PUT1A</i> F        | TGTCAATAATATGATGCCAAGAGT      |
| <i>PUT1A</i> R        | GAAGGTAATTGTGGAGATGTAGT       |
| <i>PUT1B</i> F        | TGGCTCTACTATTGGTCATT          |
| <i>PUT1B</i> R        | TTATCACTGGCTAATCTCATTG        |

Sequences in Red indicate the gene-specific gRNA target sequences of that guide oligonucleotide. Underlined bases indicate unique 23bp AddTag that act as a gRNA target site to carry out CRISPR-Cas9 mutagenesis DNA complementation.

## 2.2 Microbial strains

### 2.2.1 Strains and isolates

All *C. albicans* and *C. auris* isolates used in this study, unless otherwise stated, were all obtained from the Dublin Dental University Hospital (DDUH) strain collection at the DDUH, University of Dublin, Trinity College, Lincoln Place, Dublin 2. All strains used in this study, including CRISPR-Cas9 edited strains, are outlined in Table 2.1.

### 2.2.2 Growth conditions and culture media

All *Candida* spp. were routinely grown on Yeast Extract Peptone Dextrose (YEPD) agar (10g/L yeast extract [Sigma-Aldrich], 20g/L bacteriological peptone [Oxoid], 20g/L glucose [Sigma-Aldrich], and 15g/L Bacto Agar [Difco, Sparks, Maryland, USA] to make agar plates) at 30 or 37°C for 24 - 48h in a static incubator (Gallenkamp, Leicester, UK). At 24 – 48h of growth, single colonies grown on such plates were used as initial inoculum. For liquid culture, *Candida* spp. were routinely grown in YEPD broth (10g/L yeast extract [Sigma-Aldrich], 20g/L Bacteriological Peptone [Oxoid], 20g/L Glucose [Sigma-Aldrich]) in a shaking incubator (New Brunswick Scientific, Edison, New Jersey, USA) set at 200 rpm, 30 or 37°C overnight.

Nourseothricin resistant strains were grown on 200ug/ml for *C. albicans* derived strains and 250ug/ml for *C. auris* derived strains (CloNAT, Werner Bioagents, Germany). Hygromycin resistant strains were grown on 400ug/ml for *C. auris* mediator tail mutants and 500ug/ml for *C. auris* clinical isolates (Sigma-Aldrich). Yeast Nitrogen Base (YNB) (YNB 6.7g/L [Sigma-Aldrich], glucose 20g/L [Sigma-Aldrich] was used for growing *Candida* spp. in nutrient poor conditions, or when using certain drugs or reagents. YNB without Amino Acids and Ammonium Sulphate (Sigma-Aldrich) was used and paired with Yeast Synthetic Drop-out

Medium Supplements without leucine (Sigma-Aldrich) to select Nourseothricin and Hygromycin sensitive strains. All *Candida* spp. were grown aerobically unless otherwise stated. *Escherichia coli* strains were cultured in Luria broth (L broth) or L agar containing 100µg/ml ampicillin (Penbritin, GlaxoSmithKline, Rathfarnham, Dublin) at 37°C. All agar and media used in this study were sterilized via autoclaving or filtration before use.

**Table 2.2 All strains used in this study**

| Strain                                              | Genotype                                                                                                    | Parent                       | Source                          |
|-----------------------------------------------------|-------------------------------------------------------------------------------------------------------------|------------------------------|---------------------------------|
| <b><i>Candida albicans</i></b>                      |                                                                                                             |                              |                                 |
| SC5314                                              | Wild-type                                                                                                   | -                            | (Gillum <i>et al.</i> , 1984)   |
| AHY940                                              | $\Delta leu2/LEU2$                                                                                          | SC5314                       | (Nguyen <i>et al.</i> , 2017)   |
| MAY1244                                             | $\Delta leu2/LEU2$                                                                                          | AHY940                       | (Fletcher <i>et al.</i> , 2023) |
| DsyR                                                | Azole-resistant clinical strain                                                                             | DSY296                       | (Coste <i>et al.</i> , 2004)    |
| DsyS                                                | Azole-susceptible clinical                                                                                  | DSY294                       | (Coste <i>et al.</i> , 2004)    |
| <i>Tac1</i> $\Delta$                                | <i>tac1-1</i> $\Delta::hisG/tac1-2$ $\Delta::hisG$                                                          | DSY2903                      | (Coste <i>et al.</i> , 2004)    |
| <i>Tac1</i> $\Delta$ TAC1-5                         | <i>tac1-1</i> $\Delta::hisG/tac1-2$ $\Delta::hisG$<br><i>LEU2::TAC1-5</i>                                   | DSY2906                      | (Coste <i>et al.</i> , 2006)    |
| <i>med3</i> $\Delta$                                | $\Delta med3/\Delta med3, \Delta leu2/LEU2$                                                                 | MAY1244                      | (Fletcher <i>et al.</i> , 2023) |
| <i>tlo</i> $\Delta$                                 | All <i>TLO</i> s deleted, $\Delta leu2/LEU2$                                                                | AHY940                       | (Fletcher <i>et al.</i> , 2023) |
| <i>tlo</i> $\Delta$ + <i>TLO</i> $\alpha$ 1         | <i>tlo</i> $\Delta$ , <i>ADH1/adh1::P<sub>TET1</sub>TLO</i> $\alpha$ 1                                      | <i>tlo</i> $\Delta$          | (Fletcher <i>et al.</i> , 2023) |
| <i>tlo</i> $\Delta$ + <i>TLO</i> $\beta$ 2          | <i>tlo</i> $\Delta$ , <i>ADH1/adh1::P<sub>TET1</sub>TLO</i> $\beta$ 2                                       | <i>tlo</i> $\Delta$          | (Fletcher <i>et al.</i> , 2023) |
| MAY1244-TAC1-5                                      | <i>TAC1-5D<sup>977N</sup></i>                                                                               | MAY1244                      | This study                      |
| <i>med3</i> $\Delta$ -TAC1-5                        | <i>med3</i> $\Delta$ , <i>TAC1-5D<sup>977N</sup></i>                                                        | <i>med3</i> $\Delta$         | This study                      |
| <i>tlo</i> $\Delta$ -TAC1-5                         | <i>tlo</i> $\Delta$ , <i>TAC1-5D<sup>977N</sup></i>                                                         | <i>tlo</i> $\Delta$          | This study                      |
| <i>tlo</i> $\Delta$ -TAC1-5 + <i>TLO</i> $\alpha$ 1 | <i>tlo</i> $\Delta$ ,<br><i>ADH1/adh1::P<sub>TET1</sub>TLO</i> $\alpha$ 1,<br><i>TAC1-5D<sup>977N</sup></i> | <i>tlo</i> $\Delta$ (TAC1-5) | This study                      |
| <i>tlo</i> $\Delta$ -TAC1-5 + <i>TLO</i> $\beta$ 2  | <i>tlo</i> $\Delta$ ,<br><i>ADH1/adh1::P<sub>TET1</sub>TLO</i> $\beta$ 2,<br><i>TAC1-5D<sup>977N</sup></i>  | <i>tlo</i> $\Delta$ (TAC1-5) | This study                      |
| <b><i>Candida auris</i></b>                         |                                                                                                             |                              |                                 |
| 8985                                                | NCPF8985, clade I                                                                                           | /                            | (Borman <i>et al.</i> , 2017)   |
| 8977                                                | NCPF8977, clade III                                                                                         | /                            | (Borman <i>et al.</i> , 2017)   |
| <i>med2</i> $\Delta$ 8985                           | $\Delta med2$ , <i>LEU2</i> (NAT <sup>R</sup> )                                                             | 8985                         | This study                      |
| <i>med15</i> $\Delta$ 8985                          | $\Delta med15$ , <i>LEU2</i> (NAT <sup>R</sup> )                                                            | 8985                         | This study                      |

|                                     |                                                                                                                                                                                                                  |                                        |                     |
|-------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------|---------------------|
| <i>med2Δ</i> 8977                   | $\Delta med2$ , <i>LEU2</i> (NAT <sup>R</sup> )                                                                                                                                                                  | 8977                                   | This study          |
| <i>med15Δ</i> 8977                  | $\Delta med15$ , <i>LEU2</i> (NAT <sup>R</sup> )                                                                                                                                                                 | 8977                                   | This study          |
| <i>med15Δ</i> 8977 Hyg <sup>R</sup> | <i>MED15</i> , <i>Leu2::Hyg<sup>R</sup></i>                                                                                                                                                                      | <i>med15Δ</i> 8977                     | This study          |
| <i>med15Δ</i> 8977 com              | <i>MED15</i> , <i>LEU2</i>                                                                                                                                                                                       | <i>med15Δ</i> 8977<br>Hyg <sup>R</sup> | This study          |
|                                     |                                                                                                                                                                                                                  |                                        |                     |
| <b><i>Escherichia coli</i></b>      |                                                                                                                                                                                                                  |                                        |                     |
| <b>DH5α</b>                         | $\Delta(argF-lac)169$ ,<br>$\phi80dlacZ58(M15)$ , $\Delta phoA8$ ,<br><i>glnX44(AS)</i> , <i>deoR481</i> , <i>rfbC1</i> ,<br><i>gyrA96(NalR)</i> , <i>recA1</i> , <i>endA1</i> ,<br><i>thiE1</i> , <i>hsdR17</i> |                                        | New England biolabs |

### 2.2.3 Microbial strain preservation method

All microbial samples are stored using Microbank™ storage system (Pro-Lab Diagnostics, Birkenhead, Wirral, UK) in a -80°C freezer to ensure long-term viability. This system provides multiple vials that contain cryopreservative solution and porous beads. Inoculation of these vials will be carried out following manufacturer's instruction.

To reactivate cryopreserved strains, the frozen vial containing the microbe of interest will be first thawed on ice. After thawing, a single bead will be taken out from the vial and then streaked normally on a YEPD agar plate. Vial will be kept on ice and returned to the freezer as soon as possible after streaking on plate.

### 2.2.4 Cell counting – haemocytometer method

To obtain a precise number of cells in liquid medium, 1ml of overnight grown/exponential phase microbial culture was spun down at 14,000 rpm for 2 min, washed twice with 1ml of PBS and resuspended in 1ml of PBS. This suspension was diluted 1 in 100 by adding 10µl of the resuspended culture into 990µl of PBS. 10µl of the diluted cell suspension was added to the centre chamber of a clean haemocytometer (Marienfeld Superior, Germany). A light microscope (Nikon Eclipse E600) at X400 magnification was used to count cells located in the central grid of the haemocytometer, in a total of 5 separate and non-adjacent 0.20 mm x 0.20 mm squares. The obtained cell counts were then be used to standardized cells cultures to desired concentrations for various procedures.

### 2.2.5 Cell counting – spectrophotometric method

Overnight cultures of *C. albicans* were first grown in YEPD medium in a shaking incubator set at 37°C, 200 rpm. The following day, the OD<sub>600</sub> of a 100µl cell solution in 900µl YEPD dilution was measured. This measurement allowed for the reliable estimation of cell numbers per ml in liquid medium by using OD<sub>600</sub> of 0.38 being the equivalent of 1x10<sup>7</sup> cells/ml as a reference, which was then used to standardise cell counts for various microbial experimentations.

### 2.2.6 Comparison of Cell Counting methods

To find out which cell counting methods is more reliable, spot assays were used to investigate the precision of each method. In the case of more pseudohyphal and aggregating strains, it was found that the spectrophotometric method of cell counting was more reliable than haemocytometer method as it provides consistent results when performed in triplicate. Thus, for this study, spectrophotometric method of cell counting was chosen to perform microbial cell counting.

### 2.2.7 Microscopy and cell imaging

A wet mount was prepared by adding 10µl of resuspended cell culture on a clean microscopic slide and then covered with a cover slip for examination. To observe cellular morphology and obtain cell images, a Zeiss Axio microscope (Carl Zeiss AG, Oberkochen, Germany) was used. For the examination of flat bottom 96 well plates (and other versions), a Zoe Fluorescent Cell Imager (Bio-Rad) was used.

### 2.2.8 Calcofluor white staining for cell imaging

To visualize calcofluor white stained cells, a wet mount was prepared by mixing 5 $\mu$ l of resuspended overnight cell culture with 5 $\mu$ l of Calcofluor White (CFW) stain (Sigma-Aldrich, concentration 1g/L) and add the mixture onto a microscopic slide. Then, cover the mixture with a cover slip for visualization on a Zeiss Axio microscope using a UV filter (400nm).

## 2.3 DNA techniques

### 2.3.1 Genomic DNA extraction from yeast cells

*Candida* strains were grown overnight in 4ml YEPD medium in a shaking incubator set at 37°C, 200 rpm. On the following day, overnight cultures were spun down at 12,000 rpm for 2 mins and the pellet was resuspended in 200µl of breaking buffer (2% Triton X-100 [v/v], 1% SDS [w/v], 10mM Tris HCl [pH8.0], 1mM EDTA, 100mM NaCl). The resuspended solution was transferred into a 2ml screw cap tube (North Rhine-Westphalia, Numbrecht, Germany) containing 0.3g of glass beads and 200µl of phenol:chloroform:isoamyl alcohol (24:24:1) solution (Sigma-Aldrich) was added subsequently. The tube was then placed in a bead beater for 1 min to break the yeast cells. Afterwards, the mixture was centrifuged for 10mins at 14,000 rpm. The aqueous phase was removed and placed in a fresh 1.5ml Eppendorf tube (Eppendorf, Hamburg, Germany). An equal amount of chloroform:isoamyl alcohol (24:1) solution (Sigma-Aldrich) was then added to the same tube. The Eppendorf tube was centrifuged at 14,000 rpm for 2 min in an Eppendorf benchtop centrifuge (rotor FA-45-30-11) (Hamburg, Germany). The aqueous phase located in the middle was removed and placed in another fresh 1.5ml tube. 20µl of 3M sodium acetate and 400µl of 70% (v/v) ice cold ethanol was added later to the same tube. The tube was then centrifuged at 14,000 rpm for 10 mins and the supernatant was discarded, leaving a white/translucent pellet at the bottom. The pellet was washed with 70% (v/v) ethanol, air-dried for 1 to 2 mins, and then resuspended in 35µl of molecular grade water. The air-drying step is crucial and needed to be carried out with care to ensure no ethanol residue were left. The DNA concentration was examined (described in Section 2.3.3) and 100ng/µl of solutions were made for use in routine PCR (refer to Section 2.3.6).

### 2.3.2 Plasmid extraction from bacterial cells

The plasmid extraction from *E. coli* was done by using the QIAprep Spin Miniprep kit (Qiagen) and the whole plasmid extraction procedure was carried out according to the manufacturer's instructions. In the case of pCE38, a plasmid containing a hygromycin marker, plasmid minipreps yielded low concentrations of DNA. For this reason, the Thermo Scientific GeneJET Plasmid Maxiprep Kit (Thermo Fisher Scientific) was used to extract sufficient pCE38 plasmid from 100ml overnight *E. coli* culture to carry out *C. auris* cell transformation. Plasmid concentrations were examined the same way as other nucleic acids (described in Section 2.3.3) and adjusted to the required concentration. All plasmids that were used in this study were listed out in Table 2.3.

**Table 2.3 Plasmids used in this study**

| Name                       | Description                                                                                                                                                                                                                                             | Reference                       |
|----------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------|
| <b>CRISPR plasmids</b>     |                                                                                                                                                                                                                                                         |                                 |
| <b><i>C. albicans</i></b>  | Hernday CRISPR method (Nguyen <i>et al.</i> , 2017)                                                                                                                                                                                                     |                                 |
| pADH110                    | Plasmid containing A fragment for CRISPR protocol                                                                                                                                                                                                       | (Nguyen <i>et al.</i> , 2017)   |
| pADH119                    | Plasmid containing B fragment that can be amplified using unique gRNA oligonucleotide                                                                                                                                                                   | (Nguyen <i>et al.</i> , 2017)   |
| pADH137                    | Plasmid that can be digested by <i>MssI</i> to create the Cas9 cassette for CRISPR protocol                                                                                                                                                             | (Nguyen <i>et al.</i> , 2017)   |
| <b><i>C. auris</i></b>     | Modified Hernday method by Ennis <i>et al.</i> (2021)                                                                                                                                                                                                   |                                 |
| pCE27                      | Plasmid containing nourseothricin (NAT) marker and can be used to amplify A and B fragments for CRISPR protocol                                                                                                                                         | (Ennis <i>et al.</i> , 2021)    |
| pCE35                      | Plasmid with NAT marker that can be digested by <i>MssI</i> to create the Cas9 cassette for CRISPR protocol                                                                                                                                             | (Ennis <i>et al.</i> , 2021)    |
| pCE41                      | Plasmid containing hygromycin (HYG) marker and can be used to amplify A and B fragments for CRISPR protocol                                                                                                                                             | (Ennis <i>et al.</i> , 2021)    |
| pCE38                      | Plasmid with HYG marker that can be digested by <i>MssI</i> to create the Cas9 cassette for CRISPR protocol                                                                                                                                             | (Ennis <i>et al.</i> , 2021)    |
| <b><i>TLO</i> plasmids</b> |                                                                                                                                                                                                                                                         |                                 |
| pNIM1-TLO1/2/11            | Plasmid with NAT marker containing either <i>TLO<math>\alpha</math>1</i> , <i>TLO<math>\beta</math>2</i> , or <i>TLO<math>\gamma</math>11</i> under the tetracycline promoter with 3X-HA tag, flanked by <i>KpnI</i> and <i>SacII</i> restriction sites | (Fletcher <i>et al.</i> , 2023) |

### 2.3.3 DNA/RNA quantification and quality checking

The quantification of nucleic acids (DNA/RNA) was done using a Nanodrop spectrophotometer (Thermo Fisher Scientific) and Nanodrop 2000c software (Thermo Fisher Scientific) according to manufacturer's instructions after DNA/RNA purification procedure. The Nanodrop spectrophotometer measures the concentration of nucleic acid solution in ng/ $\mu$ l. Besides, it can detect protein and phenol contamination or carbohydrate and phenol contamination in the nucleic acid sample by providing the 260/280 nm ratio and the 260/230 nm ratio of the sample. The ideal 260/280 nm ratio indicating good quality DNA is around 1.8 and around 2 for RNA samples whereas the ideal 260/230 nm ratio indicating good quality DNA and RNA is between 2.0-2.2.

### 2.3.4 Gel electrophoresis

Agarose gels were prepared by heating up 70ml agarose solution containing 1% (w/v) Agarose (Sigma-Aldrich) and 1X TAE buffer for 2 mins twice in the microwave. Then, 5 $\mu$ l SYBR<sup>TM</sup> Safe DNA Gel Stain (Thermo Fisher Scientific) was mixed into the molten agarose mixture sufficient for a small gel (10 – 12 wells) and poured evenly on the agarose fixing tray (Sigma-Aldrich) with well combs. To make a large gel (20 – 25 wells), double the amount of components needed to make an Agarose gel (agarose solution - 140 $\mu$ l, SYBR Safe Stain - 10 $\mu$ l). Routinely prepared PCR samples (refer to 2.3.6) can be added straight into the wells as the GoTaq system contains its own loading dye unless PCR samples were prepared differently and needed to be mix with 10X loading dye prior to gel run. Gels were normally run at 70V using a power supply unit for 42 mins and the voltage and time can be adjusted accordingly. After the clear separation of gel ladder, the gels can be visualized and imaged using an Alpha Imager Mini (Alpha-Innotech, La Chaux-de-Fonds, Switzerland) under UV light.

### 2.3.5 Restriction digestion of DNA

Restriction enzymes were obtained from either Promega, Thermo Fisher Scientific or New England Biolabs (NEB) and DNA digestion was done following the manufacturer's instruction. Double digestions were carried out under an ideal condition that was determined by NEB calculator to ensure complete double digestion. After digestion, the product sizes were checked on a 1% (w/v) Agarose gel (described in Section 2.3.4).

### 2.3.6 Polymerase chain reaction (PCR) and colony PCR

Commonly used PCRs were carried out using the Go Taq® PCR Core system (Promega), unless otherwise stated. Basic PCRs were done according to the well-optimised ratios; 28.5µl molecular grade water, 10µl 5X Green buffer, 5µl MgCl<sub>2</sub> (25 mM), 1µl dNTPs (10 mM), 2µl of each forward and reverse oligonucleotide (10 mM), 0.5µl Taq polymerase, and 1µl template DNA (1ng/µl). For multiple PCR reactions, MasterMix sufficient for multiple runs were made to reduce margin of error. After DNA amplification via PCR, DNA samples was quantified using Nanodrop 2000 (Thermo Fisher Scientific).

To carry out colony PCR (yeast), singular colony was picked up from agar plate and mixed into a PCR tube containing 100µl (20 mM) Sodium Hydroxide (NaOH). Then, the NaOH solution with cells was boiled at 99°C for 15mins and 2ul of the boiled mixture can be used as DNA template for basic PCR (using the Go Taq® PCR Core system).

### 2.3.7 Cleanup for PCR and DNA restriction digestion

PCR products were normally subjected to a PCR clean up procedure to remove any impurities from the PCR process for further downstream applications. The PCR cleanup was done by using

the GenElute™ PCR cleanup kit (Sigma-Aldrich) based on the manufacturer's instructions. Cleaned PCR products were resuspended again in 30µl of molecular grade water and the quality of the PCR products was determined as described in Section 2.3.3. For the clean up of restriction enzyme digested products, QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) was used following the manufacturer's instruction and again, with a final resuspension of the clean product in 30µl of molecular grade water.

### 2.3.8 Sanger sequencing

Basic PCR was carried out to amplify DNA with region of interest around 1000bp. The PCR product with a set of relevant amplification primers was then sent to Source Bioscience (Nottingham, England) to perform Sanger sequencing. Sequence files were provided in .seq and .abi file formats. Both sequence files can be viewed with ApE (a free plasmid editing software) and compared/analysed with *Candida* genomic sequences available on The *Candida* Genome Database (<http://www.candidagenome.org/>). The reference genome for *C. albicans* is SC5314 strain and B9J08 (clade I) for *C. auris*.

## 2.4 RNA techniques

### 2.4.1 RNA extraction

*Candida* strains were grown in 4 ml YEPD medium overnight in a shaking incubator set at 200 rpm, 37°C. The next day, the OD<sub>600</sub> value of the overnight culture was measured, and a fresh culture (OD<sub>600</sub> 0.1) was established in 25ml YEPD and grown to mid-exponential phase (0.6 ~ 0.8) with shaking at 200 rpm, 37°C. When the desired OD<sub>600</sub> was achieved, 10ml of exponential phase culture was spun at 4000 rpm for 5 mins. The supernatant was removed, and the pellet was resuspended with 1ml sterile water, spun at 13,000 rpm for 3mins, and supernatant was removed once again. Then, 600 µl of RLT containing β-ME (10µl β-ME per 1ml Buffer RLT) was added to the pellet and transferred into a screw cap tube containing 0.25g glass beads. The tube was then placed in a bead beater and subjected to 4 x 30s pulses, with 1 min incubation on ice after each pulse. After cell breaking, the tube was pelleted at 14,000 rpm for 2 mins. The supernatant was transferred to a new 1 ml Eppendorf tube and an equal volume of 70% ethanol (around 400µl) was added. RNeasy Mini Kit (Qiagen) was then used to extract RNA from this mixture containing lysed cells after mechanical disruption, with “on-column” DNase I treatment included at the beginning of the RNA extraction process using the RNase-Free DNase Set (Qiagen).

### 2.4.2 cDNA synthesis for qRT-PCR

After obtaining clean, DNA-free RNA using the RNeasy Mini Kit (Qiagen), the concentration of RNA (in ng/µl) in the samples were measured using a Nanodrop 2000 (see Section 2.3.3). RNA samples were then all standardized to a fixed concentration of 1000 ng in 10 µl.

The Superscript IV Reverse Transcriptase kit (Invitrogen) was used to convert RNA into cDNA in this study. 10µl of each standardized RNA samples (100ng/µl) was mixed with 1µl

dNTPs and oligo(dT)<sub>15</sub> primer (Promega) in a PCR tube and incubated in a SimpliAmp thermocycler (Applied Biosystems) at 65°C for 5 min. After incubation, each PCR tube was cooled down for 1 min on ice and the following solutions were added accordingly: 4 µl First Strand buffer, 1 µl SuperScript IV enzyme, 1 µl DTT, 1 µl RNase Out, and 1 µl of molecular grade water. The samples were then incubated at 50°C for 10mins and then 80°C for 10 mins. A final 1 µl of RNase was added to all cDNA samples and incubated for 20 mins at 37°C and cDNA samples can be stored at -20°C or used it immediately for qRT-PCR.

#### 2.4.3 Quantitative Real-Time PCR (qRT-PCR)

A MasterMix for qRT-PCR consisted of (per sample): 7.5 µl SYBR Green, 0.375 µl of each forward and reverse primers (10 mM), and 5.75 µl of molecular grade water. A 14µl volume of MasterMix solution was added to 96 PCR well plates and 1 µl cDNA (prepared following Section 2.4.2, diluted 1:10 in molecular grade water) was added later. All qRT-PCR primers were listed in Table 2.1.

qRT-PCR reactions were done in an AB7500 Real Time PCR machine (Applied Biosystems) at the default settings for SYBR reactions. Each biological replicate was split into three technical replicates to reduce margin of error. The *ACT1* housekeeping gene was used as a endogenous control to determine C<sub>T</sub> values. Comparative C<sub>T</sub> method was used to analyse the data. The initial data was exported to Microsoft Excel and C<sub>T</sub> values were first calculated, analysed, and finally graphically represented using GraphPrism ver. 10 (San Diego, California, USA).

## 2.5 Transformation of *Candida* species

### 2.5.1 Electroporation method

The electroporation method used to transform *C. albicans* and *C. auris* is modified from (Staib *et al.*, 2001). First, a single *Candida* colony was taken from a YEPD agar plate (after 24 – 48 incubation) to inoculate 50ml YEPD medium in a 250 ml conical flask and incubated overnight in a shaking incubator set at 200 rpm, 37°C. On the following day, the OD<sub>600</sub> value of the overnight culture was measured and equalized to an OD<sub>600</sub> of 1.6 ~ 2.0 in a 50ml falcon tube. The equalized culture was spun down and washed once in sterile water at 4000 rpm for 5 mins. The cell pellet was then resuspended in 8ml sterile water, 1ml 10X TE, 1ml (1 M) Lithium Acetate (LiAC) and incubated in the shaking incubator for an hour at 200 rpm, 37°C. A 250 µl volume of 1M Dithiothreitol (DTT) was added later to the cell suspension for a further 30 mins incubation. After the short incubation, 20 ml of sterile water was added into the cell suspension and spun down as before. The supernatant was discarded, and the pellet was washed with 10ml of ice-cold sterile water and spun down again as before. Supernatant was discarded again, and 10ml of cold sorbitol was added to the pellet and spun down for 2mins at 4000 rpm. The supernatant was removed, and the pellet was further spun down as before to remove excess sorbitol. After complete removal of excess supernatant, the pellet was resuspended in 50 µl 1 M sorbitol and kept on ice. 40 µl of the cell slurry was taken and mixed with 5µl of DNA (100 ~ 200 ng/µl), for transformation. For a negative control, the same volume of molecular grade water was mixed with 40 µl of cell slurry in another tube. The cell-DNA mixture was then transferred to a 0.2 cm electroporation cuvette (Bio-Rad) and electroporated at 1.5 kV for 5 ms in a Bio-Rad MicroPulser<sup>TM</sup> electroporator (Bio-Rad Laboratories, Hercules, California, USA). Prior to electroporation, it was ensured that the cell-DNA mixture was settled evenly at the bottom of the electroporation cuvette and that the cuvette was dry. Following electroporation, 1 ml of YEPD medium was added directly into the cuvette and the cell suspension was

transferred into a sterile fresh 1ml Eppendorf tube. The cells were then be recovered by incubating in an Eppendorf Thermomixer incubator shaker at 37°C for 3 hours without shaking (tubes were inverted every 30 minutes to prevent cell settling). After the recovery period, 200µl of the well-mixed culture was plated on a sterile, prewarmed (30°C) YEPD agar plate supplemented with antibiotic (NAT or HYG) and incubated in a static incubator for 2 ~ 3 days at 30°C. The selection for NAT resistant *C. albicans* strains require a YEPD plate containing NAT 200 µg/ml and for *C. auris* NAT 250 µg/ml. For the selection of HYG resistant *C. auris* strains, 400-500 µg/ml of HYG was used. Transformants were then screened by carrying out colony PCR (described in Section 2.3.6) to identify desired transformants containing the NAT/HYG marker. Colonies were also plated on YNB plates without leucine to recycle the remaining antibiotic marker and restore leucine prototrophy. Loss of the resistance marker was confirmed by culture on antibiotic containing YEPD.

### 2.5.2 Heat shock method

The heat shock method used to transform *C. albicans* (Dr. Leenah Alaalm, personal communication) was also optimised for *C. auris* transformation. First, a single colony of *Candida* strain from YEPD plate (24 ~ 48h incubation) was taken to inoculate 10ml of YEPD medium for overnight incubation in a shaking incubator set at 200 rpm, 37°C. The next day, the OD<sub>600</sub> of the overnight was determined and a fresh 10 ml culture was inoculated at OD<sub>600</sub> 0.3. The culture was then grown in the shaking incubator set at 200 rpm, 37°C until the OD<sub>600</sub> reached between 4 ~ 8. An amount of culture was transferred to a sterile Eppendorf tube based on the formula: volume of culture = 10ml/ (1.5 X OD<sub>600</sub> value). The culture was spun down into a pellet at 2500 rpm for 2 mins. The supernatant was discarded, and the pellet was washed twice with 1ml sterile water. The supernatant was removed and 40 µl of sterile water was added for resuspension. An 800 µl volume of 50% (w/v) PEG3350 was added to the cell suspension

and 50 µl DNA (plasmid or DNA) was subsequently added. The following solutions were then added in the following order to the tube: 100 µl 10X TE, 100 µl 1M LiAC pH 7.4, 40 µl 5 mg/ml salmon sperm DNA (boiled for 5 mins and kept on ice), and 20 µl 1M DTT. The mixture was incubated at 30°C for 1 hour and transferred to 42°C for 15 minutes (30 minutes for *C. auris*) without shaking. The transformation mix was spun down to form pellet and 600 µl YEPD media was added after the removal of supernatant. The cell suspension was incubated in a static incubator for 3 ~ 4 hours and 200 µl of cell suspension (120 µl for *C. auris*) was spread on prewarmed (30°C) YEPD agar plates supplemented with antibiotic (NAT or HYG). Plates were incubated for 2 ~ 3 days at 30°C until transformants could be selected for colony PCR. Colonies were also plated on YNB plates without leucine to recycle the remaining antibiotic marker and restore leucine prototrophy. Loss of the resistance marker was confirmed by culture on antibiotic containing YEPD.

## 2.6 Protein techniques (Western Blot)

### 2.6.1 Protein extraction from yeast cells

*Candida* strains were grown overnight in 4ml YEPD medium (unless otherwise stated) in a shaking incubator set at 37°C, 200 rpm. On the following day, the OD<sub>600</sub> value of the overnight culture was measured and a fresh 50ml YEPD culture (OD<sub>600</sub> 0.1) was inoculated and grown to mid-exponential phase (0.6 ~ 0.8) in the shaking incubator set at 200 rpm, 37°C. The downstream procedures were performed at 4°C to prevent protein degradation. The culture was spun down at 4000 rpm for 10min at 4°C. The supernatant was then removed and the pellet was washed twice with ice cold 1X PBS. After the washing steps, the pellet was resuspended in 250µl standard protein extraction lysis buffer (50mM HEPES pH7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X100 + 1 Roche Complete Protease Inhibitor Cocktail pill) and transferred to a screw cap tube filled with 0.05µg glass beads. The tube was then placed in a bead beater and subjected to 5 x 30s pulses, with 1min incubation on ice after each pulse. The tube was spun for 10 mins at 14,000 rpm, 4°C and the supernatant was transferred to a fresh 1.5 ml Eppendorf tube (kept on ice). 25µl of the supernatant was mixed with 25µl protein loading dye (0.35 M Tris-HCl, 10% [w/w] SDS, 36% Glycerol, 0.012% Bromophenol blue, 5% β-ME) and then boiled for 5 mins at 99°C. Protein samples were flash frozen in liquid nitrogen for a few seconds and stored at -80°C for future use.

### 2.6.2 SDS-PAGE for protein size separation

Polyacrylamide gel (stacking and running gel) was made using solutions obtained from Bio-Rad. 15% running gel (2.8 ml water, 3 ml 40% Acrylamide, 2 ml 1.5 M Tris pH8.8, 80 µl 10% SDS, 80 µl 10% APS and 8 µl TEMED, APS and TEMED added last) were first made and poured into the gel mold. The running gel was then fixed using 2-propanol solution before solidification. After the running gel was solidified, 2-propanol solution was removed and the

gel was washed with water and dried with tissue. 6% of stacking gel (2.9 ml water, 0.75 ml 40% Acrylamide, 1.25 ml 0.5 M Tris pH6.8, 50 µl 10% SDS, 50 µl 10% APS and 5 µl TEMED) was added onto the solidified running gel and a gel comb was inserted. After the stacking gel was solidified, gel comb was removed and 10µl of boiled protein samples were loaded in each well and 5µl of PageRuler™ Prestained NIR protein ladder (Thermo Fisher Scientific) was loaded alongside. The gel was run in 1X running buffer (3.03g Tris base, 14.25g Glycine, 1g SDS in 1L water) at 90 V for 30 mins and later at 120V for around 2 hours until even ladder separation was observed.

#### 2.6.3 Protein transfer from gel to PVDF membrane

After SDS-PAGE was completed, the gel was soaked in 1X cold transfer buffer (3 g Tris base, 14.4 g Glycine, 200 ml methanol and 800 ml water). A PVDF membrane was prewetted in methanol for 30s before use to activate the membrane. The gel sandwich for protein transfer was prepared accordingly (from negative to positive): wet foam pad, damp filter paper (Thermo Fisher Scientific), gel, prewetted PVDF membrane, damp filter paper, wet foam pad. During the gel sandwich making procedure, the gel and PVDF membrane were flattened using a small plastic ruler. The cassette was closed and placed in a tank (containing ice for cooling) and submerged in cold transfer buffer. The transfer process was done at 300 mA for 1 hour. Successful protein transfer can be indicated by the appearance of multiple visible coloured protein markers on PVDF membrane.

#### 2.6.4 Immunoblotting for Western Blot

The PVDF membrane was covered with Ponceau S stain to check for protein transfer or washed with PBS-T (0.1% Tween 20) straight away. After washing, the membrane was blocked with

5% milk (Bio-Rad) in PBS-T for 1 hour with agitation at room temperature. The membrane was then washed with PBS-T for 5 mins and probed with 2.5  $\mu$ l primary antibody (rabbit anti-HA tag ab9110, Abcam) in 5% milk containing PBS-T for 1 hour with agitation. After probing, membrane was washed twice for 10min in PBS-T. The membrane was probed with 1 $\mu$ l secondary antibody (Amersham™ ECL™ Anti-Rabbit IgG [Fisher Scientific]) in 5% milk containing PBS-T for 1 hour with agitation. The membrane was then washed twice for 15 mins in PBS-T and washed in sterile PBS for 15 mins.

#### 2.6.5 PVDF membrane fluorescent imaging

The fluorescent signal on the membrane was developed by fully covering the membrane with Pierce™ ECL Western Blotting Substrate (1 ml each) for 5min with agitation. Initial exposure was performed and imaged using Chemidoc imaging system (Bio-Rad), and longer exposures were performed if imaging was not clear.

## 2.7 Phenotypic techniques

### 2.7.1 Growth rate measurement (liquid)

*Candida* strains were grown overnight in 4ml YEPD medium (unless otherwise stated) in a shaking incubator set at 37°C, 200 rpm. On the following day, the OD<sub>600</sub> value of the overnight culture was measured. Cells were washed in sterile water and a suspension was prepared in fresh YEPD at an OD<sub>600</sub> of 0.1 in triplicate (120 µl for each condition tested) in a 96 well flat bottom plate. The plate was incubated in FLUOstar Omega Microplate reader for 24h at 200 rpm, 37°C and the Microplate reader will measure and record the OD<sub>600</sub> value of all wells every 1 hour. Growth data was then exported to Microsoft Excel and the growth values were calculated manually, and graphically represented using GraphPrism ver. 10 (San Diego, California, USA).

### 2.7.2 Broth microdilution assays

A 96 flat bottom well plate was prepared containing a 2-fold dilution series of a specific stressor/antifungals from columns 2 to 11 in a total volume of 100µl. The first column (no. 1) acted as negative control (no cells, just media) and the last column (column 12) acts as positive growth control (only cells without stressors/antifungals). *Candida* strains were first grown in 4ml YEPD medium overnight in a shaking incubator set at 200 rpm, 37°C. The next day, the OD<sub>600</sub> value of the overnight culture was measured and standardized to 1x10<sup>6</sup> cells/ml in RPMI or YEPD medium as indicated. The serial dilution of specific stressors/antifungals was then carried out by adding 200 µl of stressors/antifungals into column 2 and 100 µl of medium into columns 3 to 12. Column 2 was then 2-fold diluted by transferring 100 µl to the next column until column 11. A 10 µl volume of standardized culture was then added into columns 2 to 12 after removing 10 µl of solution from the plate, making a total of 100µl in each well. The plate was incubated in a static incubator for 24 hours at 37°C. After 24 hours, the growth of each

well was measured and determined by FLUOstar Omega Microplate reader. Initial data was then exported to Microsoft Excel and the minimum inhibitory concentration (MIC) values were calculated manually, and graphically represented using GraphPrism ver. 10 (San Diego, California, USA).

### 2.7.3 Spot plate assays

*Candida* strains were grown overnight in 4ml YEPD medium in a shaking incubator set at 37°C, 200 rpm. On the following day, the OD<sub>600</sub> value of the overnight culture was measured, washed in sterile water once, and diluted in sterile water to a cell density of  $2 \times 10^6$  cells/ml using spectrophotometric cell counting method described in Section 2.2.5. Serial 10-fold dilutions were performed to a final dilution step containing  $2 \times 10^2$  cells/ml. The series of diluted cell suspensions were all plated onto YEPD agar plates in volumes of 4µl using a multichannel pipette. YEPD agar plates contained various stressors/antifungals of interest and was compared with YEPD agar plates without any stressors/antifungals to observe inhibition of growth. All plates were incubated in a static incubator for 2 days at 30°C or 37°C. The plates were imaged using a Flash and Go plate visualiser connected to a PC.

### 2.7.4 E-test assays

*Candida* strains were grown overnight in 4ml YEPD medium in a shaking incubator set at 37°C, 200 rpm. On the following day, the OD<sub>600</sub> value of the overnight culture was measured, the cells were washed in sterile water once, and diluted in sterile water to a cell density of  $1 \times 10^6$  cells/ml using spectrophotometric cell counting method described in Section 2.2.5. A sterile swab was dipped into a diluted cell mixture ( $1 \times 10^6$  cells/ml), rotated against the tube wall, and then streaked all over the RPMI plate twice, rotating the plate 30° clockwise each time. The

plate was left without lid for 5 mins to remove moisture. After drying, a sterile forceps was used to take out a E-test strip containing antifungal of interest and the strip was placed upwards (visible scale) on the inoculated agar plate. The plate was incubated for 2 days at 37°C or 30°C. All plates were imaged using a Flash and Go plate visualiser connected to a PC.

#### 2.7.5 Disk diffusion assays

*Candida* strains were grown overnight in 4ml YEPD medium in a shaking incubator set at 37°C, 200 rpm. On the following day, the OD<sub>600</sub> value of the overnight culture was measured, washed in sterile water once, and diluted in sterile water to a cell density of  $1 \times 10^6$  cells/ml using spectrophotometric cell counting method described in Section 2.2.5. Similar to the E-test assay, a sterile swab was dipped into a diluted cell mixture ( $1 \times 10^6$  cells/ml), rotated against the tube wall, and then streaked all over the RPMI plate twice, rotating the plate 30° clockwise each time. The plate was left without lid for 5 mins to remove moisture. After drying, a sterile forceps was used to take out a disk containing antifungal of interest and the disk was placed upwards (visible scale) on the inoculated agar plate. The plate was incubated for 2 days at 37°C or 30°C. All plates were imaged using a Flash and Go plate visualiser connected to a PC.

## **Chapter 3**

CRISPR-Cas9 gene editing in *Candida albicans* and *Candida auris*

### 3.1 Introduction

#### 3.1.1 The discovery of CRISPR-Cas system

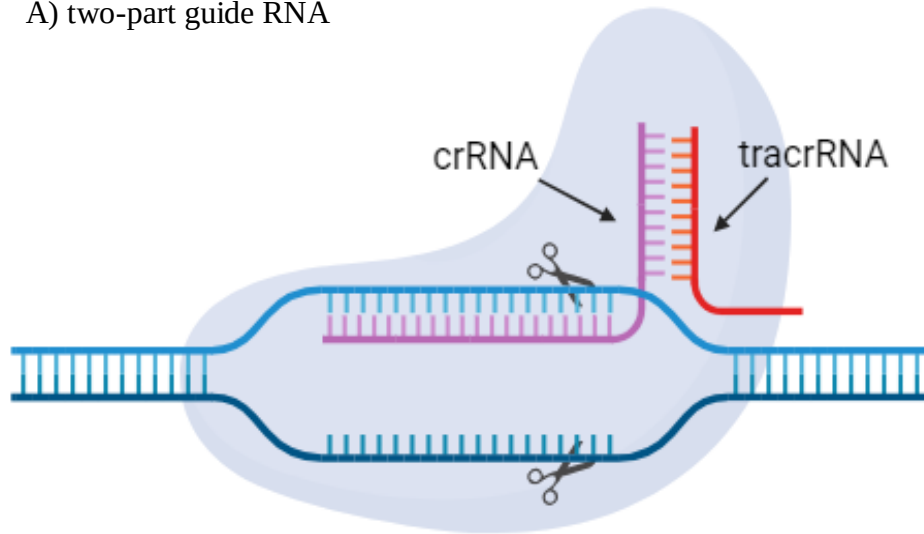
CRISPR, known as clustered regularly interspaced short palindrome repeats, were first discovered in the DNA sequence of a common bacterium, *Escherichia coli*, by Ishino *et al.* (1987) in Japan. However, the function of CRISPR remained unknown at that time. CRISPR was found to have a high degree of polymorphism in different strains of the same species of pathogenic bacteria and was used for molecular strain typing (Groenen *et al.*, 1993; Hoe *et al.*, 1999). A significant breakthrough in our understanding the biological function of CRISPR loci was later made by Francisco Mojica of the University of Alicante (Spain), who noticed that DNA repeats described in *Escherichia coli* were matching parts of the archaeal DNA sequence of *Haloferax mediterranei* (Mojica *et al.*, 2005). Based on her findings, Mojica become the first person to hypothesize that these unusual loci containing fragments of foreign DNA were a part of an “immune system” of bacteria and archaea. Later studies discovered the presence of genes associated with CRISPR repeats containing motifs characteristic of helicases and nucleases, suggesting their involvement in DNA metabolism (Jansen *et al.*, 2002). Currently, CRISPR repeats have been described in most archaeal and nearly half of the studied bacterial genomes, but not in eukaryotic or viral DNA sequences.

Proteins associated with the CRISPR system were known as Cas proteins and among all of them, the most studied ones are the proteins involved in directional cutting of foreign DNA (in some cases, RNA). The most studied protein is the nuclease Cas9 (originally named as Cas5), which contains characteristic HNH (His-Asn-His) motifs that are common to nucleases (Bolotin *et al.*, 2005). The same study by Bolotin *et al.*, 2005 also discovered a specific pattern in the nucleotide sequences on one side of the described spacers of the CRISPR, now known as the

PAM (protospacer adjacent motif) sequence, which if present in a foreign DNA fragment will target it for destruction by Cas proteins.

The final remaining puzzle required for the whole CRISPR-Cas9 system to work efficiently is the presence of the sgRNA (single guide RNA), a combination of crRNA (a short sequence that targets PAM sequence) and tracrRNA, a RNA fragment that binds to the Cas9 protein (Figure 3.1.1). The initial transcription of the CRISPR locus was found to produce a pre-crRNA precursor molecule consisting of several spacers and repeats, which is later cleaved into individual fragments. Later studies revealed that the actual crRNA length is around 20 nucleotides in size which complements the target DNA that targets the Cas9 enzyme to attack foreign DNA. The other sgRNA component, the tracrRNA was later discovered to have association with crRNA and was involved in the mechanism of target DNA cutting by Deltcheva *et al.*, 2011. The tracrRNA is required for triggering pre-crRNA processing by RNase III and activating crRNA-guided DNA cleavage by Cas9. Most gene-editing systems employ sgRNA (single guide RNA), a combination of crRNA and tracrRNA, which greatly promoted the practical use of CRISPR-Cas9 system in various gene editing applications (Jinek *et al.*, 2012).

A) two-part guide RNA



B) single guide RNA

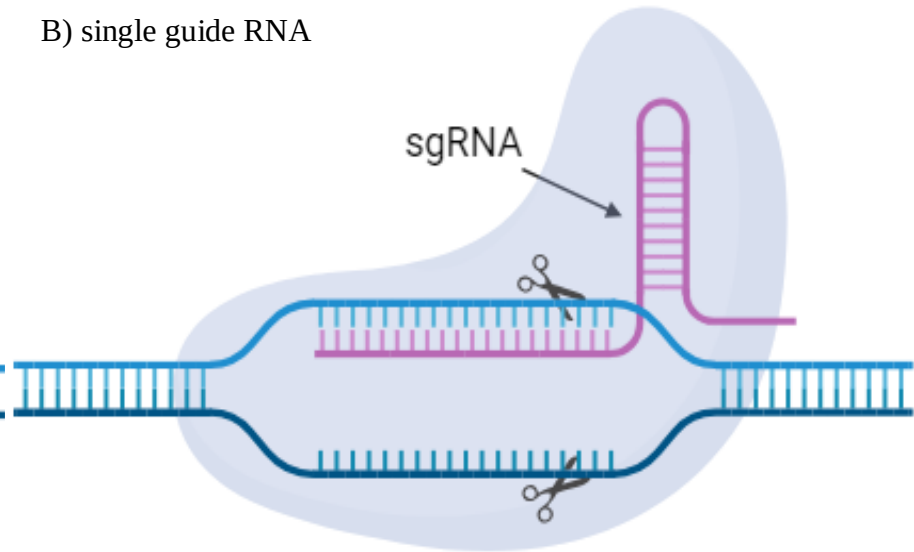


**Figure 3.1.1. Diagram of two-part guide RNA and single guide RNA structures.**

A) The two-part guide RNA consists of a duplex of a crRNA (purple) and a tracrRNA (red). B) The single guide RNA consists of a sgRNA (purple) which is the fusion of crRNA and tracrRNA.

### 3.1.2 CRISPR-Cas9 gene editing

The CRISPR-Cas9 method that was applied in this study belongs to type II CRISPR system that uses Cas9 to cut off desired DNA targets. Cas9, the major component of the type II CRISPR system, is an endonuclease with two different domains, with one containing an HNH nuclease domain that cleaves the complementary DNA strand and the other containing RuvC-like domain that cleaves the non-complementary DNA strand. The type II system also relies on either a crRNA/tracrRNA pair or a chimeric sgRNA that can recognize a PAM containing DNA target sequence via the crRNA to recruits and activate Cas9 to cleave the DNA target (Jinek *et al.*, 2012) (Figure 3.1.2).

After the double-stranded break (DSB) is created by Cas9 at the DNA target with the PAM, the damaged DNA can be repaired via 2 mechanisms: homology directed repair (HDR) and non-homologous end joining (NHEJ). NHEJ repairs DSBs by joining the DNA fragments through an enzymatic process that does not require exogenous homologous DNA. However, this process is prone to small random insertions or deletions at the cleavage site. Conversely, HDR is more precise and uses a homologous DNA template to repair cleaved DNA. CRISPR-Cas9 gene editing can take advantage of HDR mechanisms to introduce a DNA template (commonly known as a repair template) with homology to the predicted DSB site but also containing specific mutations. However, a large amount of repair template is required for HDR to be carried out (Asmamaw and Zawdie, 2021). A schematic of the HDR mechanism after DSB introduction by Cas9 is shown in Figure 3.1.3.

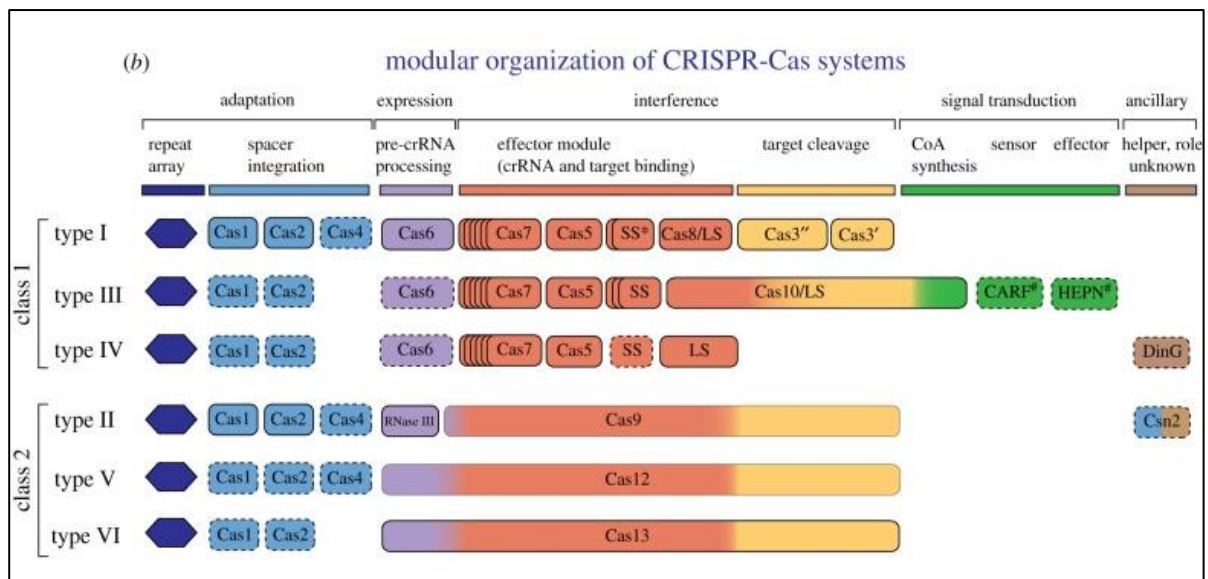


**Figure 3.1.2. The classification of CRISPR-Cas systems.**

CRISPR-Cas systems were split into 2 classes and 3 types in each class. Class 1 has multiple effector proteins whereas Class 2 only has a singular large effector protein. The colour highlights the function of a particular protein at different stages of CRISPR-Cas system. Figure adapted from (Koonin and Makarova, 2019).

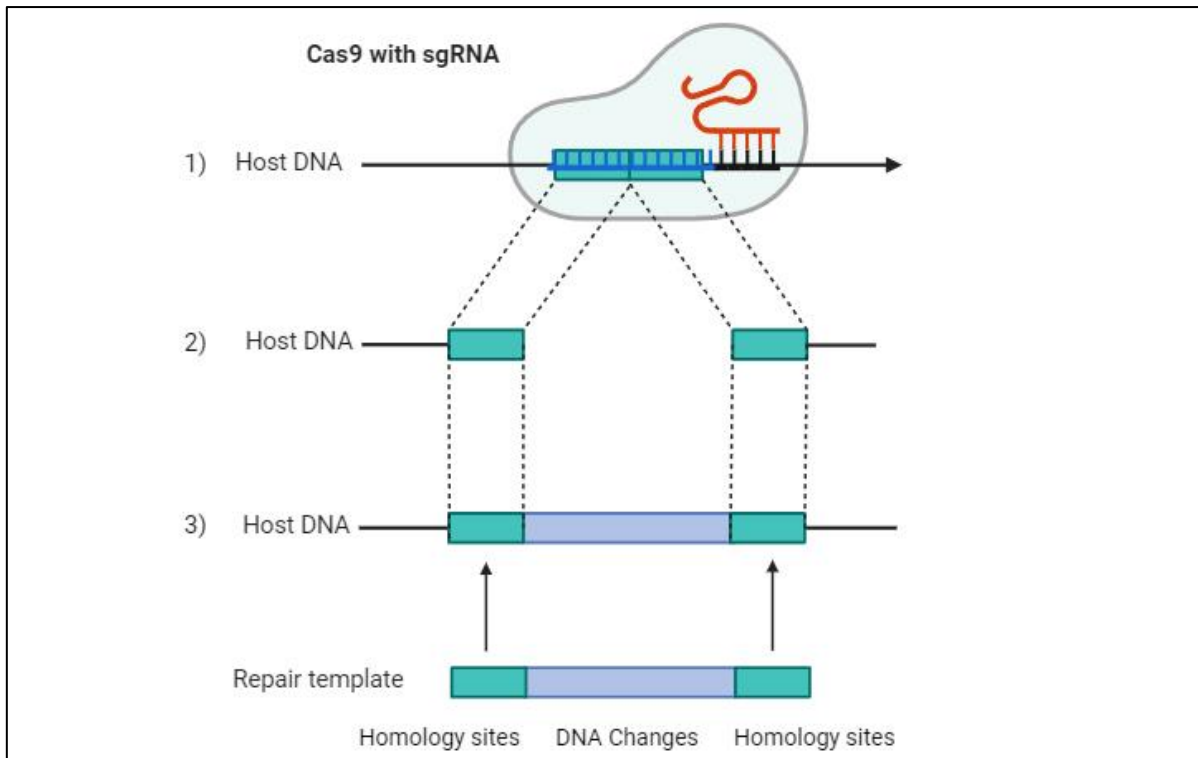


**Figure 3.1.3. The basic HDR mechanism after CRISPR-Cas9 on one DNA strand.**

The general concept of CRISPR-Cas9 editing relying on HDR after Cas9 excision at the target DNA is outlined in this figure. The HDR mechanism used for the insertion of repair template was done in 3 steps. 1) The Cas9 complex with sgRNA (Red is crRNA) recognise and bind to its target DNA. 2) Cas9 will then introduce DSB at the target DNA. 3) The repair template with 2 homology sites (green) at both ends and DNA with changes (blue) will be used to stitch the cleaved DNA via HDR.

### 3.1.3 CRISPR-Cas9 systems for *Candida albicans* and *Candida auris* genome editing

The first application of CRISPR-Cas9 to gene editing in *C. albicans* was published in 2015, in which a Cas9 nuclease and sgRNA system from *S. cerevisiae* was optimised for use in *C. albicans* (Vyas *et al.*, 2015). The adaptation was carried out by modifying the *CAS9* nuclease encoding gene to avoid the misincorporation of serine amino acids into the nuclease enzyme of *C. albicans* due to alternative CTG codon usage. A CRISPR-Cas9 plasmid system was designed to integrate into the *C. albicans* genome containing the *CAS9* gene and the endogenous RNA polymerase III promoter, *pSNR52*, which was used to express the cloned sgRNA. This “solo system” was shown to have a high transformation frequency of 60 ~ 80%. This system could generate homozygous mutations in this diploid yeast, and was shown to be capable of mutating several copies of a multigene family in a single transformation (Vyas *et al.*, 2015). The publication of this novel CRISPR-Cas9 system paved the future pathway of CRISPR-Cas9 editing in *C. albicans*.

### 3.1.4 The LEUpOUT CRISPR-Cas9 system for gene editing in *C. albicans* and *C. auris*

In this study, we opted to use the LEUpOUT CRISPR-Cas9 system for *C. albicans* (Nguyen *et al.*, 2017) and *C. auris* (Ennis *et al.*, 2021) as they utilise the same CRISPR-Cas9 plasmid constructs and contain a recyclable marker. This system was also used in our laboratory by Fletcher *et al.* to delete the *TLO* gene family in *C. albicans* (Fletcher *et al.*, 2023).

The LEUpOUT CRISPR-Cas9 system works the same way in both *C. albicans* and *C. auris*. The system works by introducing two overlapping DNA fragments, one with the gRNA cassette and a second encoding Cas9, into the cell *via* transformation. Both cassettes encode overlapping halves of the nourseothricin resistance gene *NAT1*, which are combined *in vivo* by homologous recombination to form a functional recyclable marker. The cassette also has homology to the

chromosomal *LEU2* gene to allow the integration of the full-length CRISPR-Cas9 cassette into the *LEU2* gene. Integration of the cassette into the *LEU2* gene should render the transformants *NAT<sup>R</sup>* and auxotrophic for leucine. To achieve this in *C. albicans* requires a *LEU2/Δleu2* heterozygous background whereas any *C. auris* strain can be used as it is a haploid yeast. To recycle the cassette, the LEUpOUT method is employed where *LEU<sup>-</sup>/NAT<sup>R</sup>* transformants are grown on media without leucine to select for reconstitution of the *LEU2* gene which would necessitate excision of the whole CRISPR-Cas9 cassette containing the *NAT1* marker, resulting in clean *LEU<sup>+</sup>/NAT<sup>S</sup>* transformants that can be transformed again.

The whole LEUpOUT CRISPR-Cas9 schematic workflow for *C. albicans* (Nguyen *et al.*, 2017) and *C. auris* (Ennis *et al.*, 2021) is described in Figure 3.1.4. Several plasmids were designed specifically for *C. albicans* and *C. auris* to generate gRNA and Cas9 cassettes. Both *C. albicans* and *C. auris* LEUpOUT systems shared the same gRNA and Cas9 construct that integrates into *LEU2* and is recycled via the LEUpOUT method. All the DNA fragments required for CRISPR-Cas9 can be amplified using the given plasmids (Table 2.3). The “A fragment” contains half of the *NAT1* marker and the *SNR52* promoter (to drive gRNA expression) whereas the “B fragment” contains the unique gRNA that targets the gene of interest and the remaining half of the *NAT1* marker. The unique gRNA is incorporated into the oligonucleotide used for B fragment amplification. Both A and B fragments are stitched via overlapping PCR to form the “C fragment” (gRNA cassette; Figure 3.1.4). The linear Cas9 fragment was obtained by digesting the Cas9 plasmid with the *MssI* restriction enzyme. The gRNA cassette and the Cas9 fragment are then transformed together into *Candida* cells. The gRNA cassette and Cas9 fragment are joined by HDR mechanisms via the overlapping marker sequences and will be integrated into the singular *LEU2* via HDR. The whole recyclable cassette can subsequently be “flipped out” after growing positive transformants on media without leucine, which selects for

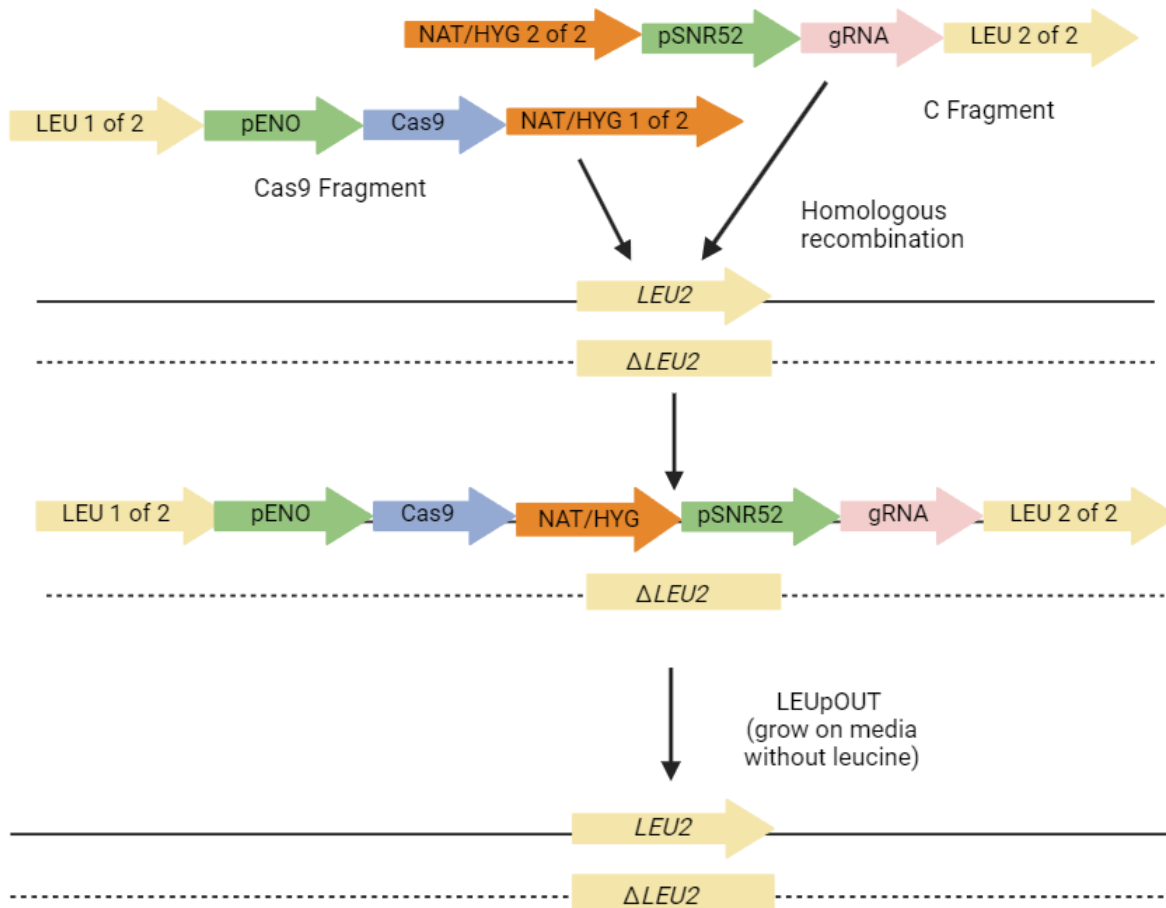


**Figure 3.1.4. CRISPR-Cas9 LEUpOUT system for *C. albicans* and *C. auris* genome editing.**

The whole CRISPR-Cas9 LEUpOUT system designed by (Nguyen *et al.*, 2017) for *C. albicans* and further adapted by (Ennis *et al.*, 2021) for *C. auris*. Both the Cas9 fragment and the C fragment (gRNA cassette) were co-transformed into *C. albicans* (heterozygous *LEU2/leu2Δ* strain) and *C. auris*. Homologous recombination allows the fusion of the two fragments via overlapping NAT/HYG marker sequences (each contain half of NAT/HYG gene). The whole CRISPR-Cas9 construct was then integrated into *LEU2* and later removed by selection for *LEU2* prototrophic revertants following growth on leucine free media.

revertants that have restored the *LEU2* locus by excision of the cassette.

### 3.1.5 Aim of this study

The aim of this study is to investigate Mediator tail function in *C. albicans* and *C. auris*. To study the role of Mediator tail in the function of the Tac1 transcription factor, we used CRISPR-Cas9 to introduce the homozygous *TAC1*-5 point mutation into *C. albicans* strains (WT, *tlo* $\Delta$  and *med3* $\Delta$ ) to render the Tac1 protein hyperactive. We then investigated the function of hyperactive Tac1 in the *tlo* $\Delta$  and *med3* $\Delta$  backgrounds compared to WT.

For *C. auris*, CRISPR-Cas9 was used to delete the *MED2* and *MED15* Mediator tail subunit encoding genes, orthologous to *C. albicans* *TLO2* and *MED15*, to find out if these genes were also involved in pathogenicity and drug resistance in *C. auris*.

## 3.2 Materials and Methods

### 3.2.1 Design and construction of the CRISPR-Cas9 oligonucleotides

The CRISPR-Cas9 LEUpOUT system designed for *C. albicans* (Nguyen *et al.*, 2017) was used to introduce a homozygous *TAC1*-5 SNP into the *C. albicans* strains WT MAY1244, *tlo* $\Delta$  and *med3* $\Delta$ , all of them are *LEU/leuA* derivatives of the WT strain SC5314. The gRNA was designed to generate the *TAC1*-5 allele, introducing an A to G substitution at nucleotide position 2929 in *TAC1*. A unique 20 bp gRNA (ACAATAATTTGGGGATTAA; position 2929 shown in bold) was identified in *TAC1* and was adjacent to the PAM sequence TGG. This sequence was entered into the “gRNA oligonucleotide calculator” that can be found in the supplementary material of the LEUpOUT paper (Nguyen *et al.*, 2017) to incorporate flanking oligonucleotide sequences required for cassette amplification and stitching (*TAC1*-5 gRNA - CGTAAACTATTTTAAATTTG**ACAATAATTTGGGGATTAA**AGTTTGTAGAGCTAGAAATAGC). To repair the cut in the *TAC1* gene and introduce the A to G substitution, a repair template homologous to the guide RNA was generated. To amplify this repair template, two oligonucleotides (57 bp each) spanning the gRNA were designed that overlapped by 20 bp at their 3' end and contained the A to G substitution at nucleotide position 2929. Each primer also had an additional 37 bp of unique upstream and downstream sequence at the 5' end. Both overlapping primers were combined in a PCR reaction to generate a 94 bp double stranded fragment identical to the MAY1244 *TAC1* allele apart from the A to G substitution at nucleotide position 2929.

To delete *MED2* and *MED15* in *C. auris* strains NCPF8985 (clade I, weakly aggregative) and NCPF8977 (clade III, aggregative), the adapted CRISPR-Cas9 LEUpOUT system for *C. auris* was used (Ennis *et al.*, 2021). The *C. auris* orthologues of *MED2* (B9J08\_001249) and *MED15* (B9J08\_003619) were first identified in the *C. auris* B8441 reference genome as the closest

orthologues of *C. albicans* *TLO2* and *MED15*. Unique 20bp gRNAs targeting the *C. auris* *MED2* and *MED15* genes were designed by entering the sequences of *MED2* (B9J08\_001249) and *MED15* (B9J08\_003619) into Benchling (<https://www.benchling.com/>) as described by Ennis *et al.* (2021). An ideal gRNA was identified with the highest “On-Target Score” (>60+) and an “Off-Target Score” as close to 50 as possible. The unique gRNAs designed to target *MED2* and *MED15* were then entered into the gRNA oligonucleotide calculator to append flanking sequences required for cassette amplification and stitching PCR reactions (Ennis *et al.*, 2021). The *MED2* and *MED15* repair templates were designed to delete the ORFs and introduce the sequence 5'- CGAGACGAGTGCTCGACATGAGG (referred to as the ‘AddTag’) which facilitates gene complementation after gene knockout. To facilitate this, 200 bp of upstream sequence was amplified with primers MED repair F1/R1 (Table 2.1) and 200 bp of downstream sequences were amplified with primers MED repair F2/R2 (Table 2.1). The 3' ends of primers R1 and F2 also incorporated the AddTag sequence which allowed fusion of the upstream and downstream PCR fragments to generate a ~400 bp repair template that contains 200bp homology to the upstream and downstream regions of *MED2* or *MED15*. Introduction of these repair templates to repair the DSBs generated by the Cas9 enzyme was expected to replace *MED2* and *MED15* with the non-functional 23bp AddTag. The subsequent complementation of the *MED15* deletion was carried out using a gRNA that targets the AddTag sequence in combination with a *MED15* complementary repair template containing the *MED15* ORF and ~200bp upstream and downstream DNA sequence. The hygromycin resistance *HYG* marker was used instead of *NAT1* for *MED15* complementation.

### 3.2.2 The generation of the gRNA cassette

To generate the gRNA cassette for *C. albicans*, A and B fragments were amplified from plasmid pADH119 and pADH110, respectively (Figure 3.2.1). Primers AHO1096 and AHO1097 (listed in Table 2.1) were used to amplify the A fragment using the Phusion PCR system (Thermo-Fisher Scientific) in a 50 µl reaction mix (1x Phusion HF buffer, dNTPs [200µM each], 0.25 µl Phusion High-Fidelity DNA polymerase, 0.5 µM of each oligonucleotide primer [AHO1096 and AHO1098}, and 1 ng of template DNA (pADH110) using the following conditions: initial denaturing at 98°C for 30s, denaturation at 98°C for 20s, annealing at 58°C for 20s, amplification at 72°C for 30s for 30 cycles, with a hold at 4°C until retrieval and storage at -20°C.

The B fragment incorporating the unique gRNA sequence was amplified from plasmid pADH119 using the custom-designed gRNA oligonucleotide and the AHO1097 primer (shown in Table 2.1) via touchdown PCR. Touchdown PCR was carried out in a 20 µl volume (1x Phusion HF buffer, dNTPs [200 µM each], 0.2 µl Phusion High-Fidelity DNA polymerase, 0.5 µM of each oligonucleotide primers [AHO1097 and custom gRNA oligonucleotide], and 0.2 ng of template pADH119) using the following conditions: initial denaturing at 98°C for 30s, denaturation at 98°C for 20s, annealing at 65°C for 20s, amplification at 72°C for 30s, and cycling between the last three steps for 10 cycles and the annealing temperature was reduced by 1°C after each cycle. After the PCR run, samples were hold at 4°C until retrieval and storage at -20°C.

The C fragment was generated by fusing A and B fragments via stitching PCR (Figure 3.2.3). The amount of C fragment generated from this protocol is sufficient for 2 transformations. The stitching and amplification of C fragment was carried out in 100 µl volumes (1x Phusion HF

buffer, dNTPs [200µM each], 0.5 µl Phusion High-Fidelity DNA polymerase and 1µl each of the A and B fragment) using the following conditions: initial denaturing at 98°C for 30s, denaturation at 98°C for 20s, annealing at 58°C for 20s, amplification at 72°C for 1min for 5 cycles, with a hold at 4°C until the downstream PCR amplification step. After the stitching PCR, the C fragment was amplified with the addition of 0.5µl of each oligonucleotide (100 µM AHO1097 and custom gRNA oligonucleotide) and further rounds of PCR amplification as follows: initial denaturing at 98°C for 30s, denaturation at 98°C for 20s, annealing at 66°C for 20s, amplification at 72°C for 1min for 30 cycles, with a hold at 4°C until retrieval and storage at -20°C.

For the generation of a gRNA cassette adapted for *C. auris*, A and B fragments were fused together to form a C fragment with similar methods described above (Figure 3.2.2). Both A and B fragments (*NAT1* marker) can be amplified from plasmids pCE27 and pCE35, respectively, using primers AHO1096 and CJNO3235 to amplify the A fragment and the gRNA oligonucleotide and AHO1097 to amplify the B fragment. The stitching PCR to generate and amplify C fragment was the same as described for *C. albicans* using CJNO3755 as the C fragment forward primer together with universal C fragment reverse primer CJNO3080 to amplify the C fragment.

After PCR amplification of C fragments, all samples were cleaned up as described in Section 2.3.7 prior to cell transformation.

### 3.2.3 The generation of the Cas9 cassette

The Cas9 cassette for *C. albicans* was released from plasmid ADH137 and the Cas9 cassette for *C. auris* from plasmid pCE35 (NAT) following *MssI* digestion. The plasmid digestion was performed in a total volume of 10 µl, with 0.5 µl *MssI* enzyme, 1x FastDigest Buffer, and 2µg of plasmid DNA. The digestion mixture was incubated at 37°C for 3 hours to ensure complete plasmid digestion. The release of linear Cas9 cassette was shown in Figure 4.1.3 (A) and (B). After plasmid digestion, the size of the desired linear Cas9 cassette was verified via gel electrophoresis (see Section 2.3.4) and subjected to PCR fragment cleanup (see Section 2.3.7).

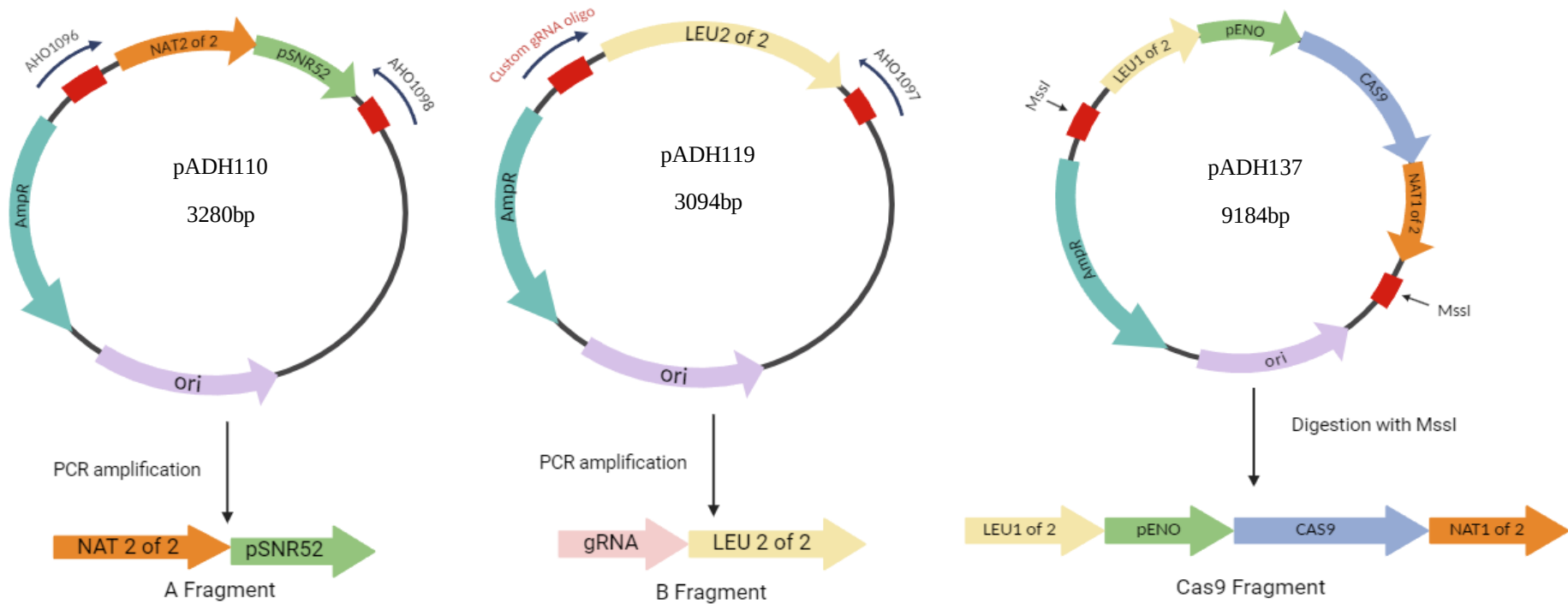


**Figure 3.2.1. Plasmid with NAT marker designed for *C. albicans* CRISPR-Cas9 LEUpOUT method.**

*C. albicans* specific plasmids (*NAT1*) containing Cas9, A and B fragments can be obtained either via PCR amplification or enzymatic digestion.

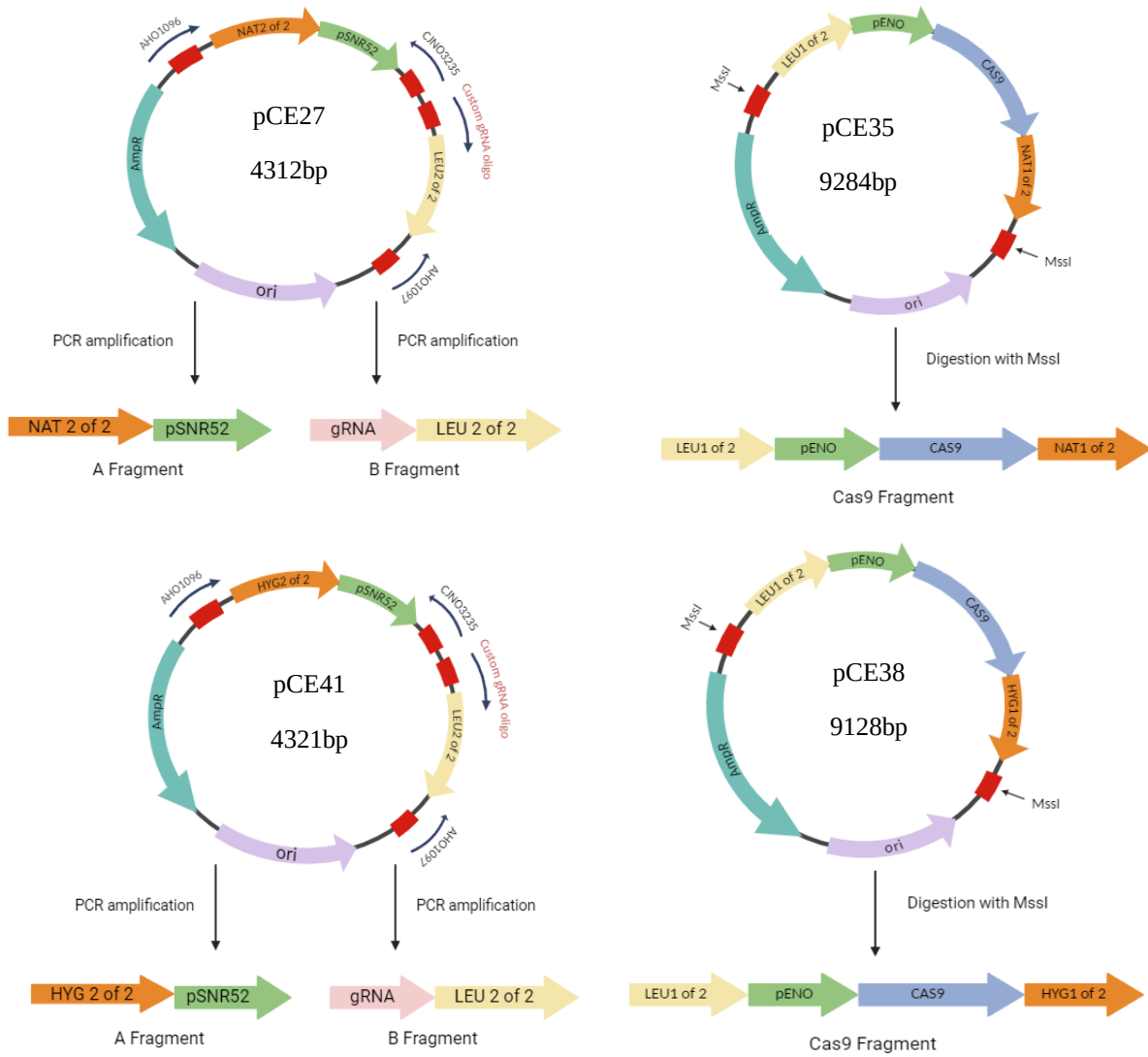


**Figure 3.2.2. Plasmid with NAT/HYG marker designed for *C. auris* CRISPR-Cas9 LEUpOUT method.**

pCE27 and pCE35 plasmids consist of the nourseothricin (NAT) marker whereas pCE41 and pCE38 plasmids have the hygromycin (HYG) marker. *C. auris* specific plasmids (NAT/HYG) containing Cas9, A and B fragments can be obtained either via PCR amplification or enzymatic digestion.

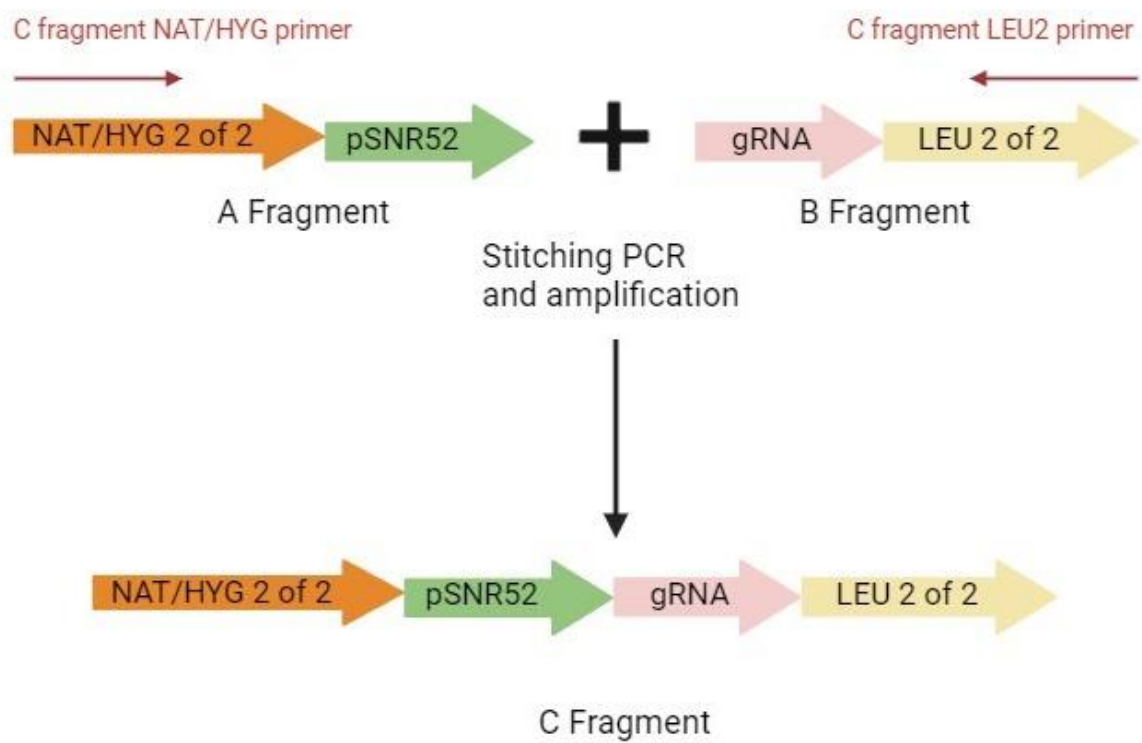


**Figure 3.2.3. The formation of C fragment (gRNA cassette) for CRISPR-Cas9 LEUpOUT system.**

DNA ligation process via PCR of A and B fragments (NAT/HYG) to form C fragment (gRNA cassette).

### 3.2.4 Transformation of the Cas9 and gRNA cassette into *C. albicans* and *C. auris*

The transformation of the CRISPR-Cas9 system into *C. albicans* was performed using the electroporation method (see Section 2.5.1). Transformation of the CRISPR-Cas9 system into *C. auris* was carried out using the heat shock method (see Section 2.5.2). Complementation of *MED15* in NCPF8977 was done using the electroporation method (see Section 2.5.1). For the electroporation method, 500-1000 ng of Cas9 cassette, gRNA cassette and repair template were transformed into *C. albicans* and *C. auris*. For the heat shock method, ~3000 ng of Cas9 cassette, gRNA cassette and repair template were transformed into *C. auris* in a 50 µl total volume. After transformation, positive *C. albicans* transformants were selected on YEPD agar with 200 µg/ml nourseothricin (NAT200) whereas *C. auris* transformants were selected onto YEPD agar with NAT250 or 400 µg/ml hygromycin (HYG400).

### 3.2.5 Confirmation of desired *C. albicans* and *C. auris* transformants

*C. albicans* transformants with homozygous *TAC1*-5 mutations were confirmed via Sanger sequencing (Section 2.3.8). The DNA sequence around the homozygous *TAC1*-5 mutation was amplified via the primers *TAC1* ampF and *TAC1*-5 R (Table 2.1) yielding a 440bp DNA product which was sent for Sanger sequencing. The deletion of *MED2* and *MED15* in *C. auris* strain 8977 were verified via colony PCR (see Section 2.3.6) using primers that flank the *MED2* gene (Med2 out F1 and Med2 out R1; Table 2.1) or *MED15* gene (Med15 out F1 and Med15 out R1; Table 2.1). Due to the larger size of the *MED15* gene, additional checks were carried with a primer that binds upstream of the ORF in conjunction with a primer that binds to internal (deleted) sequences of the *MED15* ORF (Med15 up2 F1 and Med15 up2 R2; Table 2.1). Sanger sequencing was also used to confirm *MED2* and *MED15* deletions in *C. auris* strain 8985 (Section 2.3.8) as colony PCR was unable to clearly identify positive transformants of 8985. In this case, forward and reverse oligonucleotides were designed to amplify the *MED2* and

*MED15* sequences with positive transformants yielding an amplicon of approximately 1kb which was sent for Sanger sequencing (primers listed in Table 2.1).

The complementation of *MED15* in strain 8977 was verified via colony PCR (see Section 2.3.6) using two sets of primers, which one of which flanks the *MED15* ORF (Med15 out F1 and Med15 out R1) and another that spans the internal ORF sequences and downstream flanking sequences of *MED15* (Med15 dn1 F1 and Med15 dn1 R1). All the primers designed to verify all *C. albicans* and *C. auris* transformants were listed in Table 2.1.

### 3.2.6 Recycling of the CRISPR-Cas9 system from the *LEU2* gene

The recycling step was carried out on the same day as colony PCR was performed to detect positive transformants. Positive *C. albicans* transformants were streaked on YNB plates without amino acids or ammonium sulphate (leucine negative media) and incubated at 30°C for 2-3 days. Positive *C. auris* transformants were plated on YNB without amino acids or ammonium sulphate supplemented with Yeast Synthetic Drop-out Medium Supplements (Sigma-Aldrich) and incubated at 30°C for 2-3 days. Colonies that grew on plates without leucine and exhibited nourseothricin or hygromycin sensitivity were considered as desired mutants that had excised the CRISPR-Cas9 components from the *LEU2* locus.

### 3.3 Results

#### 3.3.1 Confirmation of homozygous *TAC1*-5 point mutations in MAY1244, *tlo* $\Delta$ and *med3* $\Delta$ mutants

CRISPR-Cas9 generated transformants with homozygous *TAC1*-5 alleles were verified by Sanger sequencing (see Section 2.3.8) using primers TAC1 ampF and Tac1-5R (Table 2.1) that amplify DNA sequences around *TAC1* codon 977 (product ~500bp). From a total of approximately 20 transformants from each transformation, one positive homozygous *TAC1*-5 mutant and one heterozygous *TAC1*-5 mutant were identified following Sanger sequencing. Sanger sequencing chromatograms showed the presence of homozygous *TAC1*-5 alleles in *C. albicans* MAY1244, *tlo* $\Delta$  and *med3* $\Delta$  mutants, indicated by the single base pair substitution from A to G at position 2929 bp in the *TAC1* gene (Figure 3.3.1).

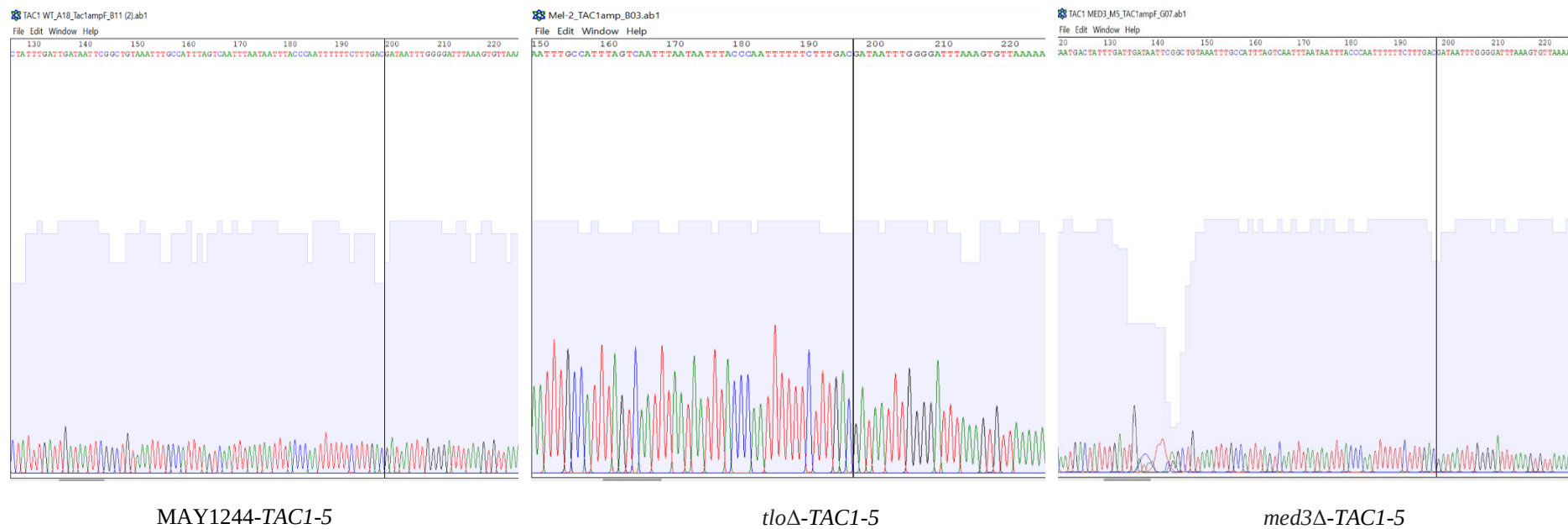


**Figure 3.3.1. Sanger sequencing data of *C. albicans* WT (MAY1244), *tloΔ* and *med3Δ* null mutants with *TAC1-5* mutation.**

Homozygous *TAC1-5* was shown in WT (MAY1244), *tloΔ* and *med3Δ* null mutants. The base after the vertical black line in each panel corresponds to position 2929 in the *TAC1* ORF having undergone an A to G substitution in each case.

### 3.3.2 Confirmation of the deletion of *MED2* and *MED15* in *C. auris*

The deletion of *MED2* and *MED15* in *C. auris* strain 8977 was verified via colony PCR (Figure 3.3.2). Using primers that flanked *MED2*, we showed that the wild-type generated a PCR product of the expected size of ~2000 bp whereas the *med2*Δ8977 exhibited a truncated DNA product size of 847bp (Fig. 3.3.2, Lanes 1 & 2). The deletion of *MED15* was also confirmed via PCR using flanking primers and primers that target an internal region (Med15 up2 primer set). Using primers that flanked *MED15*, we showed that the wild-type generated a PCR product of the expected size of ~4000 bp whereas *med15*Δ8977 exhibited a truncated DNA product size of 847bp (Fig. 3.3.2, Lanes 5 & 6). Due to the weak amplification of the wild-type gene in strain 8977 (Lane 5), we confirmed the absence of the *MED15* ORF in the *med15*Δ8977 mutant using an internal primer set (Med15 up2 F1/R1, Table 2.1). Using these primers, the WT strain yielded a product similar to the predicted size of 999bp whereas the *med15*Δ mutant did not yield an amplification product. The later complementation of *MED15* in *med15*Δ8977 was also confirmed via colony PCR. Using the *MED15* flanking primers, the complemented *med15*Δ8977 mutant yielded an identical sized band compared to WT 8977 (Fig. 3.3.2, Lanes 7 & 8). Multiple weak bands were present in lane 7 and 8 possibly due to impurities in colony PCR. The complemented *med15*Δ8977 mutant was further confirmed using another *MED15* internal primer set Med15 dn1 F1/R1 (downstream internal region, Table 2.1), which the complemented *med15*Δ8977 mutant yielded an identical sized band compared to WT 8977 (Figure 3.3.2).

PCR confirmation of the deletion of *MED2* and *MED15* in *C. auris* strain 8985 produced more ambiguous results. For this reason, the structure of the *med2*Δ and *med15*Δ loci were confirmed via Sanger sequencing. The Sanger sequencing data confirmed the presence of the AddTag sequence in place of the *MED2* and *MED15* ORFs in strain 8985, surrounded by the

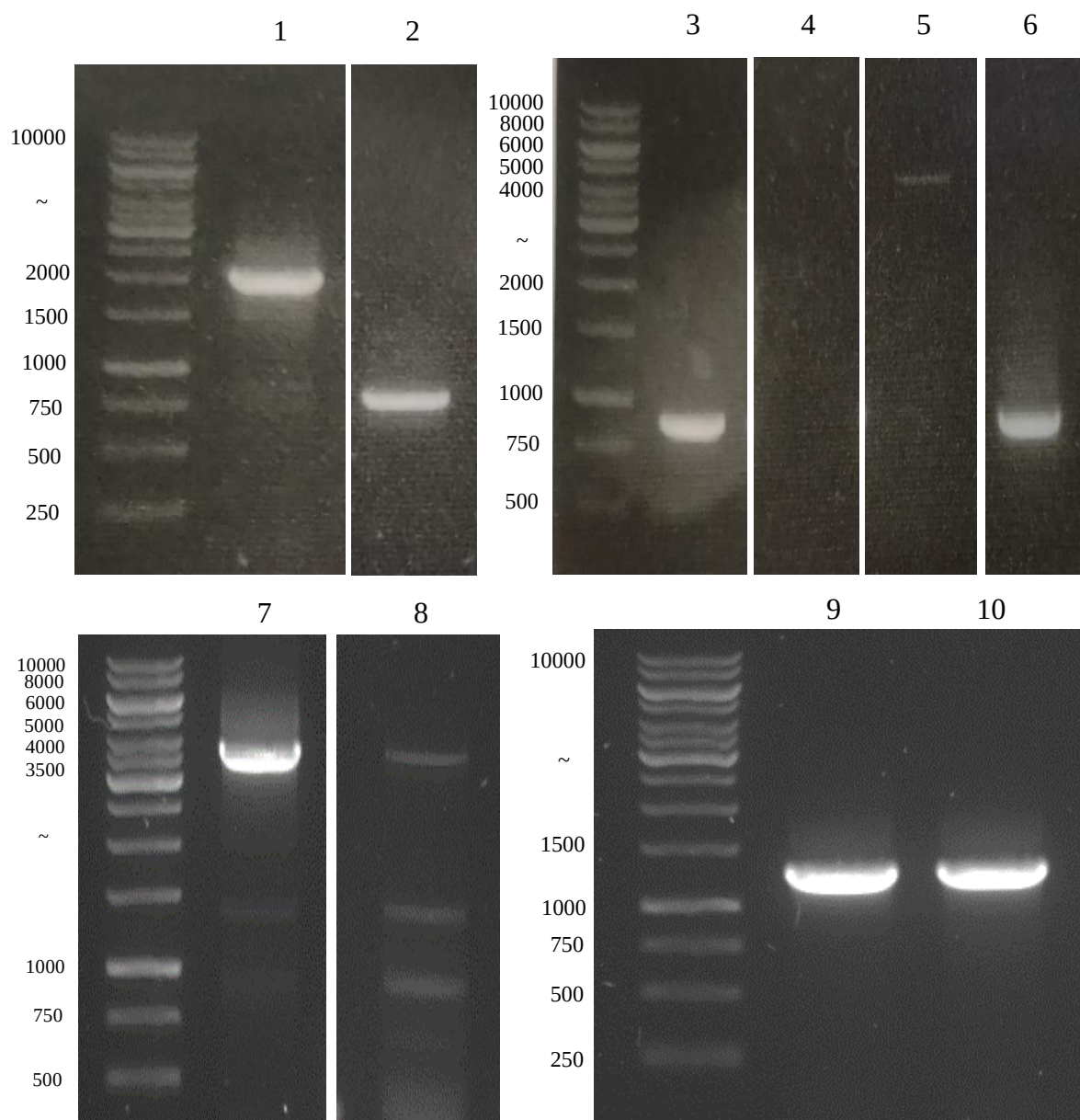


**Figure 3.3.2. Colony PCR to determine *MED2* deletion, *MED15* deletion, and *MED15* complementation in *C. auris* 8977.**

The DNA band size of each mutant amplified with corresponding oligonucleotides was visualized via gel electrophoresis. The lanes are labelled as: 1) 8977 (*MED2* flanking primers), 2) *med2Δ* 8977 (*MED2* flanking primers), 3) 8977 (*MED15* internal primers Med15 up2), 4) *med15Δ* 8977 (*MED15* internal primers Med15 up2), 5) 8977 (*MED15* flanking primers), 6) *med15Δ* 8977 (*MED15* flanking primers), 7) 8977 (*MED15* flanking primer2), 8) *med15Δ* 8977 com (*MED15* flanking primers), 9) 8977 (*MED15* internal primer Med15 dn1), 10) *med15Δ* 8977 com (*MED15* internal primer Med15 dn1).

```

      * 10 * 20 * 30 * 40 * 50 * 60 * 70
433 AGAAAACGCACTCAACTTTCTTAAGCGAGCCAATTGACAAAGGTACGTCAGAAACAATTTCAAGAGTAATTT
505 TGGATTTTTCGCTTCGGATACGTCGTATATATTGTTGTCAGTACTGGCTACTTGTGATCTGGTAGGACTAGTT
577 TCAGTCATGGCCAGGGTT CGATCCTCAGCTGAAGCCAAGGCATATCTCAACAATTGACCGAAGTGGTCTCAA
649 AGAGAGTTGGACCATTTCGAGGTACTCGAATCATCATAATGCAAAGCACGAAAAATTAAGATTAGTTCTCTA
721 ATTGTGTCTACGAGTTTCAAGATGAACAACATTCCCTCTCTAGAATTTAAGACTTTTAGAATCTTTCTCCTC
793 CTACTTACTAACCCTGGAGCCAGTTACTTTTCAGGCGGAATGGCTGCGAAACAGCTACATTAGGTCTGTCAG
865 TGGCAGTCAAATCAGCAAACGGCACCAACATCAAACAAAGAGGAGAGAACAATTTGCTTGAACCATT
937 ATTCCATTTCTTCTTTTCAATTTGCCACAATTCTTCAGAACTTTTTTTTTTTTTTTTTTCCCTCCATTCTCTCC
1009 ACTTGGCCTGTCTTCCCTCCTCCTTCATACTTCGCACCAATGTGCCAGACCAACGACCACTCGAGATATC
1081 ATAGCCATCTATAAAGCCCATTACTACCAGCGGCT CGAGACGAGTGTCTCGACATGAGG GGGCTGCCAATGGT
1153 TAGACCCCCATACAGTGGTTCTAATCGAGCTCGGAAAGACTTAGAAAGATACAACGCTGAAGAACTAATGAA
1225 GTTGAAACCGAAAAGAACATCTGAGGTCAATTTCTACTACTGGCTTAACTCACTTGCAATTGTGGCTCTCCAG
1297 TATATGGGCCTGCCATTTTTCGCAGCCA CTACAAAAGTGCAGAAAGTGTACAAGCGCCATTCTCAACACGG
1369 ATGGCCACCTCTATTATTATCTTCACTTAGA TCAAAGGTTTCACACGACTTCAC AAAATCAAGTGAGACGTTG
1441 AAGTTCTTTTCATCTGTCTGATGGAATTGTTCCCATATAACAGGAGAGGAGCGCTGGCCAATGAGAAGAACG
1513 TTCGCGGTCTGTCGTGCACGCACCCAAACCCACTCTCGGTCTAGCTTTTCCAAATCTCAAAGCTTGACAAG
1585 ACCACCGTCTAACGCACAACAAAACCACAAACA

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(*MED2* deletion in 8985 Sanger sequencing data)

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      * 10 * 20 * 30 * 40 * 50 * 60 * 70 *
1 AGCAGTAGAAGAAGAAAATGTGACCAAAAAGAGACTTCAGCATAATTACAAACAATCGCATACTTTAGGCTTCT
76 CAATCAAACAATATTTTCGTACTGCTCGATTGCTCC GTTGCCCATGTGTACCTTTTCAACAGGGTCCCACTTT
151 TGTGTGTCTCTGGGGTTTCGCAGGGCTGCCGCAGGGCATTGTGGAAAGAGAGACCCACATGAAGAAACACCAATG
226 CAGAATAGAATCGACAGCAGCAAATAACTCAGTATATAAGAGCTTTCTCAATTGCCTGTAAAAATGCAAGTTCTA
301 TCCAATTTTTTTCATCGTTGTCTCTTTGAAATCCCTGCTTCCCGCTCGAGAGCTTTGCTGGAAATCTCGGGGCCA
376 AATGTTTCTGCGCTCACCACCAATGGTTTTTCAGACAACAAGCACCCTGGACACTTTGAAGTGACGGATTGAAA
451 GTTCCAGAAACCAAGTCTAGTGTTCATCTACAGAATCTTGGGCTGAAAAATTCCTCGTGTCTTTTCTCTTC
526 CTGCTCTTCCGCCACAATGCCTCGTGTGGTCCCCGCAGGCTGTCAACCAACCTTGCACTTTTTCAATTAGCAACCG
601 CCCCTGGCAAAACCACCCACTTTTTGCAACCCACGAGAAATCAGAATGGCAAAATTCGGACTTTTTGTAGGGT
676 TGGACCTGTTTCTTGTAATAATTACAACAAATCACTACTTTTCAAATTTTCGTTTCAGGACCATGGGTATATTACT
751 TCCTCTTCATGGCTCCCGCACAGAGGTCTCAGGCTCCCTCAAGCTTCCATTTTCGGTCCCGCTCGCACCTTTTT
826 TTTTTTTTTTTTTTTTTTTTTTTTTTTTATTTTACTTTTATATTTTTTTAGCCCACTCTCTCACGATCCTTACCGT
901 ACTTTTTTCTTACCTTCACCTTCCTCAAACCTAACCCAAACCTTCACCCATCGTCATCTTCTTTTCATAGTATCC
976 TGCTGCGCTTTTCGCCTCCACCATC cgagacgagtgctcgacatgagc TGAGATATTTACTAGAAAGACGCAATCA
1051 TATATATTTGGAATGTTAAAAGTACGACGAAAGAAATTTGCCCATGTCGATCCAATGAGGTACCTGTGAGTCAGA
1126 TTGTAGATGGAGAGGGCCATACCGTTGGCGATTTTGCAGCC GTGTACGTCGCTATCTTT CAAATCGCCAGGG
1201 CACTATCAGTTAAGATCTCAATATAGGAATACATTTTCCTTCGATGACATCAGATAAAAAAATTTCACTGGCCTTT

```

(*MED15* deletion in 8985 Sanger sequencing data)

**Figure 3.3.3. Sanger sequencing data confirming the deletion of *MED2* and *MED15* in *C. auris* 8985.**

The AddTag sequence replacing *MED2* (top panel) or *MED15* (lower panel) is highlighted in red. The flanking regions of the gene used for repair template construction are highlighted in light blue. The sequences highlighted in green (top panel) show the position of the *MED2* flanking primers used for mutant screening. The sequences highlighted in yellow (bottom panel) show the position of the *MED15* flanking primers used for mutant screening.

homologous upstream and downstream sequences used for repair template construction.

To identify *med2*Δ mutants in both 8985 and 8977, 25 transformants were screened to identify a correct *med2*Δ mutant. In the case of *MED15*, approximately 1 in 4 transformants had the correct structure predicted for both *med15*Δ8985, *med15*Δ8977, and the complemented *med15*Δ8977.

### 3.3.3 The recycling of CRISPR-Cas9 components in *C. albicans* and *C. auris*

For *C. albicans*, the recycling of CRISPR-Cas9 components and the *NAT1* marker worked in all *C. albicans* strains used in this study. However, for *C. auris*, the CRISPR-Cas9 components with the *NAT1* marker failed to be recycled after *MED2* and *MED15* deletion in 2 different *C. auris* strains, 8985 and 8977. All transformants recovered growth on *LEU*<sup>-</sup> medium and remained resistant to nourseothricin. For this reason, the CRISPR-Cas9 components with the *HYG* marker were used to reintroduce *MED15* in the *med15*Δ 8977 mutant. Transformants were *HYG*<sup>R</sup>, *LEU*<sup>-</sup> and *NAT*<sup>S</sup>, indicating that the *HYG* cassette had replaced the *NAT1* cassette at the *LEU2* locus. Recycling of the *HYG*<sup>R</sup> cassette yielded *LEU*<sup>+</sup> derivatives that were shown to be complemented for *MED15* (Figure 3.3.2).

### 3.4 Discussion

The CRISPR-Cas9 LEUpOUT system was able to introduce homozygous *TAC1-5* single point mutations to all *C. albicans* strains (WT, *tloΔ* and *med3Δ*) in a single transformation. This confirms that the *C. albicans* CRISPR-Cas9 LEUpOUT system is a highly efficient system that allows the integration of DNA containing only a single base pair difference into two alleles in a single transformation with an efficiency of approximately 5%. For *C. auris*, the CRISPR-Cas9 LEUpOUT system was able to delete *MED2* and *MED15* in two different *C. auris* strains from different clades (clade I and III) and was also used to complement *MED15* in the *med15Δ* 8977 mutant. This confirms that the method is applicable to genetically diverse strains of *C. auris*. However, despite being able to generate the desired mutants, the CRISPR-Cas9 efficiency varies in different *C. auris* clades and requires optimization from the original protocol. The heat shock protocol used by Ennis *et al.* (2021) was substituted for another more efficient heat shock method (Leenah Alaalm, Personal Communication) or an electroporation transformation protocol. The heat shock transformation method worked for deletion of *MED2* and *MED15* in both clades whereas the electroporation transformation method worked for *MED15* complementation in the *med15Δ* 8977 mutant. The electroporation method (see Section 2.5.2) might be a more efficient method to use in combination with the CRISPR-Cas9 LEUpOUT system in *C. auris* as it worked more efficiently (in terms of numbers of transformants generated) compared to the heat shock protocol (see Section 2.5.1).

The main drawback of the CRISPR-Cas9 LEUpOUT system in *C. auris* was the difficulty in removal of the CRISPR-Cas9 components from the *LEU2* locus using the LEUpOUT method. Transformants were NAT resistant but could also grow on medium without leucine. This indicates that the singular *LEU2* gene was still functional in those NAT resistant mutants, suggesting that integration of CRISPR-Cas9 components and the NAT marker occurred either

upstream/downstream of *LEU2* or resulted in a duplication of the *LEU2* gene. The lack of marker gene recycling would normally make complementation of mutants difficult. However, Ennis et al. (2021) also described a second hygromycin (HYG) resistance cassette that can be used instead of the NAT marker. In this case, we used the HYG marker to introduce a gRNA to facilitate complementation of *MED15* at the native locus. The resulting *MED15* complemented transformants were HYG-resistant and NAT-sensitive and were auxotrophic for leucine. This indicates that the HYG-resistance cassette integrated into the *LEU2* locus, disrupting Leu2 function and replace the NAT marker at this locus. Finally, recycling of the HYG marker on leucine negative medium was able to generate HYG-sensitive, leucine prototrophs.

Overall, the whole CRISPR-Cas9 LEUpOUT system was proven to be useful for gene editing in *C. auris* but a more consistent gene editing and flip out system is required for future multiple gene editing. The authors of the system have since indicated that in order for marker recycling to occur efficiently, that selection on leucine medium must be carried out immediately once transformants are identified (Clarissa Nobile, Personal Communication).

## Chapter 4

Specific members of the *TLO* gene family are required for Tac1-activated fluconazole and terbinafine resistance in *Candida albicans*

## 4.1 Introduction

### 4.1.1 The emergence of azole-resistance in *C. albicans*

*C. albicans* is a pathogenic yeast, typically 5-6 µm in size with an ovoid shape and a cell wall composed of glucan, chitin and mannoprotein (Cottier and Hall, 2020). *C. albicans* is a commensal pathogen that resides on various human mucosal surfaces like the oral cavity (Nobile and Johnson, 2015) and it is the major cause of most cases of candidemia in the European region (70-90%) (Bongomin *et al.*, 2017). In recent surveillance reports, six Southern European tertiary hospitals showed a significant increase in the numbers of azole resistant *C. albicans* isolates obtained from blood cultures (from 0.7% to 4.5%), whereas another recent surveillance study conducted in Colombia revealed that 13% of the *C. albicans* clinical isolates obtained from blood cultures were resistant to fluconazole (Ceballos-Garzon *et al.*, 2023). In Ireland (2004-2018), resistance was detected in 2-5% of *C. albicans* clinical isolates obtained from blood cultures (Ryan *et al.*, 2019). Surveillance reports suggest that there is an increase in the recovery of azole-resistant *C. albicans*, which is a serious problem as azole drugs have high levels of oral bioavailability, thus making them a widely used antifungal to treat various forms of candidiasis caused by *C. albicans* (Pappas *et al.*, 2016). Multiple factors contribute to the increased incidence of azole-resistant *C. albicans* such as the wide and long-term usage of azole drugs and the fungistatic nature of azole drugs.

Currently, *C. albicans* is known to have three major azole resistance mechanisms: the alteration of drug target (Erg11), the overexpression of Erg11, and the upregulation of plasma membrane efflux pumps (Figure 1.2) (Lee *et al.*, 2021). Most *C. albicans* azole-resistant isolates can overexpress multidrug transporters encoded by either the major facilitator (MF) efflux pump *CaMDR1* (multidrug resistance 1) or the ATP-binding cassette (ABC) transporters *CDR1* (Candida drug resistance) and *CDR2* (Coste *et al.*, 2006). Furthermore, gain-of-function (GOF)

mutations in Tac1 (zinc transcription factor) is commonly responsible for the overexpression of efflux pumps in these isolates (Coste *et al.*, 2006).

#### 4.1.2 *TAC1-5*, a hyperactive *TAC1* allele that upregulates *CDR1* and *CDR2* (ABC transporters)

Tac1 is a transcription factor that regulates the expression of *CDR1* and *CDR2* by directly binding to the drug-response element (DRE) located in their gene promoter (Coste *et al.*, 2004). In azole-susceptible isolates of *C. albicans*, Tac1 activates *CDR1* and *CDR2* expression in response to a xenobiotic known as fluphenazine (FNZ) (Coste *et al.*, 2006). In azole-resistant *C. albicans* isolates, GOF mutations have been identified in Tac1 that confer hyperactive transcriptional activating activity that act to increase the expression of *CDR1* and *CDR2* and ultimately confer azole resistance (Coste *et al.*, 2006). The hyperactivation of Tac1 is not required for its sequence-specific DNA binding as Tac1 occupancy at the DRE is still observed in the absence of GOF mutations in Tac1 (Coste *et al.*, 2006).

Tac1 is a classical zinc cluster transcription factor that possesses a highly conserved Zn<sub>2</sub>Cys<sub>6</sub> motif (Jain *et al.*, 2023). Like any other zinc cluster transcription factors, Tac1 has three domains, an N-terminal DNA Binding Domain (DBD, 1 – 1320 nucleotides), a C-terminal Acidic Activation Domain (AAD 1354 – 2946 nucleotides), and a Middle Homology Region (MHR 1321 – 1653 nucleotides) (Jain *et al.*, 2023). A recent molecular dissection study of Tac1 provided some insights about how these domains interact with various components (Jain *et al.*, 2023). The Tac1-DBD is known to exist as a homodimer and it can bind to the DRE promoter site of *CDR1* and *CDR2* (Jain *et al.*, 2023). Tac1-MHR is known to be a xenobiotic binding domain that interacts with fluphenazine, resulting in Tac1 hyperactivity and upregulation of *CDR1* and *CDR2* expression (Jain *et al.*, 2023). Besides, this xenobiotic binding domain seems to play a crucial role in the activation of *CDR1* and *CDR2* as the sole presence of MHR can

induce the expression of *CDR1* and *CDR2* (Jain *et al.*, 2023). The Tac1-AAD was shown to interact with the TATA-binding protein (TBP) that plays a crucial role in transcription (Jain *et al.*, 2023). The well-studied *TAC1*<sup>GOF</sup> mutation identified in a *C. albicans* azole-resistant isolate, known as *TAC1*-5, is a single nucleotide mutation in codon 977 (A to G at nt 2929) resulting in a nonsynonymous substitution of Asn to Asp in Tac1p (Coste *et al.*, 2006). It is located within the Tac1-AAD (Coste *et al.*, 2006) and there is a possibility that the presence of *TAC1*-5 increases the interaction between Tac1-AAD with TBP and thus, increasing the transcription of *CDR1* and *CDR2*. Homozygous *TAC1*-5 was the only one found to induce high expression of *CDR1* and *CDR2* and ultimately conferred high fluconazole and terbinafine resistance in *C. albicans* (Coste *et al.*, 2006).

#### 4.1.3 Aim of this study

Based on a previous study in *C. albicans* showing that the two Mediator tail subunits, Med3 and Med15, are both required for Tac1-activated *CDR1* expression and azole resistance in *C. albicans* (Liu and Myers, 2017b), we hypothesized that *TLO* genes are required for the basal *CDR1* expression and Tac1-activated *CDR1* expression in *C. albicans*. The introduction of homozygous *TAC1*-5 (N977D, single point mutation) into *tloΔ* mutant was predicted to result in reduced fluconazole and terbinafine resistance compared to the wild-type with *TAC1*-5. To investigate this, we used CRISPR-Cas9 mutagenesis to introduce the *TAC1*-5 GOF allele into the wild-type strain (MAY1244), the *tloΔ* mutant and the *med3Δ* mutant as a comparison (See Chapter 3). Finally, we were interested in determining if individual Tlo proteins interact with hyperactive Tac1 to overexpress *CDR1* and confer fluconazole and terbinafine resistance. To investigate this, individual *TLO* genes from different *TLO* clades (*TLOα1* and *TLOβ2*) were selected and reintroduced into the *tloΔ TAC1*-5 mutant under the control of the tetracycline promoter (pTET).

## 4.2 Materials and Methods

### 4.2.1 Drug treatment for qRT-PCR analysis

*Candida* strains were grown overnight in 4ml YEPD medium in a shaking incubator set at 37°C, 200 rpm. On the following day, the OD<sub>600</sub> value of the overnight culture was measured, and a fresh culture was inoculated (OD<sub>600</sub> 0.1) in 100 ml YEPD and grown to exponential phase (OD<sub>600</sub> 0.6-0.8) in the shaking incubator set at 200 rpm, 37°C. An 80ml volume of mid-log phase *C. albicans* culture was then split equally into two conical flasks, with one being exposed to fluphenazine at a final concentration of 20 µg/ml and the other as a control (no drugs added). A 10 ml volume of each culture was then collected at given time points (0 min and 30 mins) and centrifuged at 4000 rpm for 5 mins to obtain a pellet. The pellet was then washed once in sterile water and was used for RNA extraction and qRT-PCR as described in Section 2.4.3.

### 4.2.2 Reintroduction of *TLO*α1 and *TLO*β2 using pNIM1-TLO1/2 plasmids

Individual hemagglutinin (HA)-tagged *TLO*α1 and *TLO*β2 genes under a tetracycline regulated promoter were reintroduced into *tlo*Δ-*TAC1-5* mutants using the pNIM1-*TLO* cassettes developed by Fletcher *et al.* (Fletcher *et al.*, 2023) shown in Figure 4.2.1. *E. coli* DHα5 containing either the pNIM1-*TLO1* or pNIM1-*TLO2* plasmid DNA was isolated from L -broth cultures containing 100 µg/ml ampicillin. (Section 2.3.2) and the plasmid DNA concentration was determined using a Nanodrop spectrophotometer (Section 2.3.3). The plasmid was linearised for transformation purposes using *KpnI* and *SacII* restriction enzymes (New England Biolabs). The linearized plasmid DNA was purified (Section 2.3.7) and transformed into *tlo*Δ (*TAC1-5*) mutants via electroporation method (Section 2.5.1).



**Figure 4.2.1. Cassette used to introduce *TLO* genes into *tloΔ-TAC1-5* mutants**

The *TLO* gene with a 3xHA tag under the control of a tetracycline inducible promoter, *TET1*. This cassette was integrated in the *ADH1* locus. The uninduced *TLO* gene expression from this cassette is sufficient to restore wild-type functions but can be overexpressed in the presence of doxycycline.

#### 4.2.3 Confirmation of *TLO* $\alpha$ 1 and *TLO* $\beta$ 2 reintroduction into *tlo* $\Delta$ -*TAC1*-5 mutants

The confirmation of the presence of each *TLO* gene in the *tlo* $\Delta$ -*TAC1*-5 strain was performed by conducting a colony PCR using the Pan Tlo forward primer and the HA-tag reverse primer (Table 2.3). The expression of each introduced *TLO* gene in the *tlo* $\Delta$ -*TAC1*-5 background was further verified by using qRT-PCR (Section 2.4.3) to see if the specific mRNA of the individual *TLO*s could be detected and whether this was similar to the level of expression in the *tlo* $\Delta$  pNIM1-*TLO* $\alpha$ 1 and pNIM1-*TLO* $\beta$ 2 reintegrates described by Fletcher *et al.*(2023) using primers listed under qRT-PCR in Table 2.3. Further verification was performed using Western blotting against HA-protein to visualize HA-tagged Tlo proteins (Section 2.6).

#### 4.2.4 Phenotypic analysis

Phenotypic testing was performed on these *C. albicans* strains as described in Section 2.7.

#### 4.2.5 ChIP-qPCR

The ChIP-qPCR protocol used here was adapted from the ChIP-qPCR protocol optimised for *C. dubliniensis* (Haran *et al.*, 2014).

##### 4.2.5.1 Cell growth and chemical fixation

A single *C. albicans* colony from a YEPD plate (24-48h old) was used to inoculate a 12.5 ml YEPD broth which was grown overnight at 30°C in a shaking incubator set at 200 rpm. On the following day, the OD<sub>600</sub> value of the overnight culture was measured, and this was used to inoculate a fresh 50 ml YEPD culture in a 250 ml conical flask (OD<sub>600</sub> 0.2) which was grown to OD<sub>600</sub> 1.0 with shaking at 200 rpm and 37°C. After reaching OD<sub>600</sub> 1.0, the culture was transferred to a 50 ml falcon tube and spun down at 3800 rpm for 3 mins to obtain a cell pellet. The supernatant was then discarded and the cell pellet was resuspended in 5 ml PBS. The cell mixture was spun down again and the supernatant was discarded. The cell pellet was resuspended in 36 ml PBS and 1 ml of 37% (v/v) formaldehyde was added to the mixture. The cell mixture was transferred to a 250ml conical flask and incubated in a shaking incubator set at 30°C at 200 rpm for exactly 30 mins (time sensitive). After 30 mins, 2 ml sterile 2.5M glycine was added to the cell mixture and which was incubated at 200 rpm and 30°C for 10 min. The downstream protocol after this incubation step required samples to be kept at 4°C or on ice to avoid protein degradation. After a 10 mins incubation, the cell mixture was transferred into a pre-chilled 50 ml falcon tube and spun down as before at 4°C (3800 rpm, 3mins). The supernatant was discarded and 1 ml PBS was used to resuspend the cell pellet. Samples were then transferred to screw cap tubes and centrifuged at 4°C (13000 rpm, 2 mins 30s). The supernatant was discarded and the cell pellet was flash-frozen by dipping the pellet containing tubes into liquid nitrogen followed by storage at -80°C for future use.

#### 4.2.5.2 Cell lysis and DNA fragmentation

For preparation of samples for ChIP, cells and solutions were kept on ice as much as possible to reduce protein degradation. The pellet containing formaldehyde fixed cells was defrosted on ice and 400 µl FA Lysis Buffer (50 mM HEPES pH7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate) was added to the pellet. 4 µl protease inhibitor (PI, 1:100), 3.2 µl PMSF (2 mM), and an equal amount of zirconia beads (0.5 mm in size, ~500µl) was added to the pellet. The resuspended mixture was processed for 7 cycles (30s each) using a Fastprep Homogeniser 24 (4 m/s) and cooled down for 1 min on ice between each 30s cycle. A 1 µl volume of cell lysate was then checked under the microscope to observe ideal cell breakage and additional Fastprep cycles were performed if required. The lysate was then transferred to pre-chilled tubes without disturbing the zirconia beads. The lysate was mixed with 1 ml FA lysis buffer and transferred to another pre-chilled tube to completely remove zirconia beads. The clean lysate mixture was then spun at low speed (8000 rpm) for 30s to avoid pellet formation. An 80 µl “pre-sonication” sample was removed after this centrifugation. The remaining centrifuged lysate was gently resuspended and sonicated 14 times (10s pulses at 8 microns) with 1min on ice between each sonication. Samples remained clear without excess frothing after each sonication. After sonication, samples were spun down at 13,200 rpm for 30 mins at 4°C. An 80 µl aliquot of supernatant was transferred to a tube for DNA fragment size verification and the remaining supernatant was then split into 5 O.D. units (ODUs) per tube based on this calculation formula  $5 \text{ O.D. units} = (5 \times 1000 \text{ µl}) / (\text{ODU per } 50 \text{ ml})$  and were stored at -80°C for future use. For DNA fragment size verification, the 80 µl sonicated sample was “reverse crosslinked” by mixing with 100 µl of ChIP elution buffer, 200 µl pronase and 1 µl CaCl<sub>2</sub> and incubation at 42°C for 2 h, 65°C for 6 h, followed by a 10°C hold.

#### 4.2.5.3 DNA fragment size checking of sonicated sample

After overnight reverse crosslinking, 200 µl of the overnight solution was transferred to a fresh Eppendorf tube. A 25 µl volume of 3 M sodium acetate (pH 5.2), 25 µl water, and 250 µl phenol/chloroform/isoamyl alcohol (24:24:1) were added to the sample and vortexed for 3 mins. The mixture was spun at 13,200 rpm for 5 mins and the upper layer was transferred to a new Eppendorf tube. 500 µl of ice-cold 100% ethanol (EtOH) was added to the tube which was then transferred to -20°C for 1 hour to precipitate the DNA. A DNA pellet was obtained by spinning the chilled precipitate solution at 13,000 rpm for 10 mins and the pellet was resuspended in 200 µl TE buffer. RNase A (1.5 µL of a 25 µg/µl solution) was then added and incubated for 2 hours at 37°C. Following incubation, the sample was split in half in two fresh Eppendorf tubes. One was kept as a 'backup sample' and the other underwent EtOH precipitation. For the EtOH precipitation step, 30 µl 4 M LiCl, 70 µl water, and 400 µl ice-cold 100% EtOH were added to the tube containing the DNA mixture and this was stored at -20°C for 1 hour. DNA was then pelleted by spinning the tube at 13,000 rpm for 10 mins. The supernatant was discarded and resuspended in 500 µl 70% ice-cold EtOH. DNA was pelleted again via centrifuged at 13,000 rpm for 5min. The supernatant was discarded and the DNA pellet was dried at 37°C for 5 mins. After drying, DNA pellet was resuspended in 20 µl TE buffer. DNA fragment size was determined by electrophoresis 5 µl of sample from the DNA mixture on a 1.5% TAE gel run at 75V for 1 hour. The optimum fragment size was considered 300-500bp. Sizes were estimated relative to 1kb and 100bp DNA ladders.

#### 4.2.5.4 Immunoprecipitation (IP) method

The 5 ODUs of sonicated lysate was first thawed on ice and then brought up to a volume of 500 µl with FA lysis buffer with PI (15 ml FA lysis buffer + 150 µl 100X PI, made fresh). The solution was well-mixed and 20 µl of lysate was transferred to a PCR tube as input sample

(Input). To the remaining 480  $\mu$ l lysate, either 4.5  $\mu$ l of anti-Pol II antibody or 2  $\mu$ l anti-HA antibody (Abcam ab91110) was added to the tube and sealed with parafilm. The tube was placed on rotating wheel and stored at 4°C overnight at 16 rpm. Pronase treatment of input sample was then carried out. For the input sample, 100  $\mu$ l ChIP elution buffer, 60  $\mu$ l TE (pH7.5), 20  $\mu$ l Pronase (20 mg/ml) and 1  $\mu$ l 1 M  $\text{CaCl}_2$  was added to the input sample which was incubated in a thermocycler overnight (42°C for 2h, 65°C for 6h, 10°C hold). Following overnight incubation, input samples were stored at -20°C for further use on the following day.

#### 4.2.5.5 Preparation of Antibody-Bead complexes

A master mix containing 20  $\mu$ l Protein A and 20  $\mu$ l Protein G beads (Invitrogen dynabeads) per sample was prepared in a 1.5 ml tube. 1 ml of FA lysis buffer with PI was added to the same tube containing beads. The bead mixture was rotated at 16 rpm at RT for 5 mins on the rotating wheel. The beads were then captured using a magnetic stand and the supernatant was removed. This FA lysis buffer with PI washing step was repeat two more times. After the final wash, beads were resuspended in FA lysis buffer with PI and spun on the wheel for 1 min to obtain a homogenous mixture. Then, 40  $\mu$ l of bead mixture was distributed to all sonicated lysate samples. The samples were sealed with parafilm and spun on a rotating wheel at 16 rpm, 4°C for 2 hours. After 2 hours of spinning, antibody-bead complexes were formed and washing steps were performed. Beads were captured on the magnetic stand and the supernatant was removed prior to washing steps. The washing steps started with a single wash adding 1 ml FA lysis buffer without PI followed by rotation on the wheel for 5 mins, 16 rpm at room temperature (RT), after which the beads were captured and the supernatant was removed. Three more identical washes were performed in 1ml Wash Buffer 1 (FA lysis buffer, 0.5 M NaCl). Following this, three washes were performed in 1ml Wash Buffer 2 (10 mM Tris-Cl pH7.5, 0.25 M LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% Sodium Deoxycholate). A final wash in 1 ml

TE was performed using the same conditions. Finally, 250 µl ChIP elution buffer was added to the protein-bead complexes and vortexed vigorously. The mixture was later incubated at 65°C for 20 mins and rotated at 16 rpm, at RT for 2 mins. The mixture was centrifuged at 13,000 rpm for 2 mins and the supernatant was transferred to a fresh PCR tube. This supernatant is the IP pulldown sample and was subjected to pronase treatment. For the pronase treatment, 50 µl Pronase (20 mg/ml) and 1.5 µl 1M CaCl<sub>2</sub> was added to the 250 µl IP sample. The mixture was separated into two PCR tubes (in half) and run on thermocycler overnight (42°C for 2 h, 65°C for 6h, 10°C hold). IP samples after pronase treatment can be stored at -20°C until the DNA purification step.

#### 4.2.5.6 DNA purification

All input samples and IP pulldown samples were purified with the Qiagen PCR purification kit. The amount of input sample needed to be purified was 200 µl (after pronase treatment) and 300 µl of IP pulldown sample was required. To ensure all of the DNA sample was recovered from the PCR tubes, each tube was pulsed for 1 sec at 6000 rpm for 3 times before transferring to a fresh 1.5 ml Eppendorf tube. A 1 ml volume of PB buffer was added to the Eppendorf tubes and mixed by inversion. The column was placed in 2 ml collection tube and 600 µl of sample (650 µl for IP) was added to the column. The tube was spun at 13,000 rpm for 1 min and flow through was discarded. The remaining sample was added to the column and spun down again at 13,000 rpm for 1 min. Flow through was discarded and the column was washed twice by adding 750 µl PE buffer, spun at 13,000 rpm for 1 min and the flow through discarded after each spin. The tube containing the column was spun again at 13,000 rpm for 1 min to dry the column. The dry column was then transferred to a fresh 1.5 ml tube and 50 µl Elution Buffer was added to the column to elute DNA. The tube with the column was then spun at 13,000 rpm for 1 min to elute the purified DNA samples which were kept on ice.

#### 4.2.5.7 qRT-PCR method

qPCR was carried out using the GoTaq® qPCR kit from Promega to determine relative quantities of DNA targets in IP pulldown samples and input samples. For this ChIP-qPCR, IP pulldown samples were diluted 1 in 5 whereas input samples (IN) were diluted 1 in 50 for use as DNA in ChIP-qPCR. A serial 10-fold dilution (Neat to  $10^{-4}$ ) of DNA (210 ng) isolated from *C. albicans* MAY1244-*TAC1*-5 was used as a DNA standard curve. A 0.12 µl volume of primer mix containing forward and reverse primer (50 µM), 2x Sybrmaster Mix, and 9.88 µl water was mixed with 2 µl diluted DNA for GoTaq qRT-PCR run (95°C for 2min, 40 cycles - 95°C for 15s and 60°C for 1 min). Target enrichment in the IP sample (IP/IN) was calculated by dividing the Ct values of IP pulldown samples by the Ct values of the non-IP sample (IN; input sample).

## 4.3 Results

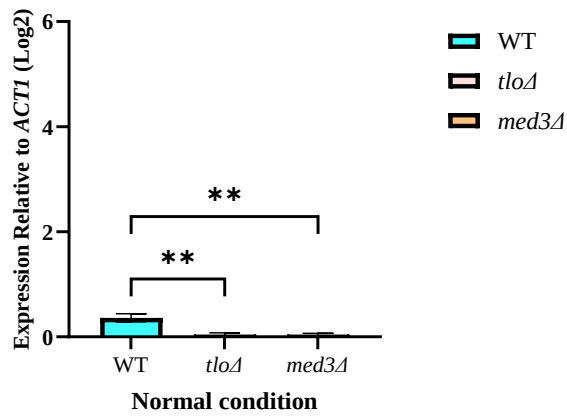
### 4.3.1 *CDR1* and *CDR2* expression in Mediator tail mutants

Before introducing a homozygous *TAC1-5* point mutation into *C. albicans* MAY1244 (WT), and the *tloΔ* and *med3Δ* mutants, qRT-PCR was carried out to quantify the basal mRNA expression values of *CDR1* and *CDR2* (Figure 4.3.1). Furthermore, wild-type and Mediator tail mutants were also exposed to fluphenazine for 30min and gene expression was detected via qRT-PCR to observe if the gene expression of *CDR1* and *CDR2* can be induced when individual Mediator tail components are absent (Figure 4.3.2). qRT-PCR revealed that both *tloΔ* and *med3Δ* mutants have significantly lower basal *CDR1* expression and lower basal *CDR2* expression (not significant) compared to MAY1244. Exposure to fluphenazine for 30 min resulted in high-level induction of the expression of *CDR1* and *CDR2* in MAY1244 but only weakly induced the expression of *CDR1* and *CDR2* in *tloΔ* and *med3Δ* mutants. The overall *CDR1* expression level was significantly higher than *CDR2* expression in all strains tested.

### 4.3.2 *CDR1* expression in *TAC1-5* mutants

The impact of introducing the *TAC1-5* mutation on *CDR1* mRNA expression in *C. albicans* MAY1244 (WT), *tloΔ* and *med3Δ* was determined via qRT-PCR (Figure 4.3.3). The presence of the homozygous *TAC1-5* mutation significantly increased the expression of *CDR1* in the WT (~4-log<sub>2</sub> fold; p <0.01) strain. However, increases in *CDR1* expression observed in both mediator tail mutants (*tloΔ* and *med3Δ*) were not deemed significant. *C. albicans* MAY1244 homozygous for *TAC1-5* had a significantly higher *CDR1* expression than all the tested strains.

(A) *CDR1* - Baseline levels



(B) *CDR1* - FNZ 30min

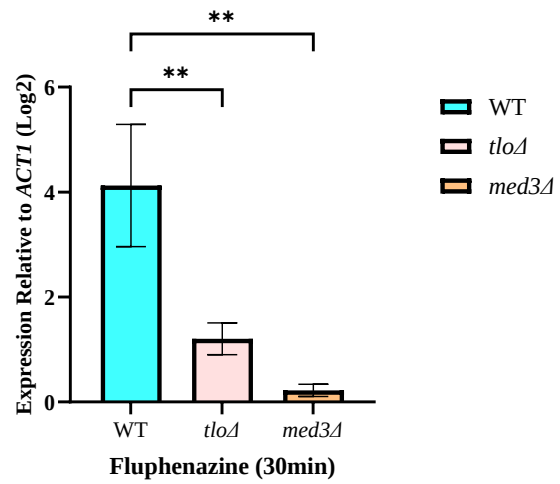


**Figure 4.3.1. *CDR1* mRNA expression in *C. albicans* wild-type and Mediator tail null mutants under different conditions determined via qRT-PCR.**

The *CDR1* expression of *C. albicans* MAY1244 (WT), *tloΔ* and *med3Δ* mutants was measured via qRT-PCR under A) normal conditions or B) in the presence of Fluphenazine (20  $\mu$ g/ml) for 30 mins. The expression of *CDR1* was normalised relative to the expression of endogenous control *ACT1* and was expressed as Log2 fold difference. Error bars were calculated based on three biological replicates and ordinary one-way ANOVA was carried out to detect any significant differences between strains (\* indicates p value < 0.05, \*\* indicates p value < 0.01).

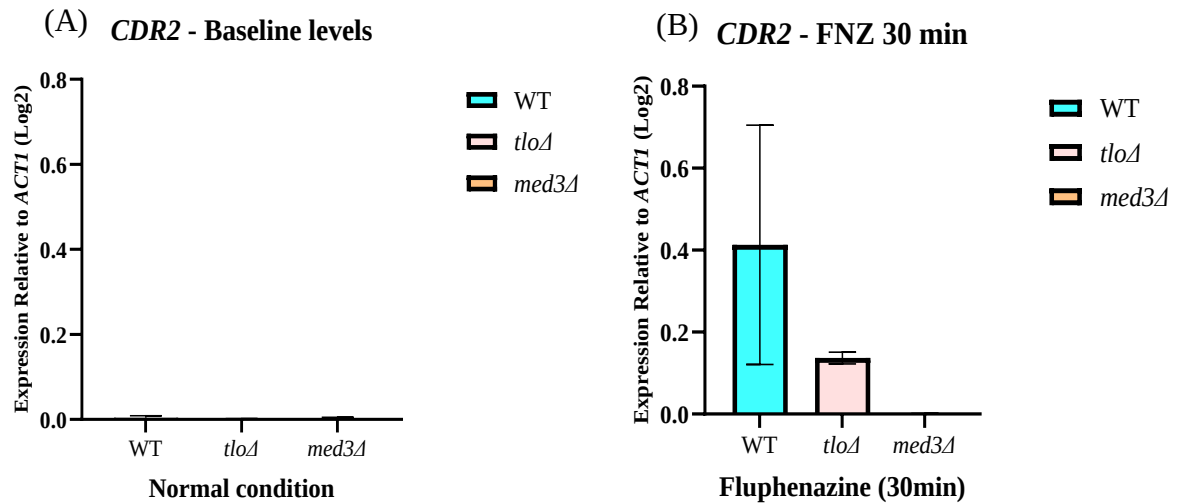


**Figure 4.3.2. *CDR2* mRNA expression in *C. albicans* wild-type and Mediator tail null mutants under different conditions determined via qRT-PCR.**

The *CDR2* expression in *C. albicans* MAY1244 (WT), *tloΔ* and *med3Δ* mutants was measured via qRT-PCR under A) normal conditions or B) in the presence of Fluphenazine (20 µg/ml) for 30 mins. The expression of *CDR2* was normalised relative to the expression of endogenous control *ACT1* and was expressed as Log2 fold-difference. Error bars were calculated based on three biological replicates and Tukey t-test were carried out to detect any significant differences between strains.

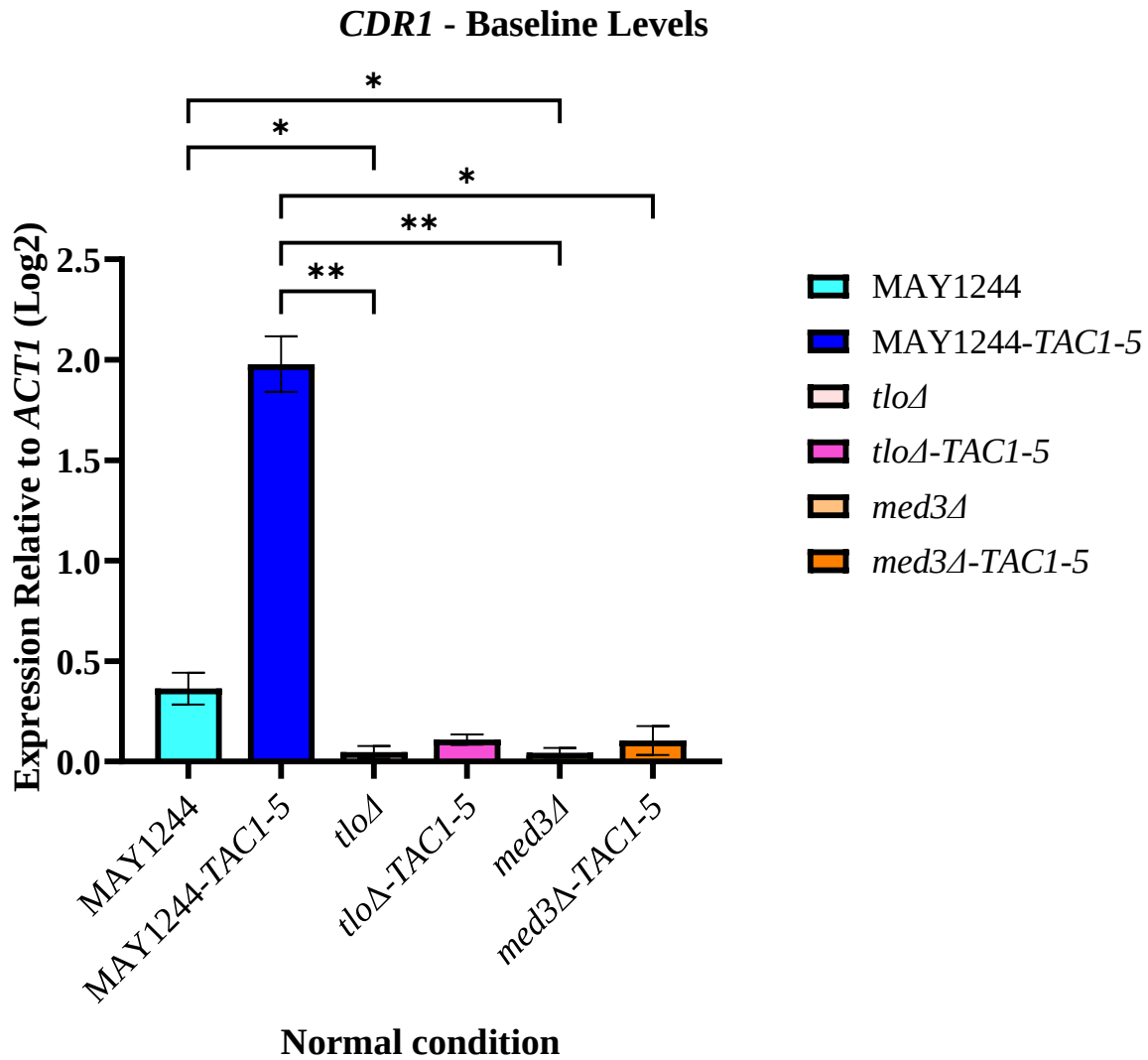


**Figure 4.3.3. *CDR1* mRNA expression in *C. albicans* wild-type and Mediator tail null mutants with/without *TAC1-5* under normal conditions determined via qRT-PCR.**

The basal *CDR1* expression of WT (MAY1244), *tloΔ* and *med3Δ* mutants with/without homozygous *TAC1-5* was measured via qRT-PCR. Each data sets were labelled in different colours and strain names. The expression of *CDR1* was normalised relative to the expression of endogenous control *ACT1* and was expressed as Log2 fold difference. Error bars were calculated based on three biological replicates and Kruskal-Wallis t-test were carried out to detect any significant differences between strains (\* indicates p value < 0.05, \*\* indicates p value < 0.01).

#### 4.3.3 Investigation of the effect of the *TAC1*-5 allele on *C. albicans* azole resistance

Broth microdilution assays were carried out in RPMI medium at 37°C for 24 h to determine the fluconazole MIC values of *C. albicans* strains with wild-type *TAC1* alleles and those expressing *TAC1*-5 (Figure 4.3.4). In these preliminary experiments, we examined the fluconazole MICs of well characterised strains known to harbour the *TAC1*-5 allele (gifts from Prof. D. Sanglard). This included a *Tac1*Δ homozygous mutant complemented with a single copy of the *TAC1*-5 allele (*Tac1*Δ + *TAC1*-5) and an isogenic pair of clinical isolates, DsyS and DsyR, where DsyS is fluconazole sensitive and DsyR is fluconazole resistant and harbours the *TAC1*-5 mutation. The *Tac1*Δ + *TAC1*-5 complemented strain had an increased fluconazole MIC of 3.9 µg/ml compared to the parental *Tac1*Δ mutant which exhibited a fluconazole MIC of 0.5 µg/ml. The *Tac1*Δ + *TAC1*-5 complemented strain also had a higher MIC value compared to the azole-sensitive clinical strain (DsyS) which possessed an MIC value of approximately 1 µg/ml. However, the *Tac1*Δ + *TAC1*-5 mutant did not surpass the MIC value of the azole-resistant clinical strain, DsyR, which had a MIC value of 250µg/ml. These data show that a single copy of *TAC1*-5 can significantly alter fluconazole susceptibility at a lower fluconazole MIC when compared to the clinical isolate DsyR as it has a homozygous pair of *TAC1*-5.

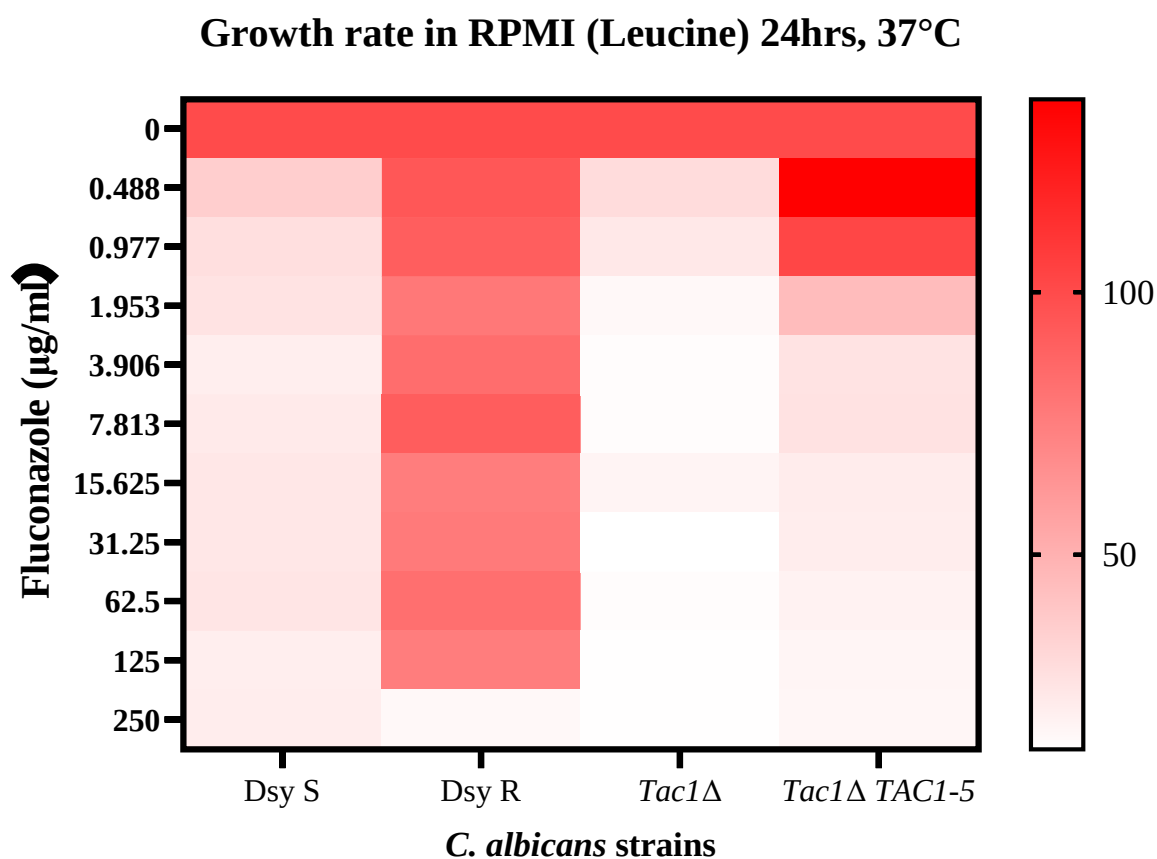


**Figure 4.3.4. Broth microdilution assay of fluconazole susceptibility in *C. albicans* strains at 37°C for 24hrs.**

Broth microdilution assay was carried out in RPMI with leucine to support the growth of all strains. All strains were labelled with their corresponding names. The absorbance value to detect cell growth was measured at 520nm. The measured absorbance value was then expressed as a percentage of the control absorbance value of each strain (100%) in RPMI medium without fluconazole after 24 h. The MIC was determined as the concentration of drug that reduced growth by 80% relative to the control.

#### 4.3.4 Fluconazole spot plate assays of *TAC1*-5 mutants

We next assessed the fluconazole sensitivity of the *TAC1*-5 mutants generated in this study. For this analysis we used a spot plate assays on YEPD medium at 30°C containing different concentrations of fluconazole (10µg/ml and 5µg/ml) to allow sufficient growth for the *tlo*Δ mutants with growth defect shown by Fletcher *et al.*, 2023 (Figure 4.3.5). *C. albicans* MAY1244 (WT) and the *tlo*Δ mutant exhibited weak growth on 5 µg/ml fluconazole whereas the *med3*Δ did not exhibit any growth on 5 µg/ml fluconazole. The presence of the *TAC1*-5 mutation resulted in increased fluconazole resistance in *C. albicans* MAY1244 (WT), *tlo*Δ and *med3*Δ mutants. *C. albicans* MAY1244 (WT) homozygous for *TAC1*-5 was shown to have the highest fluconazole resistance as it was the only *TAC1*-5 strain showing growth on the 10 µg/ml fluconazole plate. The *tlo*Δ and *med3*Δ mutants with *TAC1*-5 showed similar fluconazole susceptibility as they both exhibited strong growth on the 5 µg/ml fluconazole plate but did not grow on the 10µg/ml fluconazole plate. However, subtle fluconazole susceptibility differences might exist between *tlo*Δ and *med3*Δ mutants as only 2 different fluconazole concentrations were tested.

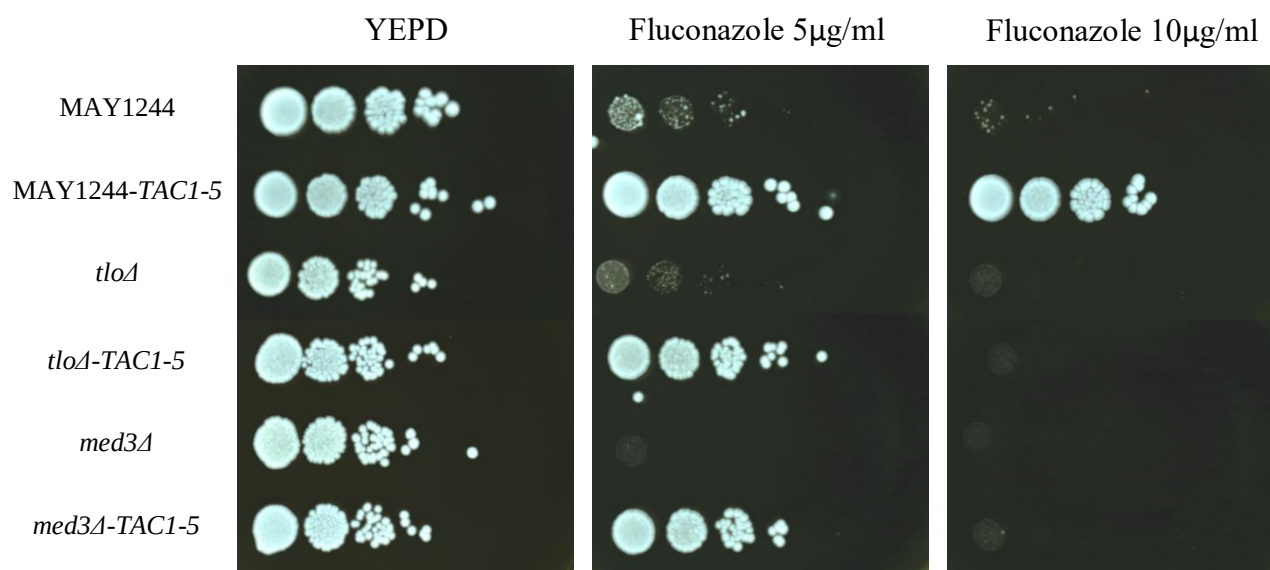


**Figure 4.3.5. Fluconazole spot plate assays of *C. albicans* MAY1244 (WT), *tloΔ* and *med3Δ* null mutants and derivatives expressing the *TAC1-5* mutation.**

Spot plate assays were inoculated with serial dilutions of overnight cultures ( $2 \times 10^6$  to  $2 \times 10^2$  cells/ml) on YEPD plates containing fluconazole (5 or 10 µg/ml) or without fluconazole as a positive control. Plates were incubated in a static incubator for 48 h at 30 °C and experiments were done in biological triplicates.

#### 4.3.5 Fluconazole disk diffusion assays of *C. albicans* MAY1244 and *tlo* $\Delta$ and derivatives expressing *TAC1-5*

The fluconazole susceptibility of *C. albicans* MAY1244 and the *tlo* $\Delta$  mutant compared to derivatives expressing *TAC1-5* was further investigated with a disk diffusion assay (Fig. 4.3.6). Plates were examined following 24 h incubation (Figure 4.3.6A) and 48 h incubation (Fig. 4.3.6B) at 30°C. 1  $\mu$ g/ml of cyclosporine A (calcineurin inhibitor) was added to suppress azole tolerance and provide a clear zone of inhibition. The introduction of the *TAC1-5* allele conferred increased fluconazole resistance to both WT and *tlo* $\Delta$  mutants as they both exhibited smaller zones of inhibition compared to their parental strains (Fig. 4.3.6A). However, MAY1244 expressing *TAC1-5* exhibited a smaller zone of inhibition compared to the *tlo* $\Delta$  *TAC1-5* derivative. In the absence of cyclosporine, the *tlo* $\Delta$  mutant was shown to exhibit a higher degree of fluconazole tolerance as it displayed a smaller and less clear inhibition zone when grown to 48h compared to MAY1244 (Fig. 4.3.6B). Interestingly, even though *TAC1-5* conferred increased fluconazole resistance to *tlo* $\Delta$ , it was found to slightly reduce the fluconazole tolerance of *tlo* $\Delta$  (Fig. 4.3.6B).

#### 4.3.6 Fluconazole E-test assays to determine the MIC values of *C. albicans* MAY1244 and *tlo* $\Delta$ and derivatives expressing *TAC1-5*

Fluconazole E-test assays were carried out at 30°C to obtain more precise fluconazole MIC values for MAY1244, *tlo* $\Delta$ , *med3* $\Delta$  and derivatives expressing *TAC1-5* (Figure 4.3.7 and Table 3.1). All three strains without *TAC1-5* (MAY1244, *tlo* $\Delta$  and *med3* $\Delta$  mutants) expressed similar MIC values between 1-2 $\mu$ g/ml (Figure 4.3.7 and Table 3.1). MAY1244-*TAC1-5* showed the highest fluconazole MIC value (16  $\mu$ g/ml) whereas *tlo* $\Delta$ -*TAC1-5* exhibited a 2-fold lower MIC (8  $\mu$ g/ml). The *med3* $\Delta$ -*TAC1-5* strain had an intermediate MIC value between the *TAC1-5* mutants (10  $\mu$ g/ml).

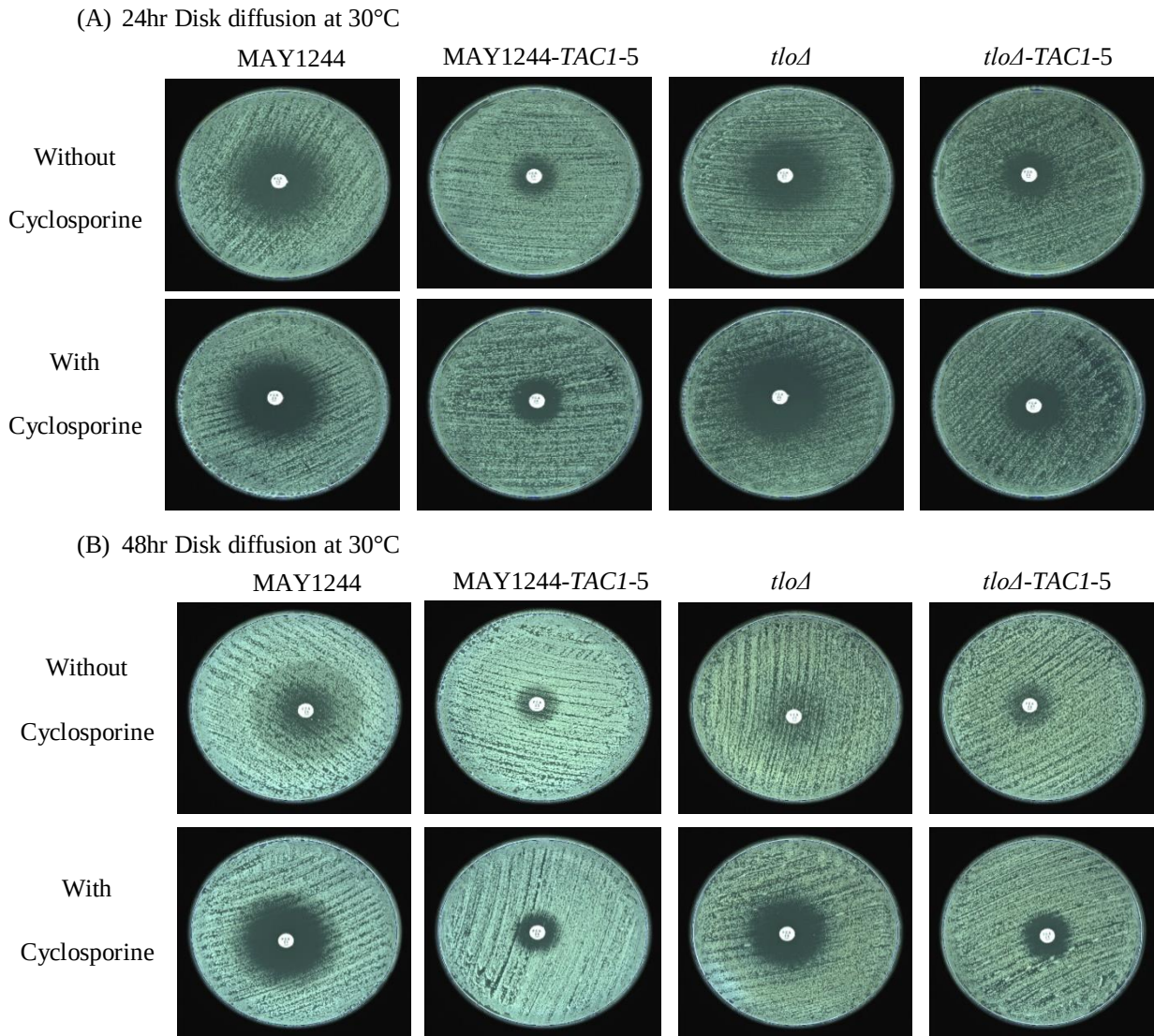


**Figure 4.3.6. Disk diffusion assays of *C. albicans* MAY1244 (WT) and *tloΔ* null mutants and derivatives expressing the *TAC1-5* mutation.**

(A) Disk diffusion assays on RPMI plates containing fluconazole (25μg/ml) with and without cyclosporine (1μg/ml). Plates were incubated for 24h at 30°C. (B) Disk diffusion assays on RPMI plates containing fluconazole (25μg/ml) with and without cyclosporine (1μg/ml) at 30°C for 48h. Both experiments were done in biological triplicate.

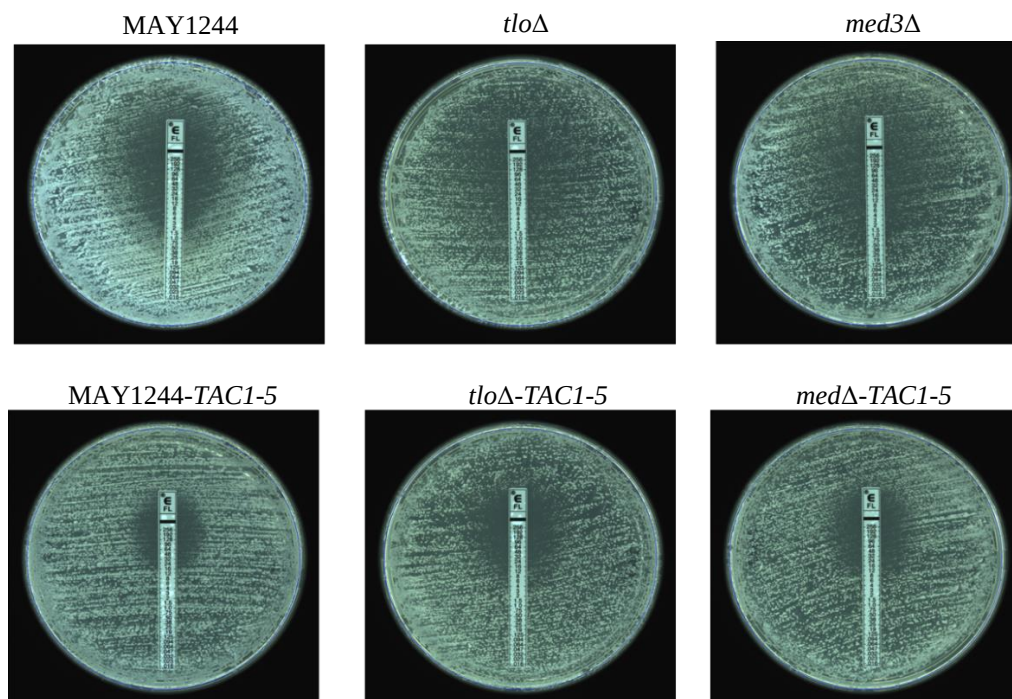


**Figure 4.3.7. Fluconazole E-test assays of *C. albicans* MAY1244 (WT), *tloΔ* and *med3Δ* null mutants and derivatives expressing the *TAC1-5* mutation.**

Fluconazole E-test were performed on RPMI plates incubated for 24h at 30°C. Experiments were done in biological duplicate and MIC values (Table 3.1) were measured as the point of 80% reduced growth.

**Table 3.1 Fluconazole E-test assay MIC values of *C. albicans* strains and derivatives expressing the *TAC1-5* mutation.**

| Strains                | Fluconazole MIC values (E- test) |
|------------------------|----------------------------------|
| MAY1244 (WT)           | 1.5                              |
| MAY1244- <i>TAC1-5</i> | 16                               |
| <i>tloΔ</i>            | 1.5                              |
| <i>tloΔ-TAC1-5</i>     | 8                                |
| <i>med3Δ</i>           | 1                                |
| <i>med3Δ-TAC1-5</i>    | 10                               |

#### 4.3.7 Terbinafine spot plate assays of *TAC1-5* mutants

Terbinafine spot plate assays were carried out to determine the terbinafine sensitivity of *C. albicans* *TAC1-5* mutants by plating them on YEPD plates for 48 h at 30°C with different concentrations of terbinafine (2.5 µg/ml and 5 µg/ml) (Figure 4.3.8). Both *tloΔ* and *med3Δ* mutants exhibited increased terbinafine susceptibility compared to MAY1244. Interestingly, *tloΔ* was more susceptible to terbinafine than *med3Δ*, showing no signs of growth on 2.5 µg/ml terbinafine plate. Similar to the fluconazole spot assays, the presence of the *TAC1-5* mutation resulted in higher terbinafine resistance in MAY1244 (WT) and the *tloΔ* and *med3Δ* mutants. However, MAY1244-*TAC1-5* was shown to have the highest terbinafine resistance as it was the only strain showing growth on 5µg/ml terbinafine plates. The *tloΔ* and *med3Δ* mutants with *TAC1-5* showed similar terbinafine resistance as they both exhibited strong growth on the 2.5µg/ml terbinafine plate and did not grow on 5µg/ml terbinafine plate (Figure 4.3.8).

#### 4.3.8 Confirmation of the reintroduction of *TLOα1* and *TLOβ2* in *tloΔ-TAC1-5* via colony PCR and qRT-PCR

The reintroduction of individual *TLO* genes (*TLOα1* and *TLOβ2*) in the *tloΔ-TAC1-5* mutant was performed by introducing cloned ORFs under the control of the pTET promoter in vector pNIM1. Experiments were performed without doxycycline according to Fletcher *et al.* (2023) who have previously shown that doxycycline induction was not required for sufficient *TLO* gene expression to restore wild-type phenotypes in the *tloΔ* mutant. Introduction was confirmed via colony PCR and qRT-PCR using primers listed in Table 2.1 (Figure 4.3.9A). qRT-PCR was carried out to further confirm the mRNA expression of *TLOα1* and *TLOβ2* in the *tloΔ-TAC1-5* background. The mRNA expression level of *TLOα1* in *tloΔ-TAC1-5* was similar to that previously reported by Fletcher *et al.* (2023) who previously expressed this construct in the parental *tloΔ* mutant. However, expression of *TLOβ2* from the same pTET

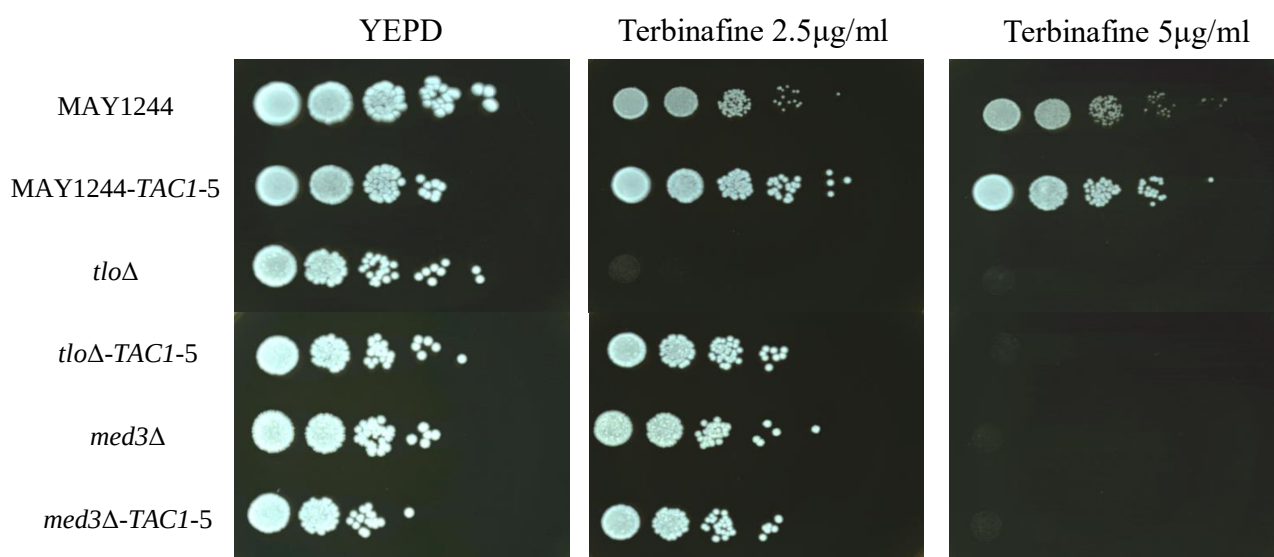


**Figure 4.3.8. Terbinafine spot plate assays of *C. albicans* MAY1244 (WT), *tloΔ* and *med3Δ* null mutants and derivatives expressing the *TAC1-5* mutation.**

Spot plate assays were carried out with serial dilutions of overnight cultures ( $2 \times 10^6$  -  $2 \times 10^2$  cells/ml) on YEPD plates containing terbinafine (2.5 and 5 μg/ml) and YEPD without terbinafine as a positive control. Plates were incubated in a static incubator for 48 h at 30 °C and experiments were done in biological triplicate.

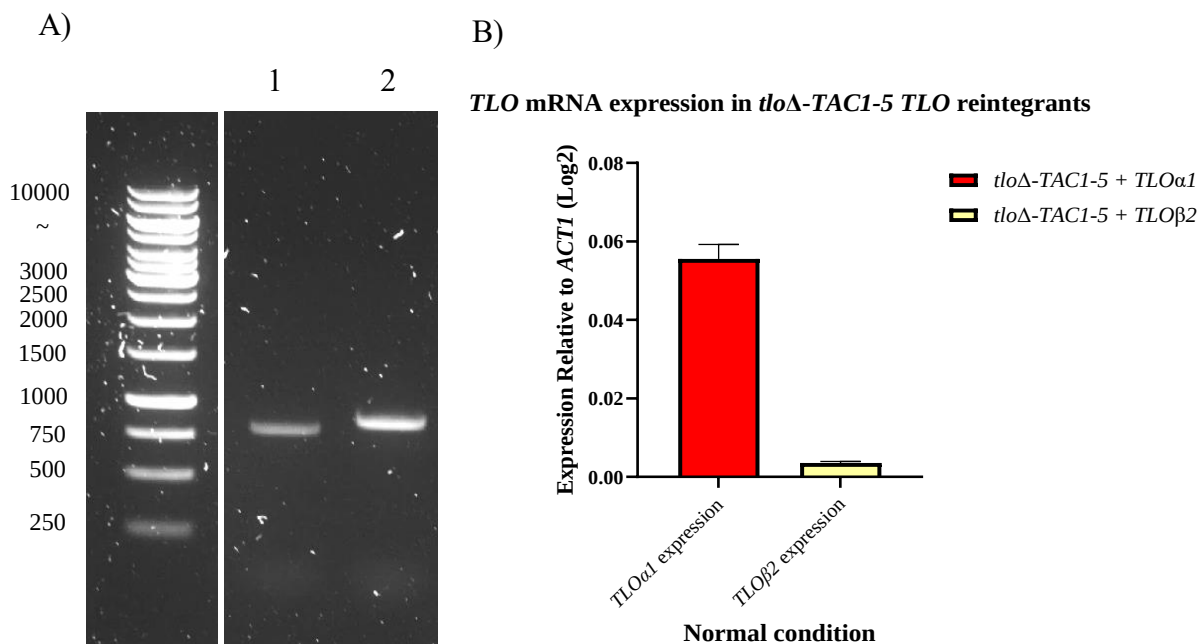


**Figure 4.3.9. Colony PCR and qRT-PCR analysis to detect the presence and expression of individual *TLO*s in the *tloΔ-TAC1-5* mutant**

A) Colony PCR confirmed the presence of *TLOα1* and *TLOβ2*. The gel lanes are labelled as followed: 1) *tloΔ-TAC1-5 + TLOα1* and 2) *tloΔ-TAC1-5 + TLOβ2*. A ‘Pan-*TLO*’ oligonucleotide was used as forward primer and the HA-tag recognizing primer (Tag R) was used as a reverse primer (Table 2.1). The predicted product sizes are: *TLOα1* – 844bp and *TLOβ2* – 913bp. B) The mRNA expression of each *TLO* (from the pTET promoter) was measured without doxycycline. The expression of *TLOα1* and *TLOβ2* was measured relative to the expression of endogenous control *ACT1* and was normalized to Log2.

promoter in *tloΔ-TAC1-5* was significantly lower than *TLOα1* and also lower than the reported levels of expression of *TLOβ2* in the *tloΔ* mutant from the same pNIM1 construct, as reported by Fletcher *et al.*, 2023 (Figure 4.3.9B).

#### 4.3.9 Cell morphology of *tloΔ-TAC1-5* strains expressing *TLOα1* and *TLOβ2*.

The cellular morphology of the *tloΔ-TAC1-5* mutant was compared to the *TLOα1* and *TLOβ2* complemented derivative to determine if they possessed a normal yeast cell morphology or a pseudohyphal phenotype normally associated with the *tloΔ* null mutant (Figure 4.3.10). Cells from 37°C YEPD overnight cultures were stained with calcofluor white and imaged as described in Section 2.2.8. The *tloΔ* null mutant as expected showed extensive pseudohyphal growth and a similar phenotype was observed in the *tloΔ-TAC1-5* mutant. The reintroduction of *TLOα1* restored a wild-type yeast cell morphology in both the *tloΔ* and *tloΔ-TAC1-5* mutants. Reintroduction of *TLOβ2* resulted in different phenotypes in the *tloΔ* and *tloΔ-TAC1-5* backgrounds. In the *tloΔ* background, *TLOβ2* restored yeast cell growth with a proportion of cells exhibiting a true hyphal phenotype. However, introduction of *TLOβ2* into the *tloΔ-TAC1-5* did not restore yeast cell growth and the *TLOβ2* derivative appeared similar to the *tloΔ-TAC1-5* parent.

#### 4.3.10 *CDR1* expression in *TLO* complemented *TAC1-5* strains

The *CDR1* mRNA expression in the *tloΔ-TAC1-5* mutant and derivatives expressing *TLOα1* or *TLOβ2* was determined via qRT-PCR (Figure 4.3.11). The reintroduction of *TLOα1* in the *tloΔ-TAC1-5* mutant restored *TAC1-5* activated *CDR1* expression to a similar level as that observed in MAY1244-*TAC1-5*. The reintroduction of *TLOβ2* partially restored the expression of *CDR1* activation by *TAC1-5*, showing a significantly higher *CDR1* expression level

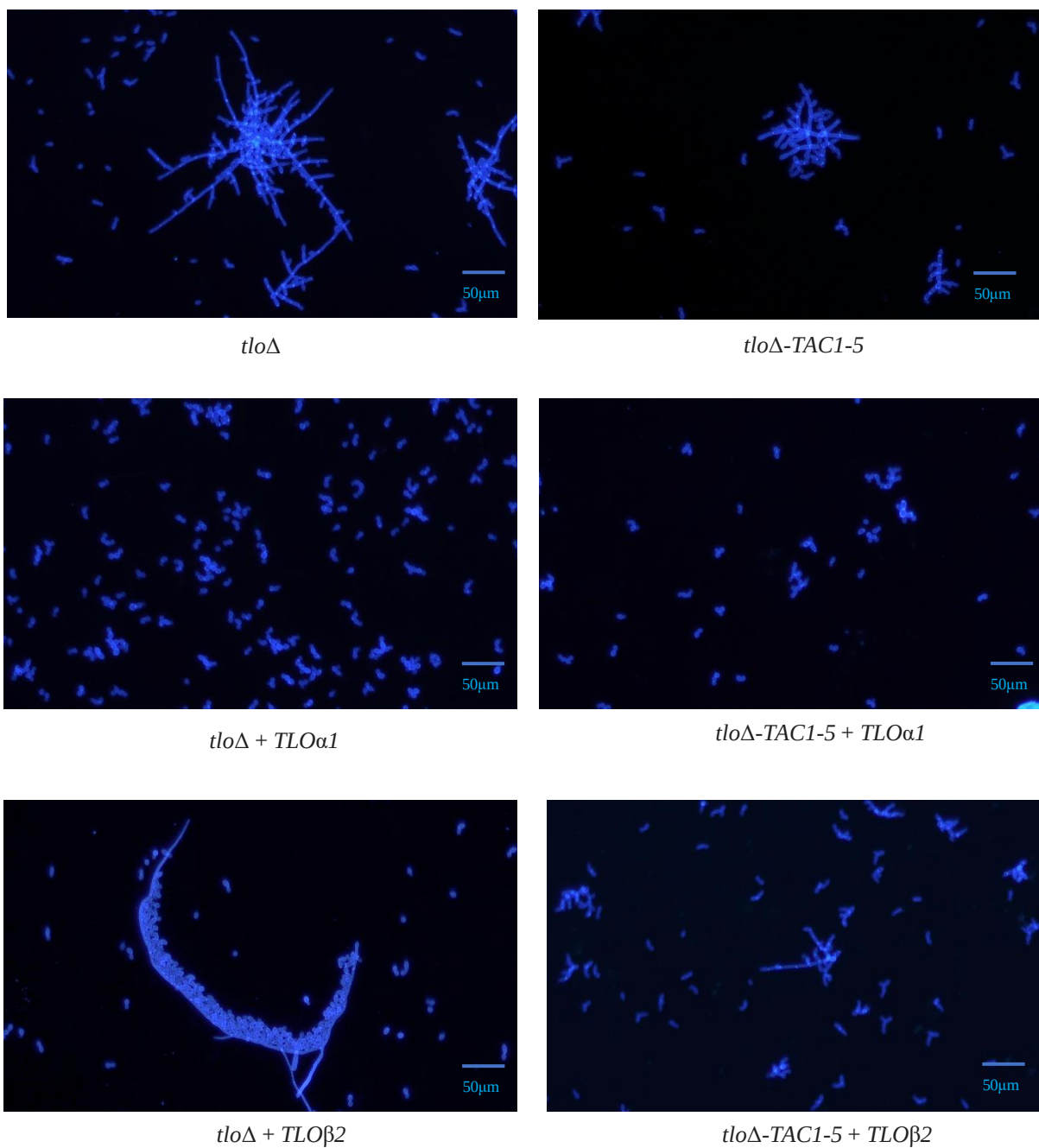


**Figure 4.3.10. Cell morphology (20X) of complemented *TLO* mutants with and without *TAC1-5***

Cells from overnight YEPD cultures (37°C) were stained with calcofluor white and visualized at 20x under Zeiss Axio microscope (UV light, 400nm).

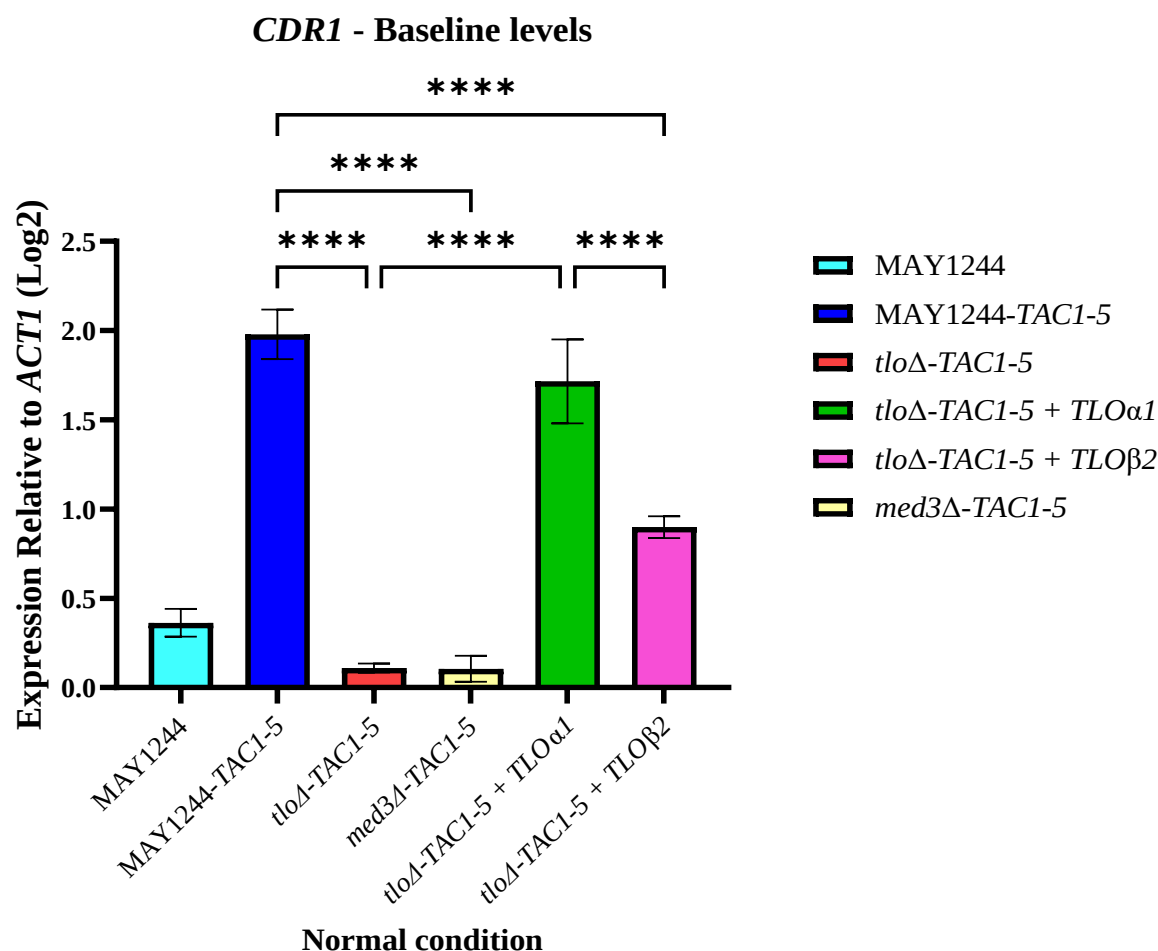


**Figure 4.3.11. CDR1 mRNA expression in *tloΔ*-TAC1-5 mutants and derivatives expressing *TLOα1* or *TLOβ2* determined via qRT-PCR.**

The *CDR1* expression of MAY1244 and the *tloΔ* mutant with WT and *TAC1-5* alleles, including derivatives expressing *TLOα1* and *TLOβ2*, at exponential growth phase (grown in YEPD broth at 30°C) was measured via qRT-PCR. The expression of each *CDR1* was normalised relative to the expression of endogenous control *ACT1* and was expressed as Log2 fold difference. Error bars were calculated based on three biological replicates and ordinary one-way ANOVA was carried out to show significant differences between strains (\*\* indicates p value < 0.01, \*\*\* indicates p value < 0.001, \*\*\*\* indicates p value < 0.0001).

compared to *tloΔ*. However, the expression of *CDR1* in the *TLOβ2* complemented *tloΔ-TAC1-5* background were significantly lower than *TLOα1 TAC1-5* and *MAY1244-TAC1-5* (Figure 4.3.11).

#### 4.3.11 Fluconazole spot plate assays of *tloΔ* and *tloΔ-TAC1-5* mutants complemented with *TLOα1* or *TLOβ2*

Fluconazole spot plate assays were carried out on YEPD agar at 37°C for 48h to determine the impact of reintroducing individual *TLOs* (*TLOα1* or *TLOβ2*) on the fluconazole sensitivity of the *tloΔ-TAC1-5* mutant (Figure 4.3.12). The reintroduction of *TLOs* from different clades showed different effects on the fluconazole susceptibility of the *tloΔ-TAC1-5* mutant. Reintroduction of *TLOα1* restored the highest level of fluconazole resistance exhibiting strong growth on the 10 µg/ml fluconazole plate whereas *TLOβ2* in the *tloΔ-TAC1-5* mutant restored intermediate levels of fluconazole resistance (Figure 4.3.12). Reintroduction of *TLO* genes to the *tloΔ* mutant without the *TAC1-5* allele did not impact significantly on fluconazole sensitivity as they did not show any growth on the 10 µg/ml fluconazole plate (Figure 4.3.12).

#### 4.3.12 Fluconazole disk diffusion assays *tloΔ* and *tloΔ-TAC1-5* mutants complemented with *TLOα1* or *TLOβ2*

The fluconazole susceptibility of the *tloΔ* and *tloΔ-TAC1-5* mutants compared to derivatives expressing individual *TLOs* (*TLOα1* or *TLOβ2*) was further investigated with a disk diffusion assay (Figure 4.3.13). Cyclosporine A was added to suppress azole tolerance and provide a clear zone of inhibition. The disk diffusion assays complemented the results of the previous fluconazole spot assays. The *tloΔ-TAC1-5* mutant expressing *TLOα1* exhibited the greatest fluconazole resistance with the smallest inhibition zone whereas the *TLOβ2* expressing

derivative exhibited an intermediate inhibition zone size. All *tloΔ* mutants without *TAC1-5* expressing individual *TLOs* (*TLOα1* or *TLOβ2*) showed similar fluconazole tolerance (growth all over disk plates without cyclosporine A) and fluconazole resistance profiles (i.e. similar inhibition zone on disk plates with cyclosporine A) (Figure 4.3.13).

#### 4.3.13 Fluconazole E-test assays to determine the MIC values of reintroduced *TLO* mutants with/without *TAC1-5*

Fluconazole E-test assays were carried out to obtain more precise fluconazole MIC values for *tloΔ* and *tloΔ-TAC1-5* mutants expressing individual *TLOs* (*TLOα1* or *TLOβ2*). (Figure 4.3.14 and Table 3.2). Similar to the results from fluconazole spot assays, the *tloΔ-TAC1-5* mutant expressing *TLOα1* exhibited the highest MIC value (16 µg/ml). The *tloΔ-TAC1-5* mutant expressing *TLOβ2* had an intermediate MIC value (12 µg/ml). The *tloΔ* mutant without *TAC1-5* and the derivatives expressing the individual *TLOs* (*TLOα1* or *TLOβ2*) all exhibited similar fluconazole MIC values approximately 1.5-2.0 µg/ml (Table 3.1 and Table 3.2).

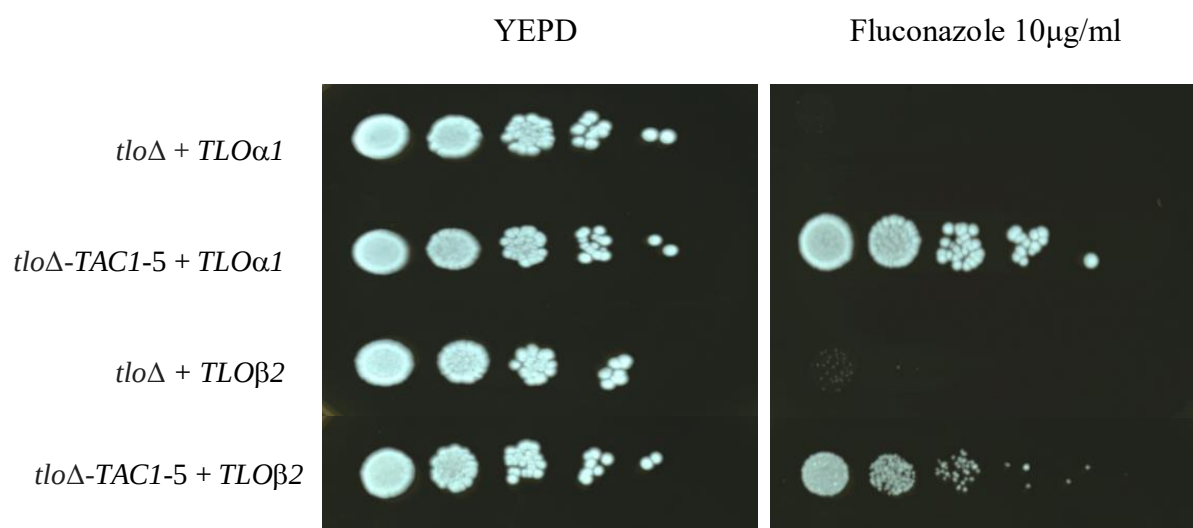


**Figure 4.3.12. Fluconazole spot plate assays of the *tloΔ* and *tloΔ-TAC1-5* mutants expressing singular *TLO*s (*TLOα1* or *TLOβ2*).**

Spot plate assays were performed using serial dilutions of overnight cultures ( $2 \times 10^6$ - $2 \times 10^2$  cells/ml) on YEPD plates containing fluconazole (10 µg/ml) or YEPD without fluconazole as a positive control. Plates were incubated in a static incubator for 48 h at 30 °C and experiments were done in biological triplicate.

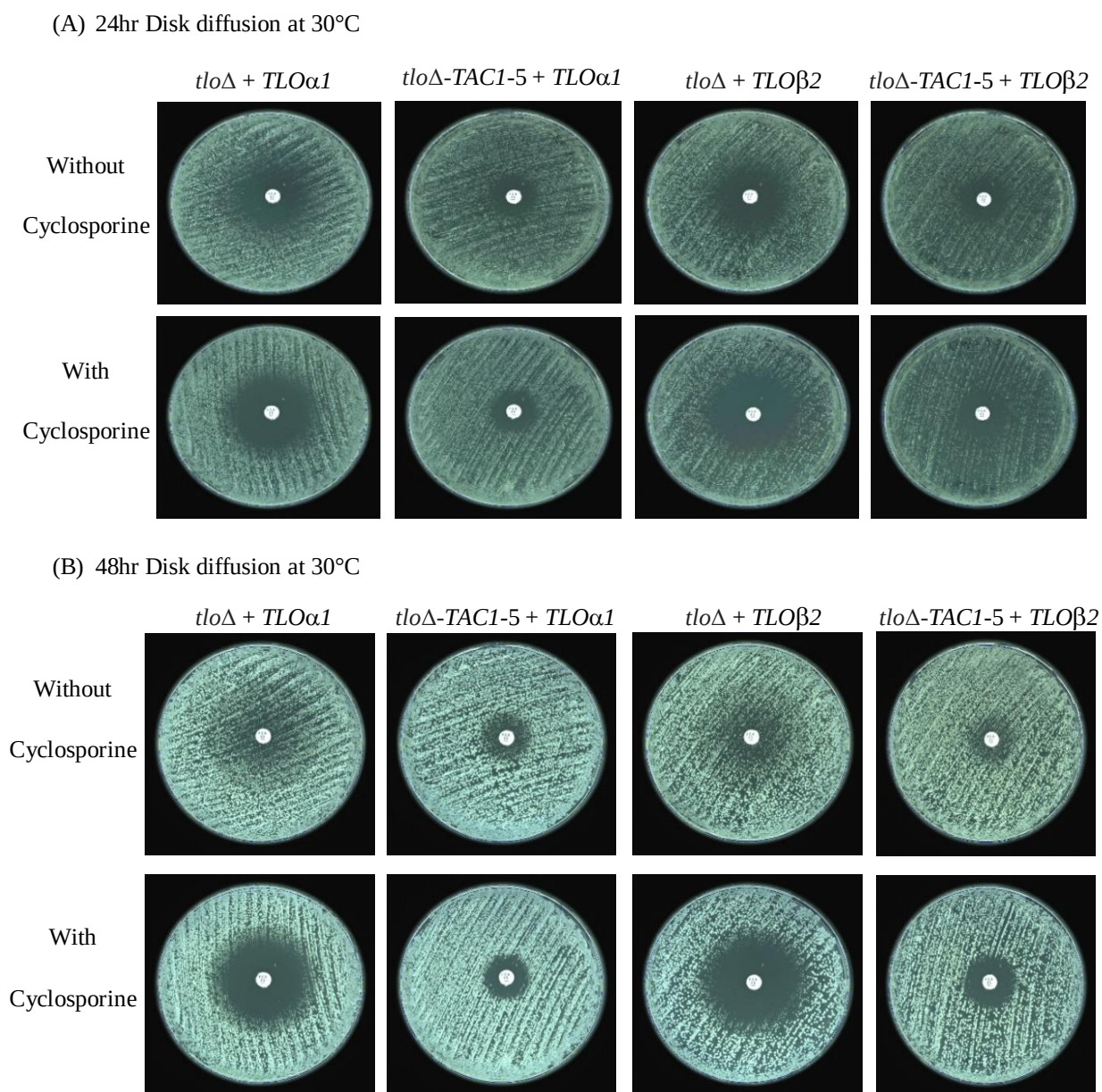


**Figure 4.3.13. Fluconazole disk diffusion assays of the *tloΔ* and *tloΔ-TAC1-5* mutants expressing individual *TLOs* (*TLOα1* or *TLOβ2*).**

Fluconazole disk diffusion assays on RPMI plates containing fluconazole (25 µg/ml) with and without cyclosporine (1 µg/ml) incubated at 30°C for (A) 24h and (B) 48h in triplicate.

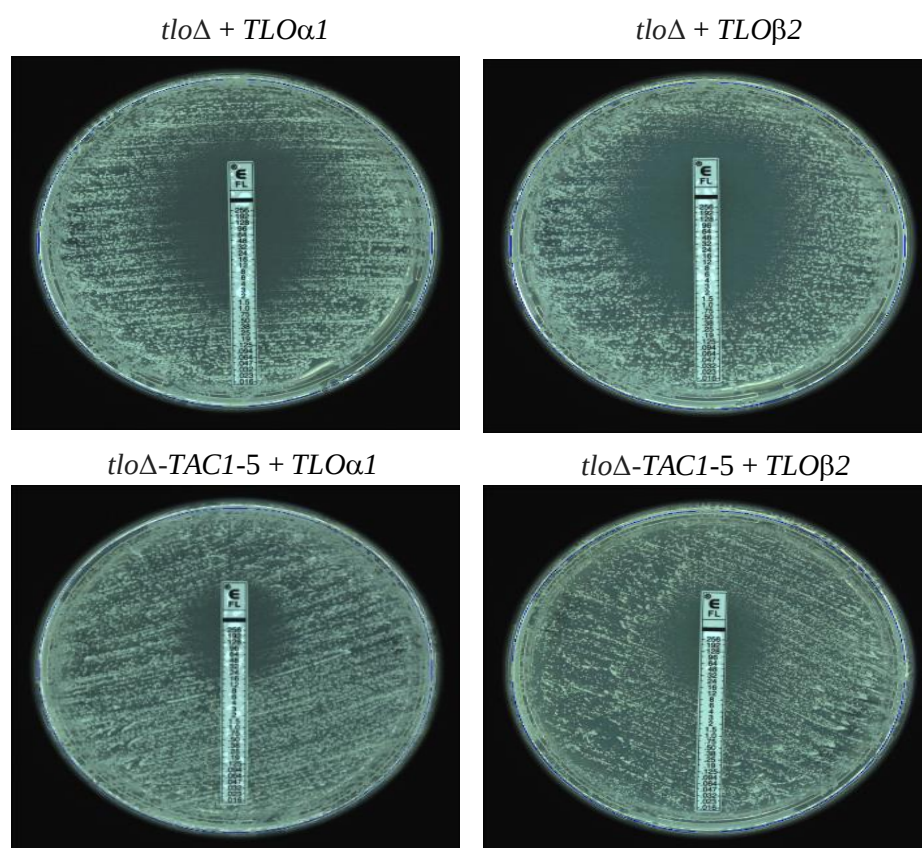


**Figure 4.3.14. Fluconazole E-test assays of the *tloΔ* and *tloΔ-TAC1-5* mutants expressing individual *TLOs* (*TLOα1* or *TLOβ2*).**

Fluconazole E-test were done on RPMI plates incubated for 24h at 30°C. Experiments were done in biological duplicate and MIC values were measured and shown in Table 3.2. down below.

**Table 3.2 Fluconazole E-test assay MIC values of the *tloΔ* and *tloΔ-TAC1-5* mutants expressing individual *TLOs* (*TLOα1* or *TLOβ2*).**

| Strains                    | MIC values (E- test) |
|----------------------------|----------------------|
| MAY1244- <i>TAC1-5</i>     | 16                   |
| <i>tloΔ + TLOα1</i>        | 1.5                  |
| <i>tloΔ-TAC1-5 + TLOα1</i> | 14                   |
| <i>tloΔ + TLOβ2</i>        | 2                    |
| <i>tloΔ-TAC1-5 + TLOβ2</i> | 12                   |

#### 4.3.14 Terbinafine spot plate assays of *tloΔ* and *tloΔ-TAC1-5* mutants expressing individual *TLOs* (*TLOα1* or *TLOβ2*).

Terbinafine spot plate assays were carried out to determine the impact of reintroducing individual *TLOs* (*TLOα1* or *TLOβ2*) in the *tloΔ-TAC1-5* mutant on its terbinafine sensitivity (Figure 4.3.15). Reintroduction of *TLOα1* in *tloΔ-TAC1-5* restored strong growth on 5 µg/ml terbinafine plates. The reintroduction of *TLOα1* in the *tloΔ* background had a weaker impact although growth was still observed at 5 µg/ml terbinafine. Restoration of *TLOβ2* in *tloΔ* and *tloΔ-TAC1-5* had dramatically different effects on terbinafine susceptibility. In the *tloΔ* background, *TLOβ2* restored growth on 5 µg/ml terbinafine plates to a similar level as *TLOα1*. However, in the presence of *TAC1-5*, *TLOβ2* appeared to increase terbinafine susceptibility in the *tloΔ* background (Figure 4.3.15).

#### 4.3.15 Western blotting to determine the expression of hemagglutinin (HA)-tagged Tlo proteins in the *tloΔ-TAC1-5* mutant

Western blotting was carried out to detect the presence of HA-tagged Tlo proteins in the *tloΔ-TAC1-5* mutant (Figure 4.3.16). Among the two Tlo proteins expressed (*Tloα1* and *Tloβ2*), only *Tloα1* protein was clearly detected in the *tloΔ-TAC1-5* mutant (Lane 1) and was similar to that detected in the *tloΔ* mutant derivative (Lane 3). However, the amount of *Tloβ2* protein produced by *tloΔ-TAC1-5* + *TLOβ2* (Lane 2) was abnormally low compared to the *tloΔ* + *TLOβ2* mutant (Lane 4).

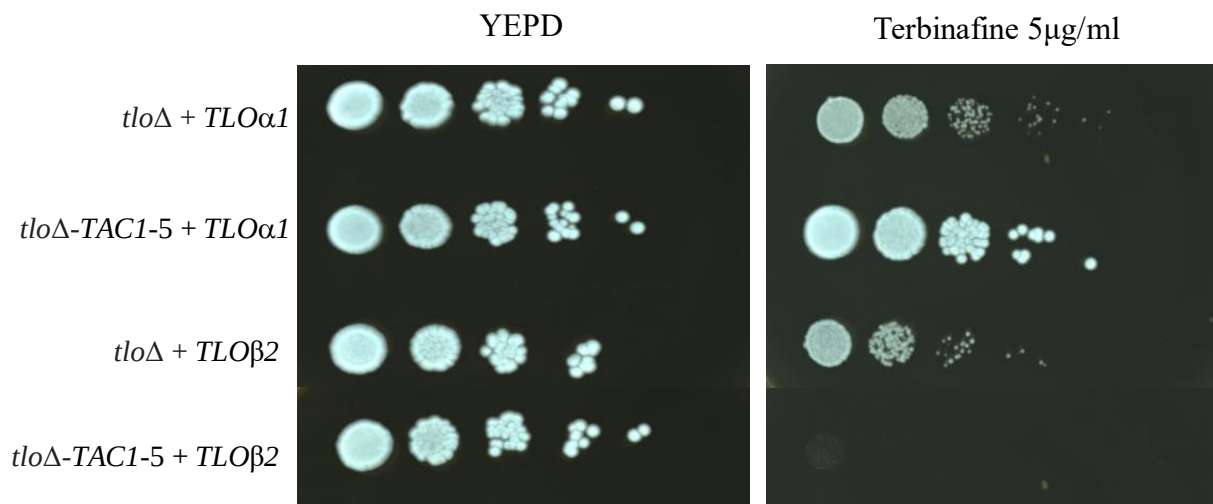


**Figure 4.3.15. Terbinafine spot plate assays of *tloΔ* and *tloΔ-TAC1-5* mutants expressing individual *TLOs* (*TLOα1* or *TLOβ2*).**

Spot plate assays were done with serial dilutions of overnight cultures ( $2 \times 10^6$ - $2 \times 10^2$  cells/ml) on YEPD plates containing terbinafine (concentration as labelled top) and YEPD without terbinafine as a positive control. Plates were incubated in a static incubator for 48 h at 30°C and experiments were done in biological triplicate.

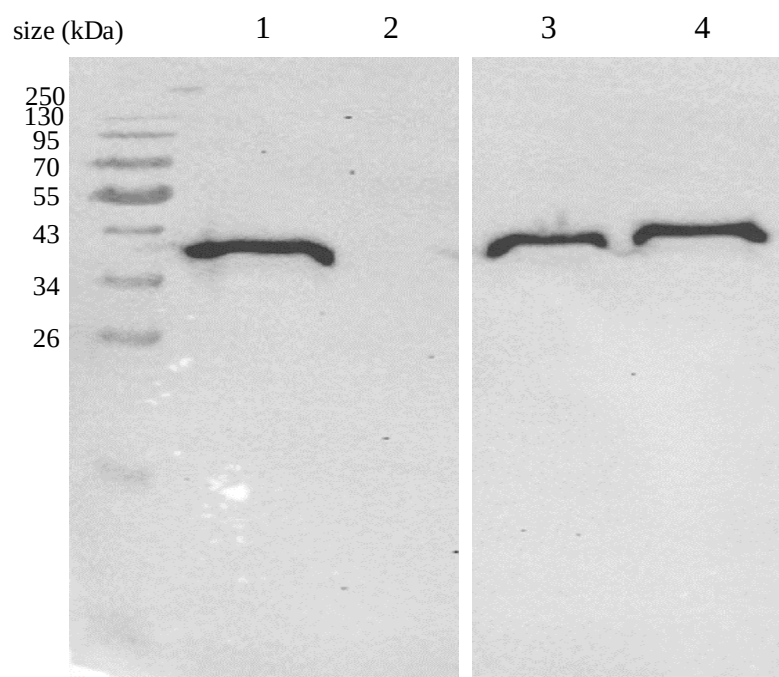


**Figure 4.3.16. Western blot analysis to detect HA-tagged Tlo protein expression in *tloΔ* and *tloΔ TAC1-5* mutants expressing individual *TLOs* (*TLOα1* or *TLOβ2*).**

Crude protein was extracted from exponential phase *tloΔ* and *tloΔ-TAC1-5* mutants expressing individual *TLOs* (*TLOα1* or *TLOβ2*) grown in YEPD media at 37°C. Anti-HA antibody (ab91110) from Abcam was used to detect HA-tagged Tlo proteins. The lanes are labelled as follows: (1) *tloΔ-TAC1-5 + TLOα1*, (2) *tloΔ TAC1-5 + TLOβ2*, (3) *tloΔ + TLOα1*, and (4) *tloΔ + TLOβ2*.

#### 4.3.16 Optimisation of DNA fragmentation by sonication for ChIP-qPCR

In order to investigate Tlo binding at the *CDR1* promoter, fragmentation of genomic DNA by sonication was optimised for ChIP-qPCR. The initial sonication trial was done for 12 rounds and 14 rounds. 14 rounds of sonication was chosen as it provided an ideal DNA fragment size of approximately 300-500bp. All sonicated samples from *TLO* reintegrated strains exhibited concentrated DNA regions of around 300-500bp on agarose gels prior to HA-tagged protein pulldown (Figure 4.3.17).

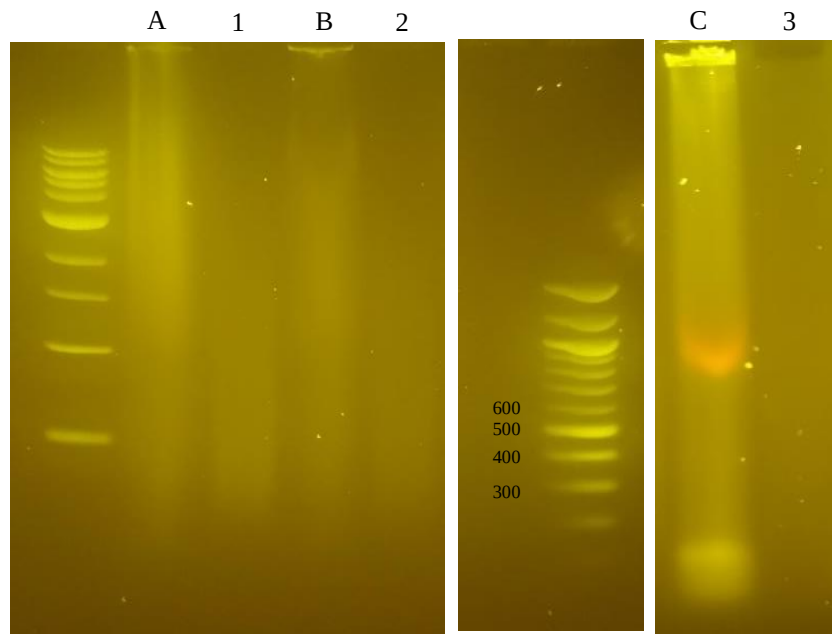


**Figure 4.3.17. Gel electrophoresis to detect DNA fragment sizes *tloΔ* and *tloΔ-TAC1-5* mutants expressing individual *TLOs* (*TLOα1* or *TLOβ2*) after sonication.**

The DNA fragments of each biological replicates of the *tloΔ-TAC1-5* complemented strains were subjected to gel electrophoresis to ensure DNA fragments size is between 300-500bp. The lanes are labelled as: (1) MAY1244 *TAC1-5*, (2) *tloΔ-TAC1-5* + *TLOα1*, (3) *tloΔ-TAC1-5* + *TLOβ2*, (A) MAY1244 *TAC1-5*, (B) *tloΔ-TAC1-5* + *TLOα1*, (C) *tloΔ-TAC1-5* + *TLOβ2*. DNA sizes are labelled from 600 to 300 bp adjacent to the DNA ladder. DNA samples in lanes 1, 2, and 3 were sonicated for 14 rounds whereas DNA samples in lanes A, B and C were not sonicated.

#### 4.3.17 ChIP-qPCR confirmation via Polymerase II pulldown

To ensure that the IP protocol was working, an RNA polymerase II (Pol II) pulldown was carried out to confirm the ChIP-qPCR method. The *PUT1* (C5\_02600W) gene of SC5314 was chosen as a positive binding site of Pol II protein based on previous ChIP-qPCR analysis (Haran *et al.*, 2014). Two primer pairs (*PUT1A* and *PUT1B*) targeting internal regions of *PUT1* were designed to detect Pol II enrichment in this region (Table 2.1). Another intergenic region of SC5314 located downstream of *PRE4* (C5\_02230W) was chosen as a negative binding site of Pol II protein based on previous ChIP-qPCR analysis (Haran *et al.*, 2014). The *NEG1* primers amplifying an intergenic region from position 496,161 bp to 496,215 bp on chromosome 5 were designed as a negative control (Table 2.1). ChIP-qPCR showed significant enrichment of Pol II at the *PUT1A* and *PUT1B* sites (normalized against *NEG1*), confirming the success of the method (Figure 4.3.18).

## ChIP Pol II pulldown

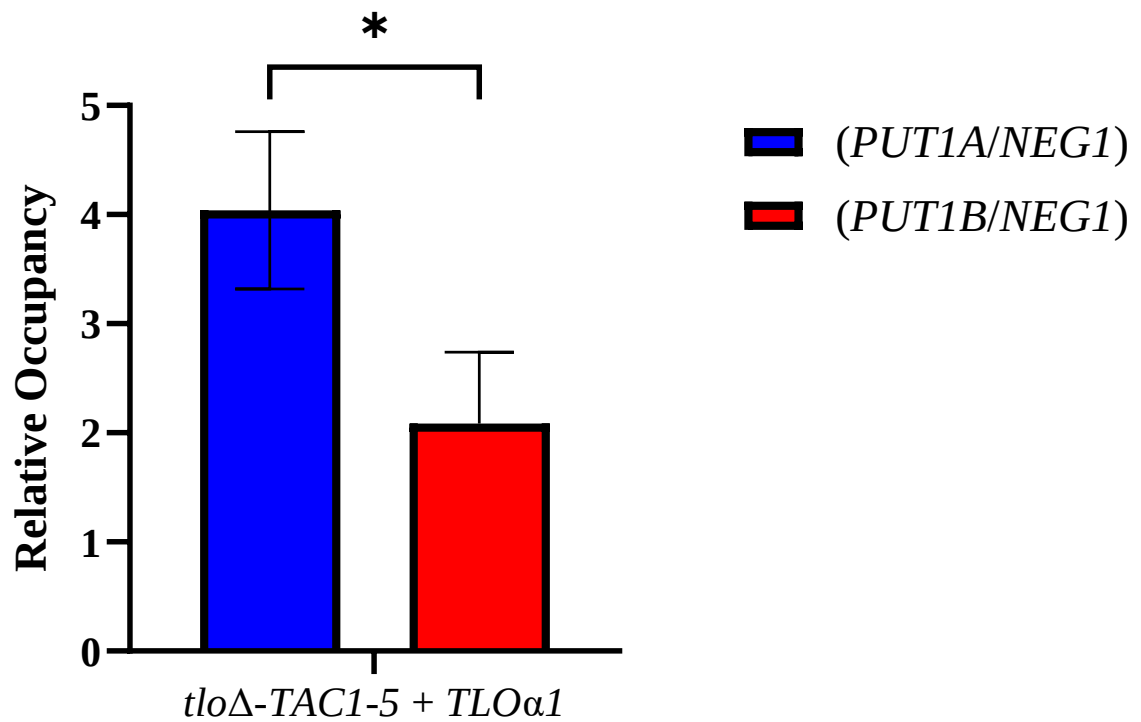


**Figure 4.3.18.** RNA polymerase II occupancy at *PUT1* regions in *tloΔ-TAC1-5 + TLOα1*

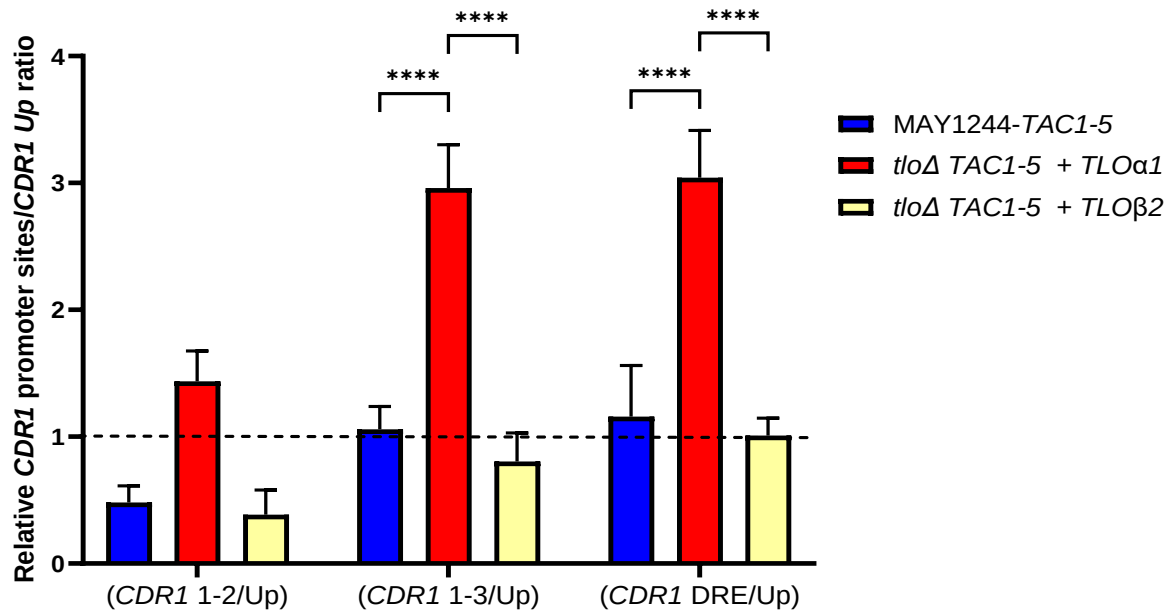
RNA polymerase II (RNAP II) occupancy at two internal regions of *PUT1* (*PUT1A* and *PUT1B*) in *tloΔ-TAC1-5 + TLOα1* yeast cells grown in YEPD. RNAP II signals (IP/IN, see 4.2.5.7) were normalized to an internal negative control region (IP/IN at *NEG1*). Error bars were calculated based on three biological replicates and t-test was carried out to show significant differences between *PUT1A* and *PUT1B* (\* indicates p value < 0.05).

#### 4.3.18 ChIP-qPCR demonstrates occupancy of Tlo proteins at the *CDR1* promoter site

ChIP-qPCR was performed after HA-tagged Tlo pulldowns to detect protein-gene interactions at the *CDR1* promoter site (Figure 4.3.19). Three upstream *CDR1* promoter sites were tested, namely *CDR1* 1-2, *CDR1* 1-3, and *CDR1* DRE (Figure 4.3.19). An upstream *CDR1* promoter site (*CDR1* Up), previously shown to lack any Mediator binding (Liu and Myers, 2017b) was chosen as a negative control. Extracts from MAY1244-*TAC1*-5 were chosen as a negative pulldown control since it did not express any HA-tag. Occupancy at each site was expressed as a ratio to *CDR1* Up levels and ratios higher than 1 were considered positive enrichment. ChIP-qPCR analysis revealed that Tlo $\alpha$ 1 from *tloA*-*TAC1*-5 + *TLO* $\alpha$ 1 showed significant occupancy at the promoter site *CDR1* 1-3 and *CDR1* DRE as it showed significantly higher ratios than MAY1244-*TAC1*-5 (approximately 1) and others. Tlo $\beta$ 2 (*tloA*-*TAC1*-5 + *TLO* $\beta$ 2) did not exhibit any significant occupancy at the *CDR1* 1-3 and *CDR1* DRE sites compared to MAY1244-*TAC1*-5. None of the Tlo proteins exhibited significant occupancy at promoter site *CDR1* 1-2, with Tlo $\alpha$ 1 showing a slightly increased ratio relative to *CDR1* Up.

A)

### Tlo ChIP occupancy ( $\alpha$ -HA) at *CDR1* promoter sites



B)

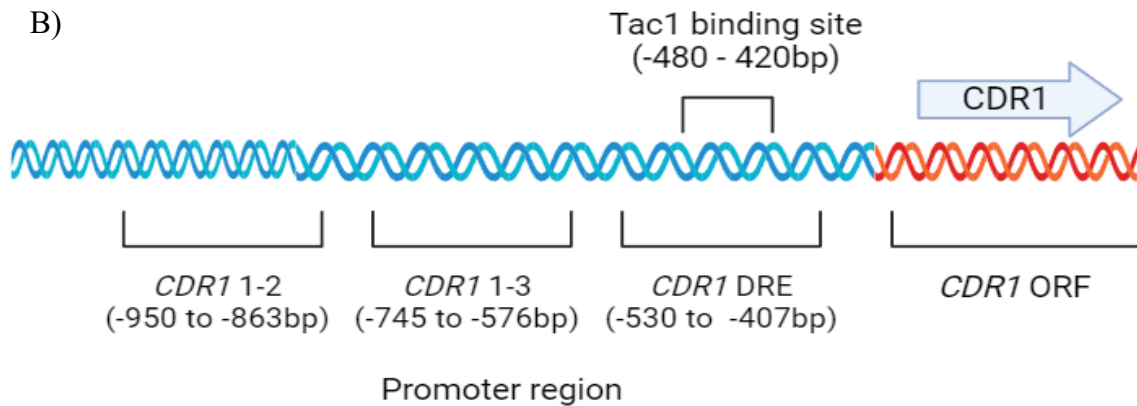


**Figure 3.21. Tlo protein (Tloα1 or Tloβ2) occupancy at different *CDR1* promoter sites**

A) The occupancy of Tlo proteins on three different *CDR1* promoter sites (*CDR1* 1-2, *CDR1* 1-3, and *CDR1* DRE) in *tlo*Δ-*TAC1*-5 reintegrated strains was determined by using anti-HA antibody to pulldown HA-tagged Tlo proteins (Tloα1 or Tloβ2). HA-tagged Tlo protein signals (IP/IN, see 4.2.5.7) at the three sites were normalized to an internal negative control region (IP/IN at *CDR1* Up) and a ratio of 1 was set as the cut off value to determine occupancy (positive = >1) as the non-HA-tagged strain (MAY1244-*TAC1*-5) exhibited ratios of approximately 1. Error bars were calculated based on three biological replicates and ordinary one-way ANOVA was carried out to show significant differences between different strains (\*\* indicates p value < 0.01, \*\*\* indicates p value < 0.001, \*\*\*\* indicates p value < 0.0001). B) Figure showing the three *CDR1* promoter sites and Tac1 binding sites at the *CDR1* promoter.

## 4.4 Discussion

This chapter primarily focused on investigating the role of the *TLO* genes, encoding the Med2 component of Mediator in *C. albicans*, in Tac1-activated expression of the *CDR1* drug efflux pump encoding gene.

### 4.4.1 *TLO* genes mediate basal and xenobiotic induced *CDR1* expression levels and are required for Tac1-activated fluconazole and terbinafine resistance

Examination of *CDR1* mRNA expression in *C. albicans* WT MAY1244 and the two Mediator tail null mutants, *tlo* $\Delta$  and *med3* $\Delta$ , showed that basal *CDR1* mRNA expression requires a functional Mediator tail. Deletion of *tlo* $\Delta$  and *med3* $\Delta$  also prevented Tac1-mediated *CDR1* induction by fluphenazine, indicating that *TLO* genes have a major role in *CDR1* mRNA expression. Furthermore, direct evidence for a *TLO* requirement for Tac1 function was presented by showing that the hyperactive Tac1 allele, *TAC1-5*, failed to increase *CDR1* mRNA expression in the *tlo* $\Delta$  null mutant. These findings also support the hypothesis that Tac1<sup>GOF</sup> mutants and xenobiotic-induced wild-type Tac1 induce *CDR1* mRNA via a similar mechanism through Mediator, indicating a general mechanism to block Tac1 hyperactivation via a small molecule screening approach. Similar results were observed in a *med3* $\Delta$ -*TAC1-5* mutant, suggesting that *TLOs* regulate *CDR1* expression by interacting with Tac1 like other Mediator tail mutants. Both Mediator tail null mutants did not significantly affect the basal *CDR2* mRNA expression and fluphenazine was unable to activate strong *CDR2* mRNA expression, supporting recent studies showing that *CDR2* is highly dependent on chromatin remodelling activity (Swi/Snf) rather than Mediator tail module (Liu and Myers, 2017b).

In this study we show that *CDR1* mRNA expression is highly dependent on the Mediator tail components Tlo or Med3. However, introduction of the *TAC1*<sup>GOF</sup> to the *tloΔ* and *med3Δ* null mutants significantly increased fluconazole MICs without significantly increasing *CDR1* mRNA levels. This finding is similar to recently published studies examining the role of Med15 in regulating *CDR1* expression (Liu and Myers, 2017b). This suggests that *TAC1*<sup>GOF</sup> mutations either regulate other genes involved in fluconazole resistance independently of Mediator tail or that *TAC1*<sup>GOF</sup> mutations might have a role in regulating Cdr1 protein levels. In this scenario, it is possible that an inhibitor of Tac1-Mediator tail module interactions, similar to the *C. glabrata* inhibitor that disrupts Pdr1-Med15 interactions, would have less efficacy in *C. albicans* *TAC1*<sup>GOF</sup> azole-resistant strains (Nishikawa *et al.*, 2016).

Unexpectedly, the fluconazole disk diffusion assay showed that the Tac1<sup>GOF</sup> mutation seemed to reduce the azole tolerance of the *tloΔ* mutant (Figure 4.3.6). Recently, the *tloΔ* mutant has been shown to exhibit high levels of fluconazole tolerance, particularly when grown at 37°C, and that this phenotype may be due to changes in *ERG* gene expression (O'Connor-Moneley *et al.*, 2024). The data shown here suggest that Tac1<sup>GOF</sup> mutations might interfere with *tloΔ* induced azole tolerance and might impact on expression of genes required for fluconazole tolerance, however further gene expression studies may be required to confirm this. Unlike the *med3Δ* mutant, the *tloΔ* mutant was also shown to be hypersensitive to terbinafine, suggesting that Tlo/Med2 and Med3 have a different regulating mechanism relating to terbinafine susceptibility and might regulate other antifungal resistance or tolerance mechanisms differently.

#### 4.4.2 The effect of singular *TLO* from different clades on Tac1<sup>GOF</sup> induced *CDR1* and Tac1-activated fluconazole and terbinafine resistance

We compared the activity of representative *TLO* genes from the alpha (*TLO $\alpha$ 1*) beta (*TLO $\beta$ 2*) clades in restoring *CDR1* expression and fluconazole susceptibility in the *tloA-TAC1-5* strain. We did not include a representative from the *TLO* gamma clade as previous studies showed that these Tlos are poorly expressed at the protein level and do not confer any phenotypic changes in the *tloA* mutant. An unexpected finding of this current study was the low level of *TLO $\beta$ 2* mRNA and protein expressed in the *tloA-TAC1-5 + TLO $\beta$ 2* strain compared to the *tloA + TLO $\beta$ 2* strain. This suggests that *TAC1-5* expression somehow downregulates *TLO $\beta$ 2* mRNA expression but not *TLO $\alpha$ 1* in *C. albicans*, suggesting that hyperactive Tac1 isoforms interact differently with individual *TLO* genes. The low *TLO $\beta$ 2* mRNA expression resulted in low Tlo $\beta$ 2 protein expression in the *tloA-TAC1-5 + TLO $\beta$ 2* mutant (as shown in Western blotting analysis). Lack of Tlo $\beta$ 2 protein could also be inferred in the cellular phenotype of the *tloA-TAC1-5 + TLO $\beta$ 2* strain. In the parental *tloA* background, the highly expressed Tlo $\beta$ 2 protein reverses the pseudohyphal morphology of the *tloA* mutant and restores a normal yeast cell morphology. However, introduction of *TLO $\beta$ 2* in the *tloA-TAC1-5* background did not significantly change the pseudohyphal morphology compared to the parent strain. Further evidence for a possible antagonistic relationship between *TLO $\beta$ 2* and *TAC1-5* was generated in the terbinafine spot plate assays which showed that the presence of *TLO $\beta$ 2* could reverse *TAC1-5* mediated terbinafine resistance in *tloA*. It may be possible that *TLO $\beta$ 2* may also antagonise the function or expression of *TAC1-5* which may explain the effects on terbinafine susceptibility. It would be interesting to determine if *TAC1-5* expression levels (mRNA or protein) are affected in strains expressing *TLO $\beta$ 2* or whether Tlo $\beta$  and Tac1 physically interact.

Based on qRT-PCR and Western blotting analysis, *TLO $\alpha$ 1* was shown to be highly expressed in the presence of *TAC1-5*, matching the results shown by Fletcher *et al.* (2023). *TLO $\alpha$ 1* also highly restored Tac1<sup>GOF</sup>-activated *CDR1* mRNA expression, implicating Tlo $\alpha$ 1 protein as the crucial protein that works with hyperactive Tac1<sup>GOF</sup> to overexpress *CDR1*. Despite the low mRNA and protein expression of *TLO $\beta$ 2*, Tlo $\beta$ 2 could partially restore *TAC1-5* activated *CDR1* mRNA expression, suggesting that low amounts of Tlo $\beta$ 2 may be sufficient to restore Tac1-activated *CDR1* mRNA expression.

#### 4.4.3 ChIP-qPCR reveals Tlo $\alpha$ 1 interactions with the *CDR1* DRE

ChIP-qPCR was carried out to show the occupancy of individual Tlo proteins at various *CDR1* promoter sites. We included the Drug Response Element (DRE) which is the site of Tac1 binding at the *CDR1* promoter. ChIP-qPCR analysis showed enrichment of Tlo $\alpha$ 1 at the DRE whereas Tlo $\beta$ 2 did not show any positive enrichment. This suggests that Tlo $\alpha$ 1 could interact with Tac1 at the DRE in the *CDR1* promoter. The low levels of enrichment of Tlo $\beta$ 2 at the *CDR1* promoter is perhaps not unexpected due to the low levels of protein detected by Western blotting analysis in the respective *tlo $\Delta$ -TAC1-5* strains.

The *CDR1* 1-3 and *CDR1* DRE regions of the *CDR1* promoter regions exhibited the greatest enrichment in Tlo $\alpha$ 1 levels whereas *CDR1* 1-2 (the furthest upstream promoter site) had very little enrichment in all Tlo pulldowns. The *CDR1* 1-3 promoter site is located at SRE2, a *cis* element that is located beside SRE1. Both SRE1 and SRE2 have been characterised as a steroid-responding region (SRR) (Prasad *et al.*, 2015). SRE1 can be induced by progesterone whereas SRE2 can be induced by progesterone and  $\beta$ -estradiol (Prasad *et al.*, 2015). The DRE promoter site has been proposed as the binding site of Tac1 to initiate induction of *CDR1* expression (Liu

and Myers, 2017b). The most possible model of the interaction between Tlo $\alpha$ 1 and Tac1 is shown in Figure 4.4.1. Tlo $\alpha$ 1 could be recruited at the *CDR1* DRE promoter site by DRE-bound hyperactive Tac1 which can then recruit other Mediator components to facilitate transcriptional activation (Liu and Myers, 2017b). The direct/indirect binding of Tlo $\alpha$ 1 at the *CDR1* DRE is not clearly verified as it is possible that Tlo $\alpha$ 1 binds indirectly to the DRE via other Mediator components. Future ChIP experiments will be required to clearly show the direct/indirect nature of Tlo $\alpha$ 1 binding at the *CDR1* DRE and *CDR1* 1-3 promoter sites and could be used to determine which Mediator components can be recruited by Tlo $\alpha$ 1 at the *CDR1* DRE promoter site.

#### 4.4.4 Summary and possible future work

To summarize, Tlo $\alpha$ 1 protein was found to interact with the DRE (the site of Tac1-binding) to upregulate *CDR1* expression and confer fluconazole and terbinafine resistance. Surprisingly, Tlo $\beta$ 2 was found to be only weakly expressed in *TAC1-5* mutants, resulting in weaker interactions with the DRE and a lesser impact on *CDR1* expression. Future studies should determine whether inhibition of alpha-Tlo, which regulates virulence related functions of *C. albicans* (Fletcher et al., 2023) in addition to *CDR1* expression, could be a novel antifungal strategy.

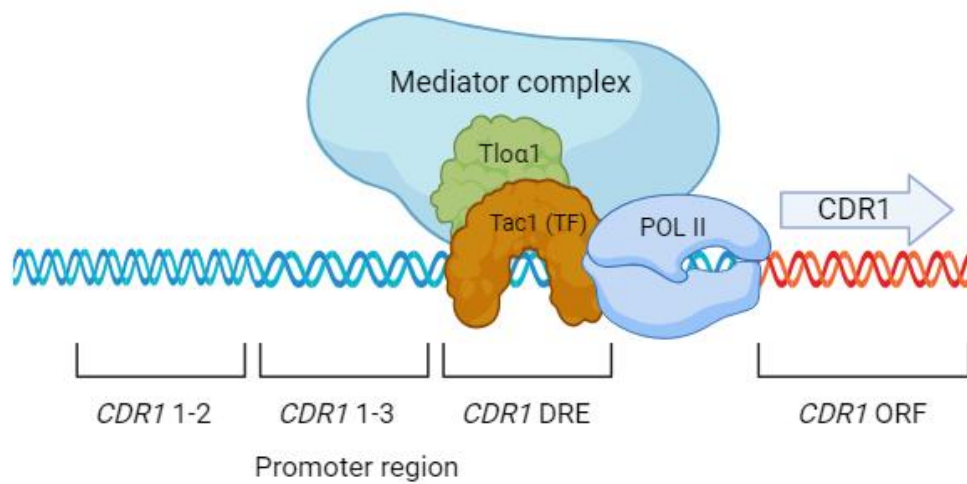


**Figure 4.4.1. The interaction model between Tloα1 with Tac1 and other components at the *CDR1* promoter.**

The 3 *CDR1* promoter regions was labelled below the brackets and *CDR1* ORF indicates the open reading frame (ORF) of *CDR1*. All protein components were shown in the figure and Tloα1 is most likely to bind at the *CDR1* DRE site together with the hyperactive Tac1 and recruits other Mediator components and Pol II to initiate *CDR1* expression.

## **Chapter 5**

Analysis of the role of Med2 and Med15 in drug susceptibility and cellular aggregation in *C. auris*

## 5.1 Introduction

### 5.1.1 Cellular aggregation mechanisms in *C. auris*

Unlike *C. albicans*, which can form true hyphae to adapt to various environments, *C. auris* can form cellular aggregates. This phenotype was found to be prevalent in certain genetic clades of *C. auris* and can aid colonization of human skin and other surfaces and acts as a unique *C. auris*-specific virulence trait (Chowdhary *et al.*, 2023). Strains which could not spontaneously aggregate have also been shown to exhibit inducible aggregation in response to antifungal drugs including azoles and echinocandins (Pelletier *et al.*, 2024).

Currently, three distinct mechanisms of aggregation have been described: (1) adhesin mediated aggregation, (2) cell separation failure and (3) extracellular matrix aggregation (Figure 5.1.1). Adhesin mediated aggregation was shown to be the result of cell-cell attachment via cell surface proteins. The agglutinin-like sequence (ALS) family adhesin of *C. auris*, *ALS4112* (B9J08\_004112), was shown to be responsible for adhesin mediated aggregation and this phenotype is media-dependent (Pelletier *et al.*, 2024). In *C. albicans*, Als proteins including Als5, promote cell-cell adhesion through the formation of amyloid aggregates in biofilms (Lipke *et al.*, 2021). The mechanism of Als4112 in *C. auris* might be similar to how Als5 promotes amyloid aggregates in *C. albicans* as the treatment of *C. auris* aggregative strain with the amyloid-inhibiting components Thioflavin-T or Congo Red partially suppressed its aggregation (Malavia-Jones *et al.*, 2023). Another study revealed that aggregation phenotypes observable in PBS can be suppressed by water, supporting the idea of amyloid-based aggregation in *C. auris* which can be disrupted by electrostatic changes (Pelletier *et al.*, 2024). The aggregation of *C. auris* might depend on another underlying cell surface proteinaceous mechanism like its close relative *C. haemulonii*, as both can be disrupted by proteinase K, trypsin, or SDS.

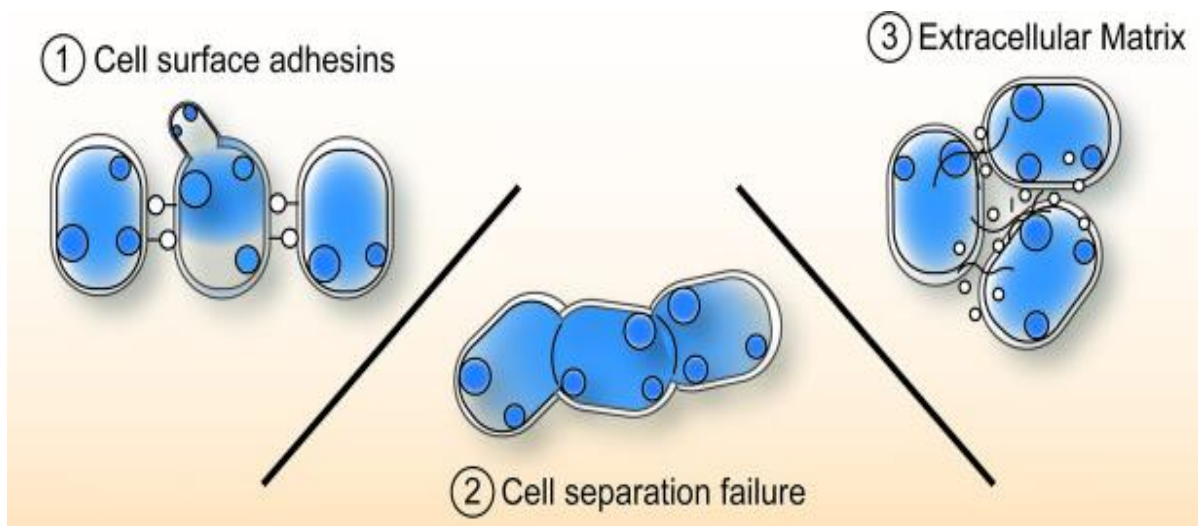


**Figure 5.1.1. Three distinct molecular mechanism of *C. auris* aggregation.**

The current three major molecular mechanism that have been discovered: 1) Cell-cell attachment due to various adhesins like Als4112 and Scf1. 2) Cell-separation defects causing cell attached to each other due to incomplete septum degradation. 3) The production of extracellular matrix to connect cells together. Figure adapted from (Santana *et al.*, 2024).

The transcriptional control of Als4112 has been shown to regulate the aggregation of *C. auris*. Overexpression of *ALS4112* was found in an aggregative strain after multiple generations grown under sub-inhibitory concentrations of caspofungin (Fayed *et al.*, 2021). However, the overexpression of *ALS4112* along with the ability to form aggregates was reduced in the absence of caspofungin. This result is redolent of Als1-mediated echinocandin-induced aggregation in *C. albicans* (Gregori *et al.*, 2011). Similarly, a study discovered a *C. auris* naturally aggregative isolate that has multiple copies of *ALS4112* which showed enhanced adherence and biofilm formation (Bing *et al.*, 2023). Another study revealed that a clade III aggregative strain that overexpressed *ALS4112* in SabDEX medium could not aggregate when *ALS4112* was deleted, showing that *ALS4112* plays a major role in aggregation (Pelletier *et al.*, 2024). Interestingly, non-aggregative strains will not upregulate the expression of *ALS4112* when grown in SabDEX but instead upregulate a different *ALS* gene that does not mediate aggregation (Pelletier *et al.*, 2024). The overexpression of *ALS4112* in a clade I non-aggregative isolate can induce aggregation, suggesting that *ALS4112* mediated aggregation is likely due to gene regulation rather than clade-specific adhesin sequences (Santana *et al.*, 2023). The same study discovered another novel, conserved, adhesin named Scf1 that also contributes to aggregation in *C. auris* from clade I to V (Santana *et al.*, 2023). The deletion of Scf1 was shown to impair *C. auris* biofilm formation, skin colonization, virulence in systemic infection, and colonization of inserted medical devices (Santana *et al.*, 2023).

The second aggregation mechanism is the formation of aggregates due to cell separation failure after budding. The cell separation failure might be due to a failure of septum degradation following cytokinesis. A genetic screen in *C. auris* identified aggregative mutants having defects in the Regulation of *ACE2* and the Morphogenesis (RAM) pathway (Santana and O'Meara, 2021). The RAM pathway is a conserved protein kinase signalling pathway that

regulates the daughter cell-specific localization by regulating Ace2, a daughter cell specific transcription factor that regulates the transcription of multiple septum-degrading enzymes like chitinases and glucanases (Saputo *et al.*, 2012). Interestingly, a report identified two *C. auris* patient isolates from urinary tract infections exhibiting a rough morphology and a strong aggregation phenotype due to cell separation defects associated with a heritable single-point mutation in *ACE2* (Tian *et al.*, 2023). The emergence of heritable and constitutive aggregation after passaging through a mammalian body might be similar to the rare cases of aggregative or elongated derivatives recovered after passage of non-aggregative parental isolates through animal hosts, although the genetic differences between these isolates remain unexplored (Yue *et al.*, 2018; Fan *et al.*, 2021). Studies revealed the transcriptional downregulation of chitinase genes in naturally aggregative *C. auris* isolates, but it is unclear whether the downregulation of chitinase genes makes a major contribution to aggregation (Brown *et al.*, 2020; Malavia-Jones *et al.*, 2023). A recent study discovered that cell separation defects might contribute to echinocandin-induced aggregation phenotypes (Pelletier *et al.*, 2024).

In the third aggregation mechanism, extracellular matrix aggregation, aggregative cells produce extracellular matrix (ECM) to connect cells and form aggregates. A single study observed the presence of ECM linkages between aggregates through SEM from cells cultured at both 37°C and 30°C (Malavia-Jones *et al.*, 2023). ECM aggregation was inducible in isolates across 4 major clades (I – IV), even in naturally non-aggregative isolates at 37°C. However, low amounts of ECM are observed in naturally aggregative isolates at 30°C, although the production of ECM in these isolates can be induced by higher temperatures of 37°C (Malavia-Jones *et al.*, 2023). The activation of ECM aggregation by higher temperatures in both non-aggregative and aggregative isolates is consistent with an observational study showing that all 19 isolates representing non-aggregative and aggregative phenotypes *in vitro* from 4 major

clades formed aggregates in infected organs in a murine model (Forgács *et al.*, 2020). All these reports suggest that all *C. auris* can form aggregates under specific conditions.

#### 5.1.2 Aim of this study

This study aimed to determine if Mediator tail plays a role in regulating antifungal susceptibility or aggregation in *C. auris*. To investigate this, we examined the *med2Δ* and *med15Δ C. auris* mutants generated by deleting the *MED2* or *MED15* genes using CRISPR-Cas9 mutagenesis, as shown in Chapter 3. As with most other yeast species, the *C. auris* genome contains only a single *MED2* and a single *MED15* gene. These mutants were generated in the non-aggregative isolate (8985) and the aggregative isolate (8977) to determine their role in different strain backgrounds.

## 5.2 Materials and Methods

### 5.2.1 Cell imaging and calcofluor white stained cell imaging under various conditions

All *Candida auris* strains were grown overnight in 4 ml YEPD media in a shaking incubator set at 37°C, 200 rpm. PBS-induced aggregation assays were carried out by washing overnight cultures twice in sterile PBS buffer (pH 7.2), resuspended in sterile PBS, and finally adjusted to a density of  $10^8$  cells/ml. All diluted samples will be vortexed for 1 min. The calcofluor white staining was carried out on the resuspended cell mixture in PBS according to Section 2.2.8.

Echinocandin-induced aggregation assays were carried out by growing *C. auris* strains in RPMI containing 4 µg/ml of anidulafungin overnight. Overnight cultures were then washed and standardized to  $10^8$  cells/ml in sterile water. All diluted samples will be vortexed for 1 min. *C. auris* cells were then visualized under a microscope according to Section 2.2.7.

### 5.2.2 Aggregation kinetic assays

All *C. auris* strains were grown overnight in 4 ml YEPD medium in a shaking incubator set at 37°C, 200 rpm. Overnight cultures were standardised to  $10^8$  cells/ml in sterile PBS buffer (pH 7.2) as described in section 5.2.1. The resuspended cell mixture was immediately transferred into a plastic cuvette (1ml/cuvette) and OD<sub>540</sub> values were measured before starting the experiment. The fungal suspension was incubated at 37°C without agitation for 2 hours, with OD<sub>540</sub> values measured every 30 minutes. Percentage of aggregation was calculated based on the reduction in OD<sub>540</sub> value using the formula:  $([OD_0 - OD_t]/OD_0) \times 100$ , where OD<sub>0</sub> is the OD<sub>540</sub> value measured at the start of the experiment and OD<sub>t</sub> is the OD<sub>540</sub> value obtained at different incubation time points. PBS without fungal cells was used as a blank. The calculated

percentage of aggregation was classified into three groups: high (more than 40%), intermediate (30-40%), and low aggregation (less than 30%).

### 5.2.3 Biofilm assays and Crystal violet biofilm staining

All *C. auris* strains were grown overnight in 4ml YEPD medium in a shaking incubator set at 37°C, 200 rpm. On the following day, the OD<sub>600</sub> value of the overnight culture was measured and standardised to 10<sup>7</sup> cells/ml (0.38 OD<sub>600</sub>). Before starting the biofilm assay, a 96 flat-bottom well plate was first precoated with 100% Fetal Bovine Serum (FBS) by adding 100µl of FBS into 96 well plates and incubated at 37°C for 40min without agitation. After FBS coating, wells were washed with 200 µl sterile PBS once and any remaining PBS was removed by tapping the plate on a tissue towel. A 100 µl volume of cell suspension (in YEPD) was then added to the wells with/without FBS precoating in triplicate and incubated at 37°C for 90min without agitation. After 90min incubation, the 100µl cell suspension was removed, and wells were washed twice with 200µl sterile PBS. A 200 µl volume of sterile RPMI (pH 7) was then added to each well and incubated for 24h in a static incubator set at 37°C.

After 24h of incubation, RPMI medium was removed, and wells were washed twice with 200µl sterile PBS. Wells containing biofilms were visualized under ZOE™ Fluorescent Cell Imager to observe the biofilm intensity after two PBS washes. Then, crystal violet staining was carried out to determine biomass and quantify biofilm formation. A 125 µl volume of 0.4% (w/v) crystal violet solution was added into wells to stain the biofilm and the wells were covered in the dark for 10 min at room temperature. Excessive dye was then removed by washing three times with 200 µl PBS. The wells were dried briefly at room temperature and 95% (v/v) ethanol was added to each well to solubilise the dye. The covered plate was left for 15 min at room

temperature. After that, the solution in the wells were mixed by pipetting up and down, and the absorbance value of each well was measured at 540nm using FLUOstar Omega Microplate reader. The results were used to determine levels of biofilm formation and were visualized using GraphPad Prism.

#### 5.2.4 Colony morphology switching assays

All *C. auris* strains were grown overnight in 4 ml YEPD medium in a shaking incubator set at 37°C, 200 rpm. On the following day, the OD<sub>600</sub> value of the overnight culture was measured and standardised to 100 cfu/ml. The cell mixture was washed with 1 ml sterile water and plated on YEPD plates containing 5 µg/ml phloxine B dye incubated at 25°C for 6 days.

Single colonies from YEPD plates (24-48h) were streaked on YEPD plates containing 5 µg/ml phloxine B dye at 30°C for 2 days. Colonies from YEPD plates containing 5 µg/ml phloxine B dye (48h) were later re-streaked on YPG (20 g/L peptone, 10 g/L yeast extract, 20 ml/L glycerol) plates incubated at 25°C for 7 days. Single white/opaque colonies taken from phloxine B plates were resuspended in 1 ml PBS, vortexed for 1min, and visualized under the microscope described in Section 2.2.7.

#### 5.2.5 Phenotypic analysis

Phenotypic testing was performed on these *C. auris* strains as described in Section 2.7.

## 5.3 Results

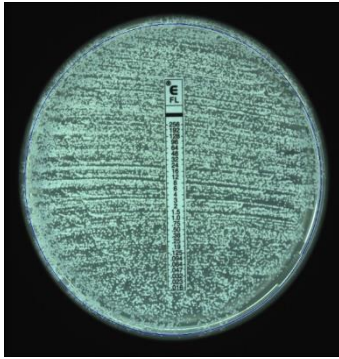
### 5.3.1 Fluconazole and caspofungin E-test assays

Fluconazole and caspofungin E-test assays were carried out as described in Section 2.7.4 to determine the fluconazole and caspofungin resistance of Mediator tail mutants (Figure 5.3.1 and Table 5.1). The Mediator tail mutants of both 8985 and 8977 showed the same fluconazole resistance as their parental strains 8985 and 8977, having a fluconazole MIC value at 256 µg/ml. The deletion of *MED3* and *MED15* did not affect the caspofungin resistance of 8985 (32 µg/ml) but did slightly reduce the caspofungin susceptibility of 8977 (0.38 µg/ml), with *med2Δ* 8977 having a caspofungin MIC value at 0.25 µg/ml and *med15Δ* 8977 having a caspofungin MIC value at 0.19 µg/ml.

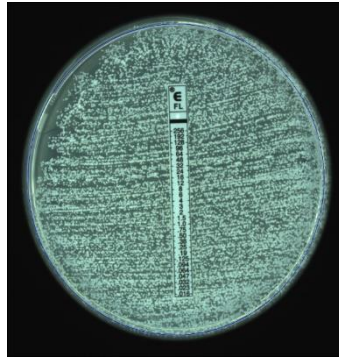
### 5.3.2 The growth rate of *C. auris* strains in YEPD with/without 10% NaCl

The growth rate of all *C. auris* strains in YEPD with and without 10% NaCl at 37°C was examined as described in Section 2.7.1 to determine the normal growth rate of Mediator tail mutants in YEPD medium and their salt tolerance (Figure 5.3.2). Mediator tail mutants of 8985 showed a slightly higher growth during exponential phase (8-19h) than the parent 8985 in YEPD medium (Figure 5.3.2A). In the case of strain 8977, the Mediator mutants exhibited a similar pattern of growth in YEPD in the exponential phase, however the *MED15* deletion mutant exhibited reduced growth during stationary phase (15-24h) compared to 8977 (Figure 5.3.2B). In the presence of NaCl, most Mediator tail mutants had similar levels of salt tolerance compared to their respective parental strain 8985 or 8977 (Figure 5.3.2C) except for *med15Δ* 8977, which exhibited less growth throughout its growth phases compared to parental 8977 (Figure 5.3.2D).

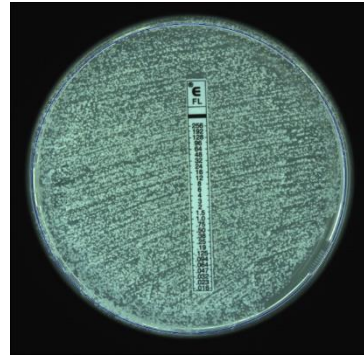
8977 (Fluconazole)



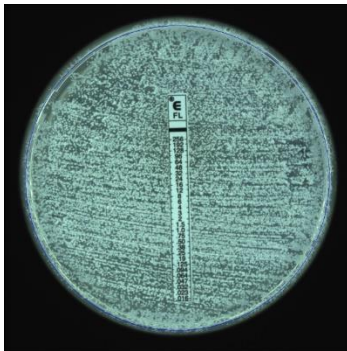
*med2*Δ 8977 (Fluconazole)



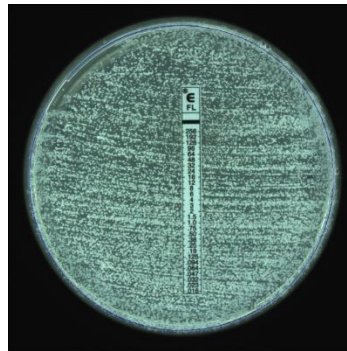
*med15*Δ 8977 (Fluconazole)



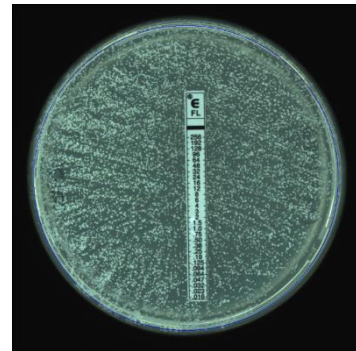
8985 (Fluconazole)



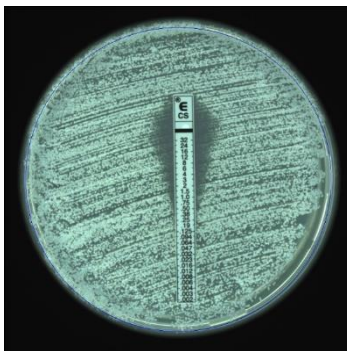
*med2*Δ 8985 (Fluconazole)



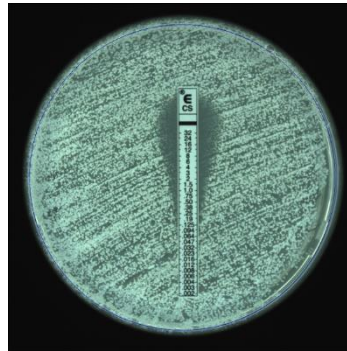
*med15*Δ 8985 (Fluconazole)



8977 (Caspofungin)



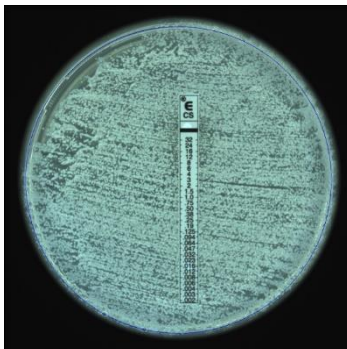
*med2*Δ 8977 (Caspofungin)



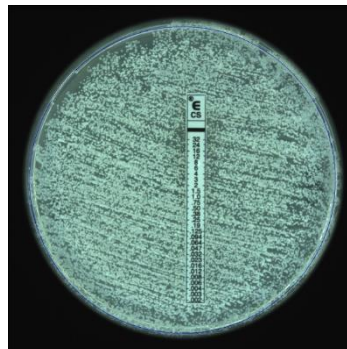
*med15*Δ 8977 (Caspofungin)



8985 (Caspofungin)



*med2*Δ 8985 (Caspofungin)



*med15*Δ 8985 (Caspofungin)

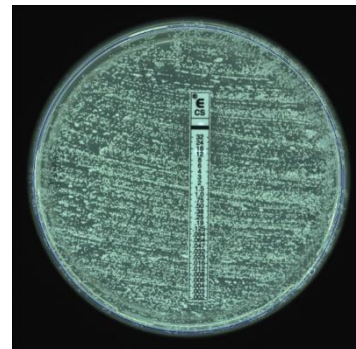


**Figure 5.3.1. Fluconazole and caspofungin E-test assays of *C. auris***

Fluconazole E-test were performed on RPMI plates incubated for 24h at 37°C. Experiments were done in biological duplicate and MIC values were measured as the point of 80% reduced growth.

**Table 5.1. Fluconazole and caspofungin E-test assays MIC values of *C. auris***

| Strains            | Fluconazole MIC values (µg/ml) | Caspofungin MIC values (µg/ml) |
|--------------------|--------------------------------|--------------------------------|
| 8977               | 256                            | 0.38                           |
| <i>med2Δ</i> 8977  | 256                            | 0.25                           |
| <i>med15Δ</i> 8977 | 256                            | 0.19                           |
| 8985               | 256                            | 32                             |
| <i>med2Δ</i> 8985  | 256                            | 32                             |
| <i>med15Δ</i> 8985 | 256                            | 32                             |

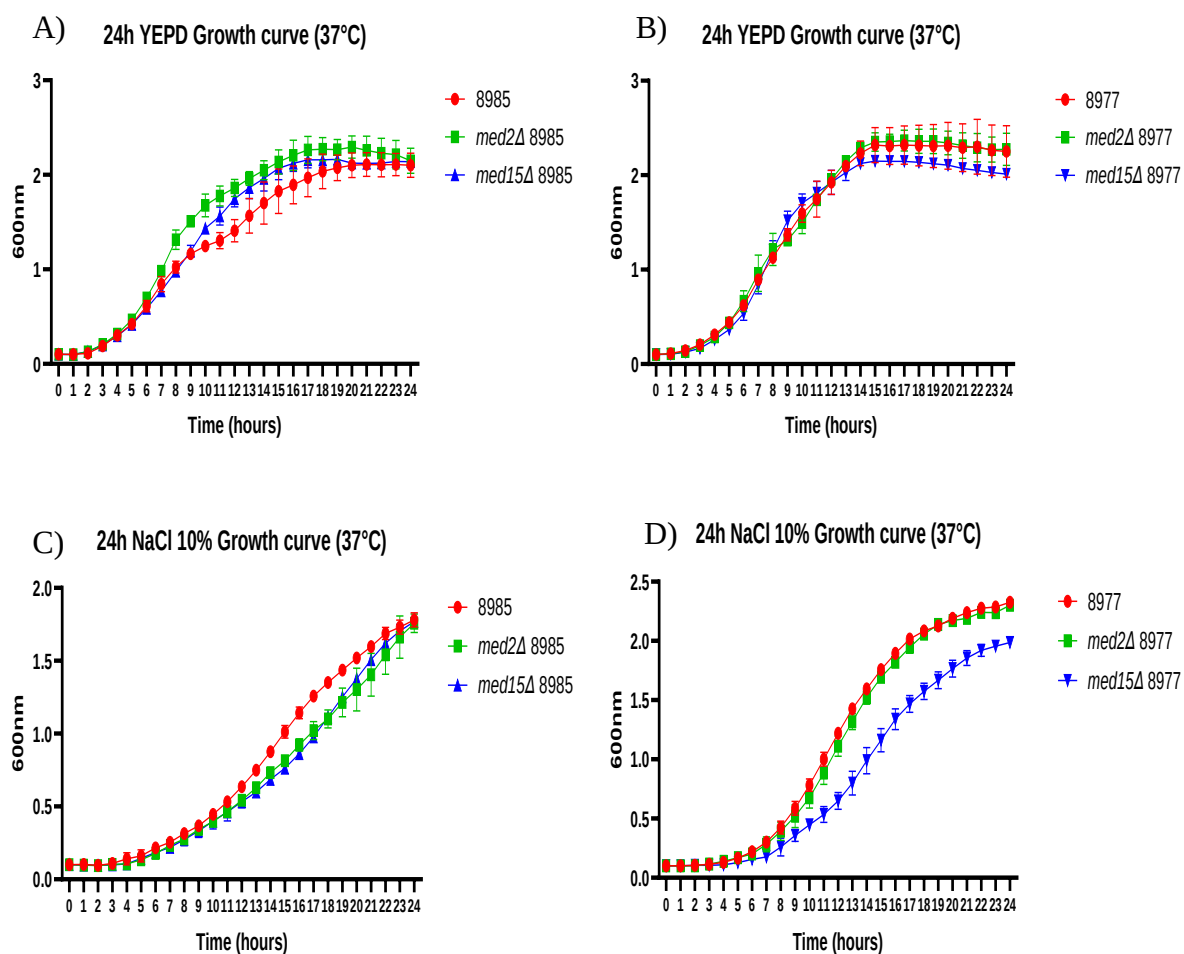


**Figure 5.3.2. The growth rate of *C. auris* in YEPD medium with/without 10% NaCl.**

Cells were grown in 96-well plates and OD600nm was measured every hour for 24 h.

### 5.3.3 *C. auris* stress resistance testing via spot plate assays

Spot plates containing YEPD with various stress reagents were prepared as described in Section 2.7.3 to determine the stress resistance of Mediator tail mutants (Figure 5.3.3). Mediator tail mutants of both 8977 and 8985 showed similar fluconazole resistance to their parental strain as no growth reduction was observed on YEPD plates containing high fluconazole concentrations (200 µg/ml). The deletion of *MED15* reduced the salt tolerance of strain 8977 as *med15*Δ 8977 displayed a slight growth reduction on 10% NaCl spot plates. The deletion of Mediator tail components did not affect the resistance of 8977 to the two tested cell wall stressors, calcofluor white and Congo red, showing similar growth compared to 8977 on spot plates containing these agents. Interestingly, the deletion of Mediator tail components, in particularly *MED15*, increased the tolerance of 8985 to cell wall stressors like calcofluor white and Congo red with *med15*Δ 8985 showing significantly increased in growth on spot plates containing calcofluor white and Congo red (Figure 5.3.3).

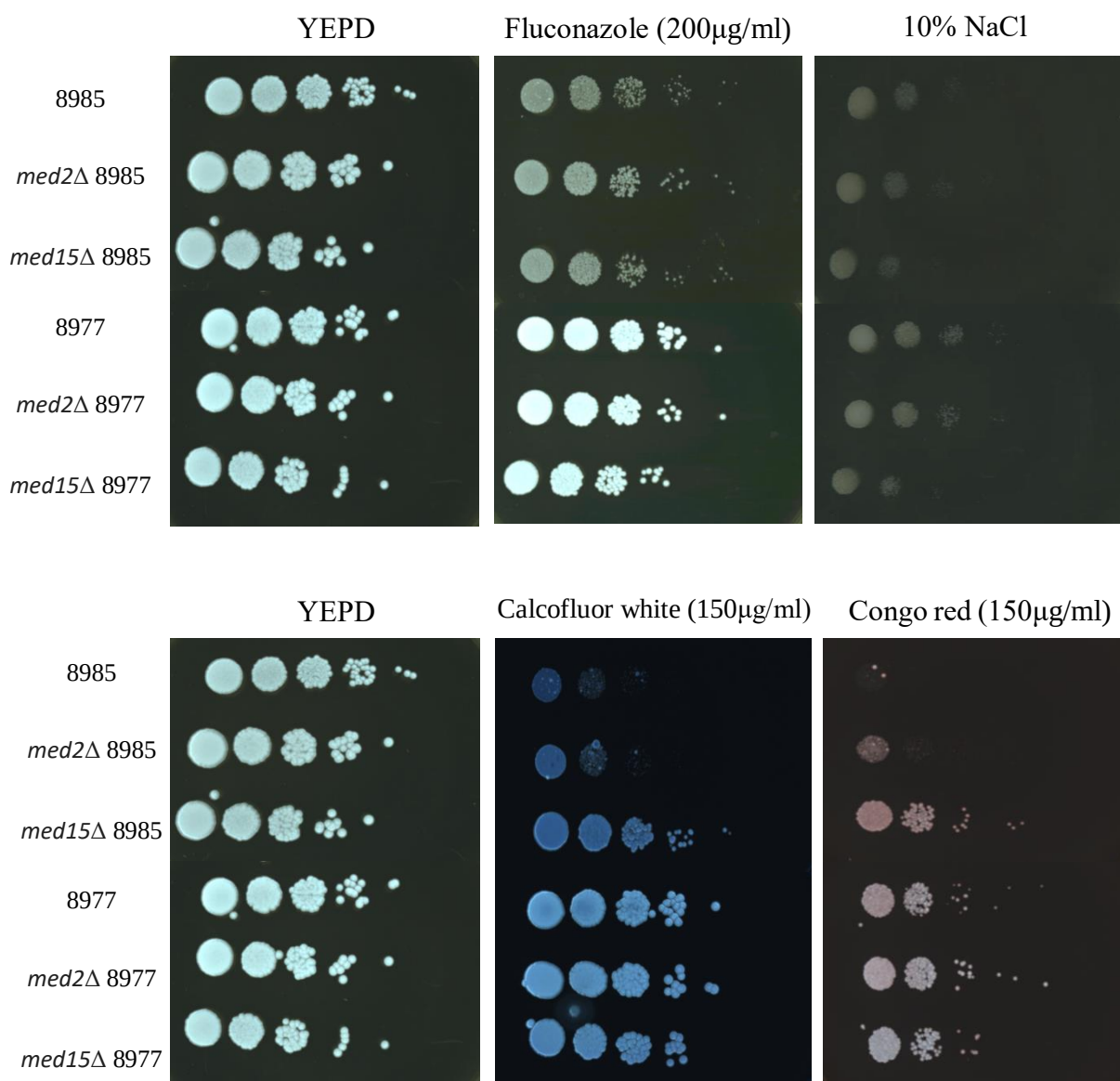


**Figure 5.3.3. Spot assays with various stress reagents to determine the stress resistance of *C. auris***

Spot plate assays were carried out with serial dilutions of overnight cultures ( $2 \times 10^6$  -  $2 \times 10^2$  cells/ml) on YEPD plates containing stress reagents (concentration as labelled top) and YEPD without any stress reagents as a positive control. Plates were incubated in a static incubator for 48 h at 30 °C and experiments were done in biological triplicates.

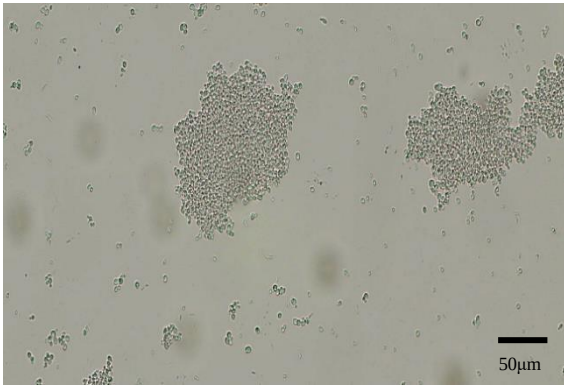
#### 5.3.4 Cell morphology of *C. auris* after PBS-induced aggregation assays

To examine cellular morphology of all *C. auris* strains (WT and *med2* $\Delta$  and *med15* $\Delta$  mutants), cell preparations in PBS were prepared as described in Section 5.2.1 and visualised with and without calcofluor white staining (Fig. 5.3.4). The deletion of *MED2* or *MED15* did not affect the aggregation of the weakly-aggregative strain 8985 which generally did not form large aggregates under these conditions. However, the deletion of *MED2* or *MED15* significantly affected the aggregation of the aggregative strain 8977. Both mutants showed reduced capacity to form large (> 50  $\mu$ m diameter) aggregates commonly observed in the parent strain 8977 (Fig. 5.3.4). The complementation of *MED15* in strain *med15* $\Delta$  8977 restored the aggregation phenotype of the mutant to the same level as 8977 (Fig. 5.3.4). Obvious cell separation defects were not observed in any of the Mediator tail mutants and all of them displayed a yeast cell morphology.

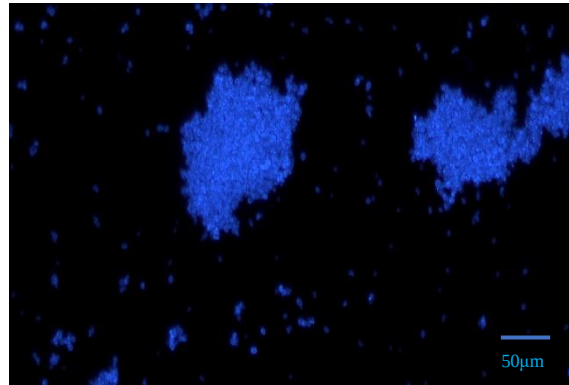
#### 5.3.5 Cell morphology of *C. auris* in echinocandin-induced aggregation assays

The cell morphology of *C. auris* strains prepared as described in Section 2.2.7 were assessed to determine if growth in the presence of echinocandins (4  $\mu$ g/ml) could induce aggregation in *C. auris* strains resuspended in sterile water (Figure 5.3.5). The aggregative strain 8977 showed a moderate level of aggregation following echinocandin exposure which was less pronounced in terms of aggregate size compared to PBS induced aggregation (Figure 5.3.5). Both Mediator tail mutants of strain 8977 exhibited similar levels of moderate aggregation to strain 8977 when exposed to echinocandins. In contrast to strain 8977, strain 8985, which was only weakly aggregative in PBS exhibited large aggregates (> 50  $\mu$ m) following exposure to echinocandins. Interestingly, both *med2* $\Delta$  and *med15* $\Delta$  mutants of strain 8985 exhibited reduced levels of aggregation compared to the parental strain 8985 (Figure 5.3.5).

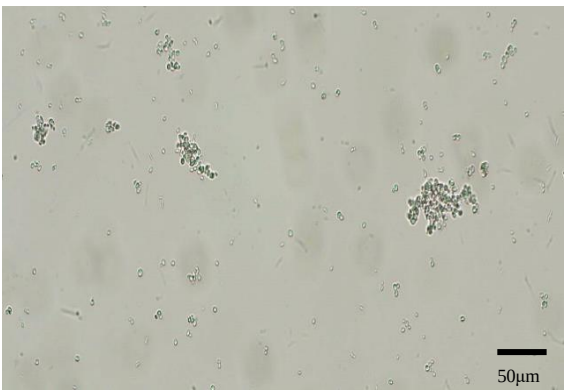
8977



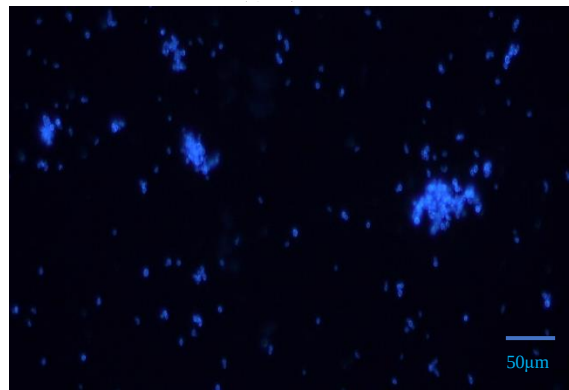
8977 (CW)



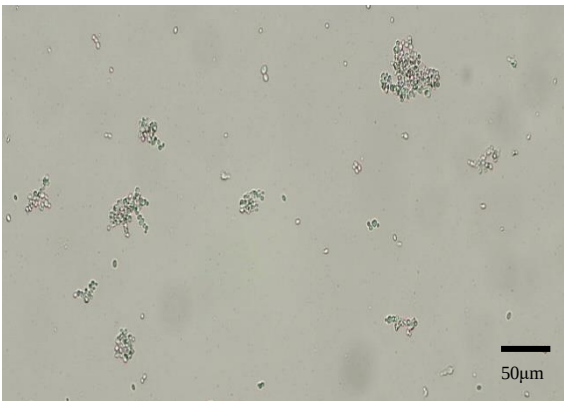
*med2Δ* 8977



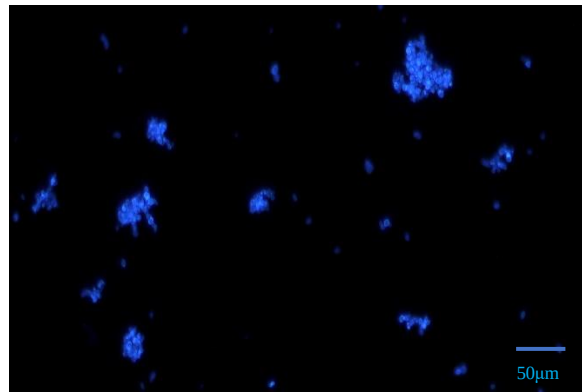
*med2Δ* 8977



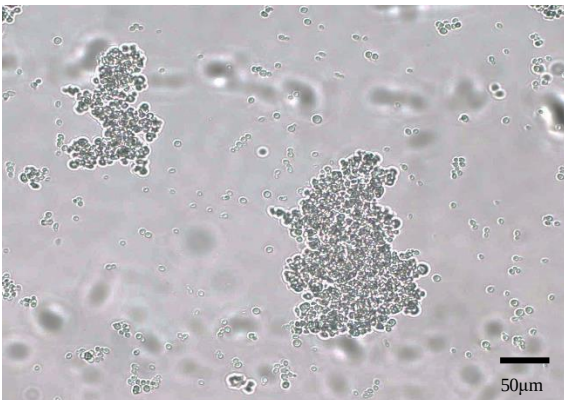
*med15Δ* 8977



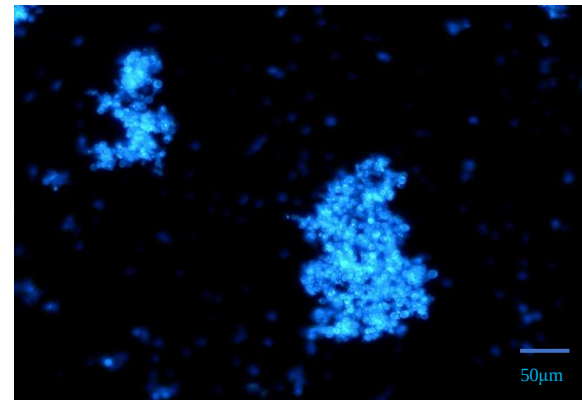
*med15Δ* 8977 (CW)



*med15Δ* 8977 com



*med15Δ* 8977 com (CW)



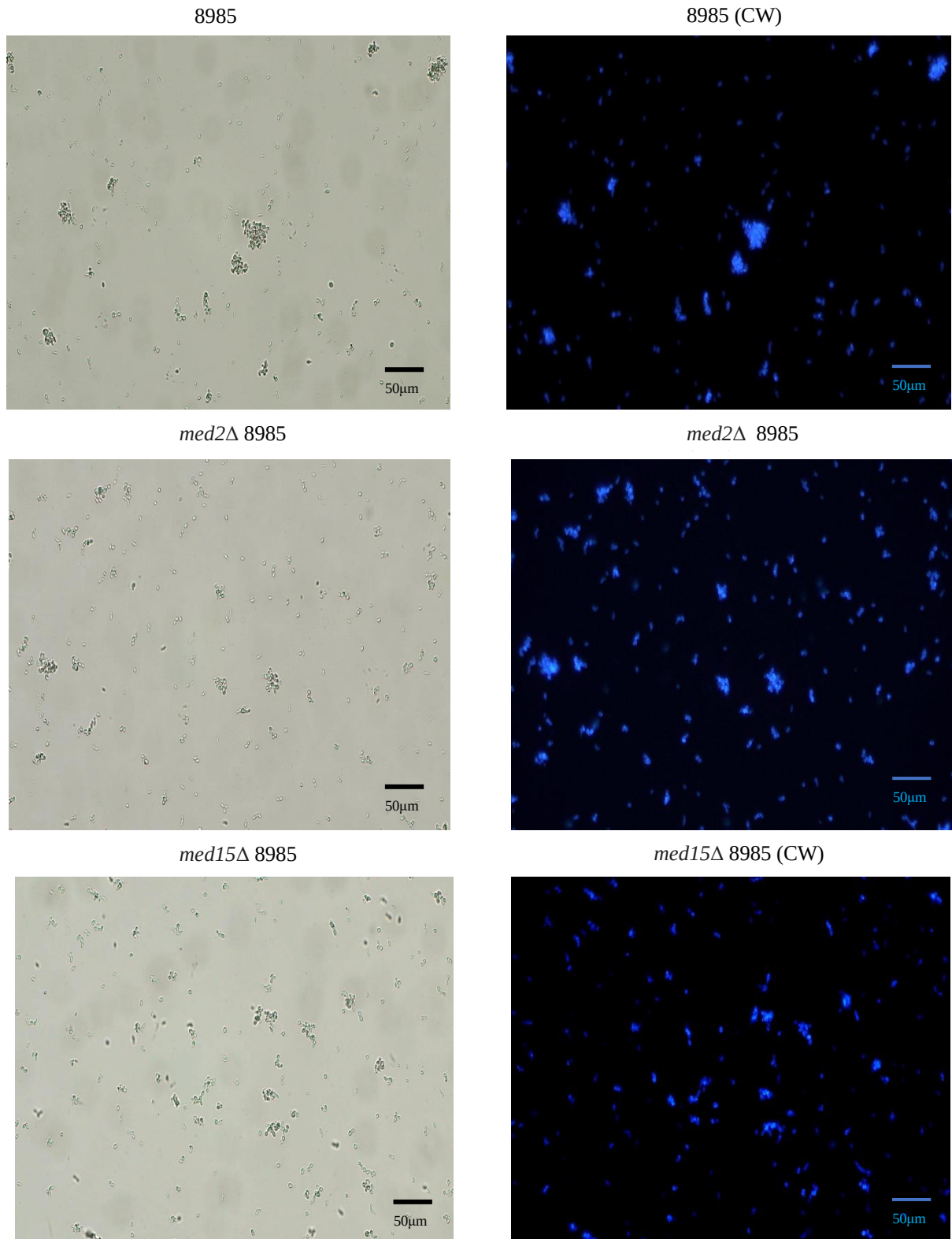


**Figure 5.3.4. Cell morphology (10x) of Mediator tail mutants of *C. auris* in PBS buffer**

Cells from overnight YEPD culture (37°C) resuspended in PBS buffer were stained with/without calcofluor white (CW) and were visualized at 100x with a Zeiss Axio microscope [light or UV (400nm) for CW stain].

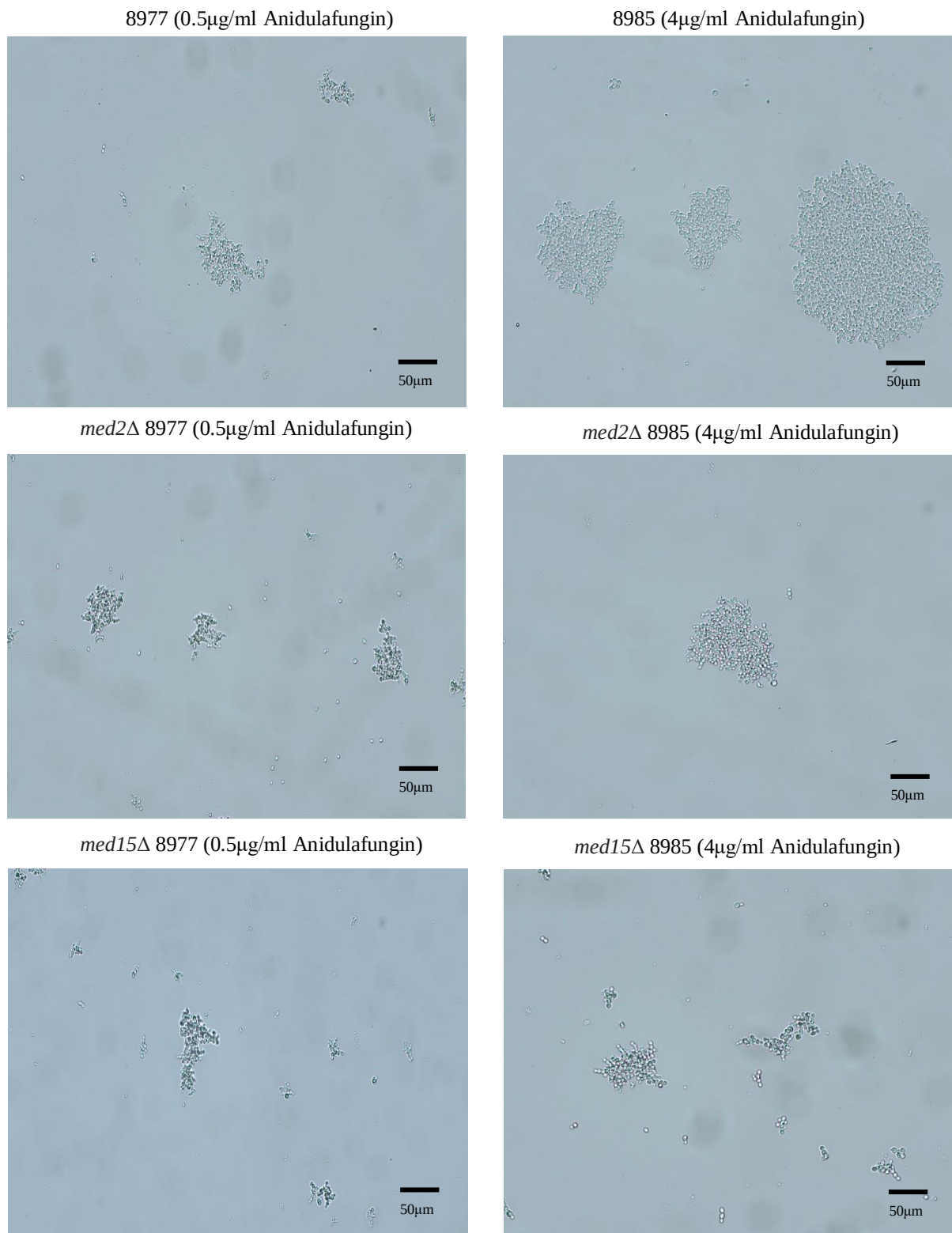


**Figure 5.3.5. Cell morphology (10x) of echinocandins induced aggregation of *C. auris* strains**

Cells from overnight YEPD cultures (37°C) containing 4µg/ml or 0.5µg/ml of anidulafungin were resuspended in water and visualized at 100x with a Zeiss Axio microscope (light).

### 5.3.6 Aggregation kinetic assays

The aggregation kinetic assays were carried out as described in Section 5.2.2 to quantify the aggregation rate of the Mediator tail mutants when induced by PBS at 37°C (Figure 5.3.6 A). The deletion of both *MED2* and *MED15* in 8977 hugely reduces the aggregation ability of 8977 as both *med2*Δ 8977 and *med15*Δ 8977 showed significantly lower levels of aggregation (<30%) compared to 8977 throughout the 3 hours incubation at 37°C (Figure 5.3.6 B). The deletion of both *MED2* and *MED15* in the weakly aggregating strain 8985 did not affect the aggregation ability of either mutant which exhibited no significant difference compared to the parent 8985 (Figure 5.3.6 A). Even though *med2*Δ 8977 and *med15*Δ 8977 showed low levels of aggregation, both mutants showed significantly higher levels of aggregation compared to the corresponding *med2*Δ and *med15*Δ mutants of strain 8985 over time (Figure 5.3.6 C & D). The complementation of *MED15* restored the aggregation ability of *med15*Δ 8977 showing high levels of aggregation similar to 8977 (Figure 5.3.6 A).

### 5.3.7 Biofilm formation assays

Biofilm formation assays were carried out as described in Section 5.2.3 to determine the biofilm formation capability of Mediator tail mutants and their parental strains in 96-well plates with and without precoating with fetal bovine serum (FBS) (Figure 5.3.7). The biomass of strain 8985 and its Mediator tail mutant derivatives showed no significant differences on hydrophobic surfaces with or without precoating with FBS. The deletion of *MED2* and *MED15* did not significantly affect the biofilm formation of 8985, showing only a slight decrease in biomass (Figure 5.3.7). Aggregative strain 8977 strain exhibited the highest biofilm density on hydrophobic surfaces among all strains tested and was significantly greater when compared to strain 8985 ( $p < 0.0001$ ; Figure 5.3.7). However, biofilm formation by strain 8977 was significantly lower on FBS coated surfaces where it was not significantly different to strain



**Figure 5.3.6. The aggregation capability of *C. auris* in PBS at 37°C**

The aggregation of each *C. auris* strain was evaluated by the reduction in optical density (at 530nm) of fungal suspensions in PBS (pH 7.2) containing  $10^8$  fungal cells/ml after 30, 60, 90, and 120 min of incubation at 37°C in inert conditions. The results were expressed as percentages of aggregation. A) The aggregation percentage of all *C. auris* strains over time. B) The differential aggregation of strain 8977 and its Mediator tail mutants C) The differential aggregation of *MED2* mutants of 8985 and 8977. D) The differential aggregation of *MED15* mutants of 8985 and 8977. Error bars were calculated based on three biological replicates and ordinary one-way ANOVA was carried out to detect any significant differences between strains (\* indicates p value < 0.05, \*\* indicates p value < 0.01).

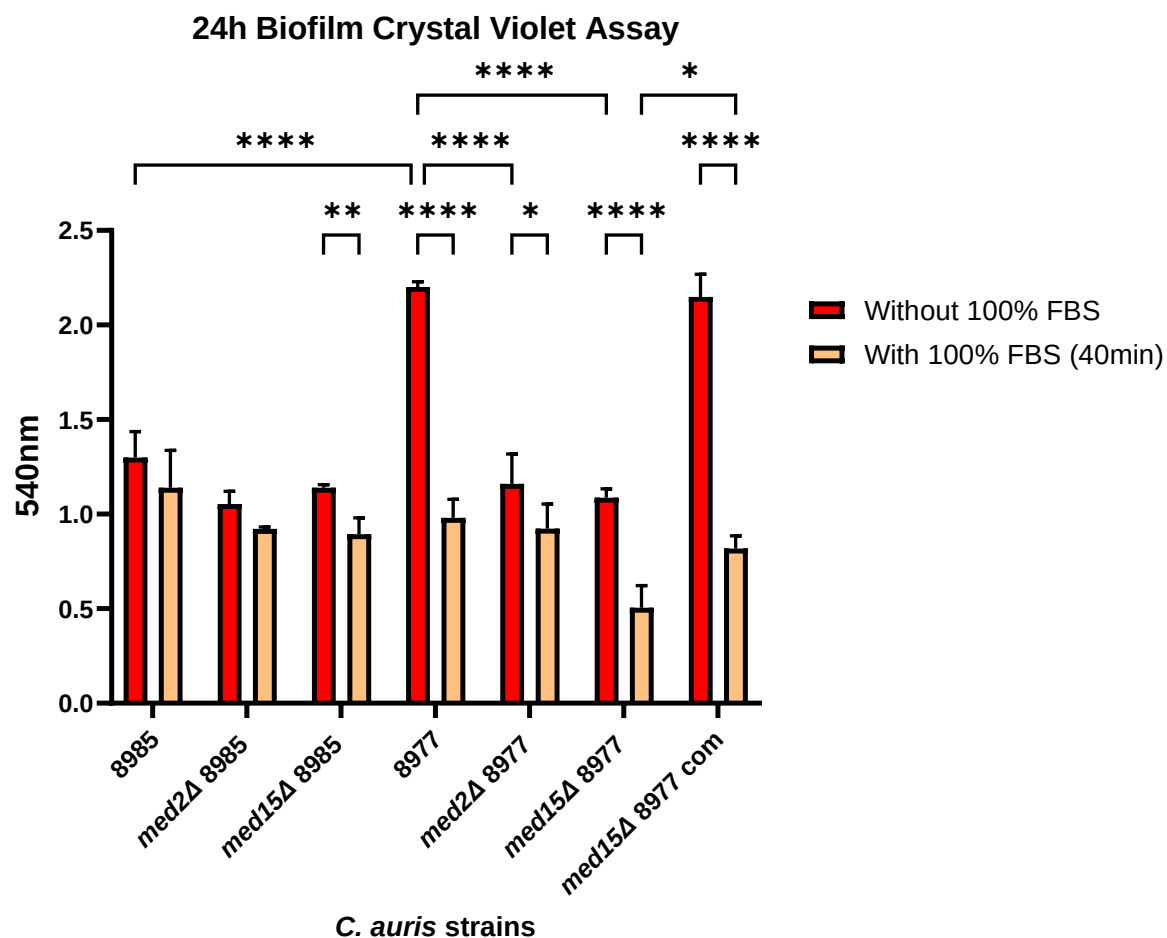


**Figure 5.3.7. The biofilm formation capability of all *C. auris* strains with/without FBS precoating**

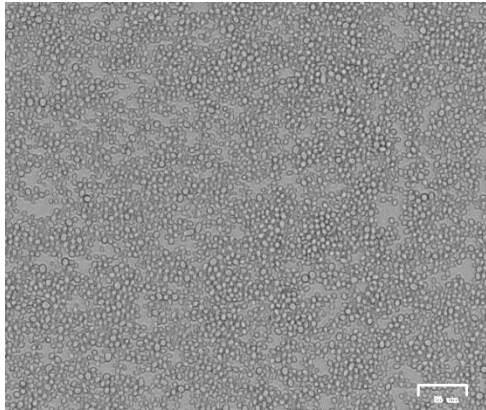
The biofilm formation capability of all *C. auris* strains on hydrophobic surfaces with/without precoating of FBS (40 min) in RPMI after 24h was analysed. Biomass of biofilm after 24h was quantified by staining with crystal violet and measuring absorbance at 540nm. Error bars were calculated based on three biological replicates and ordinary one-way ANOVA was carried out to detect any significant differences between strains (\* indicates p value < 0.05, \*\* indicates p value < 0.01, \*\*\* indicates p value < 0.001, \*\*\*\* indicates p value < 0.0001).

8985. The deletion of *MED2* and *MED15* significantly decreased the biofilm formation of 8977 on hydrophobic surfaces (Figure 5.3.7). In the case of FBS coated surfaces, the *MED15* mutant of 8977 also showed significantly reduced biofilm formation. The complementation of *MED15* restored the biofilm formation capability of *med15*Δ 8977 showing similar levels of biomass compared to parental 8977 on both hydrophobic and FBS precoated surfaces.

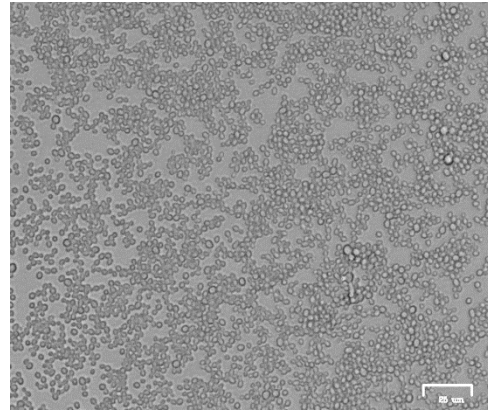
### 5.3.8 Biofilm morphology of *C. auris* strains

The biofilm morphology of *C. auris* prepared as described in Section 5.2.3 was assessed to qualitatively assess the cell density and cell morphology of Mediator tail mutants when forming biofilm (Figure 5.3.8). The Mediator tail mutants of 8985 and the parental strain 8985 retained their oval yeast cell morphology when attached on both hydrophobic and FBS coated surfaces to form biofilm. Mediator tail mutants showed slightly fewer cell attachments on FBS coated surface. The Mediator tail mutants of 8977 and the parental strain 8977 also retained their round yeast cell morphology when attached on both hydrophobic and FBS coated surfaces to form biofilm. Strain 8977 showed high levels of cell attachment on hydrophobic surfaces and a visible decrease in the number of cells attached on FBS precoated surfaces was observed. Both Mediator tail mutants of 8977 showed much fewer cells attached on both hydrophobic and FBS precoated surfaces, in particularly *med15*Δ 8977 which showed only approximately 225 cells attached to the FBS precoated surface (Figure 5.3.8). The complementation of *MED15* restored the number of cells attached on both hydrophobic and FBS precoated surfaces matching its parental strain 8977.

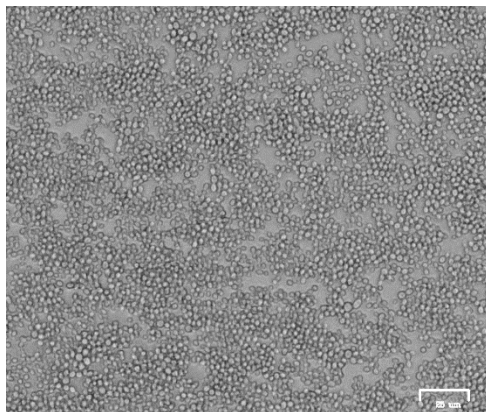
8977



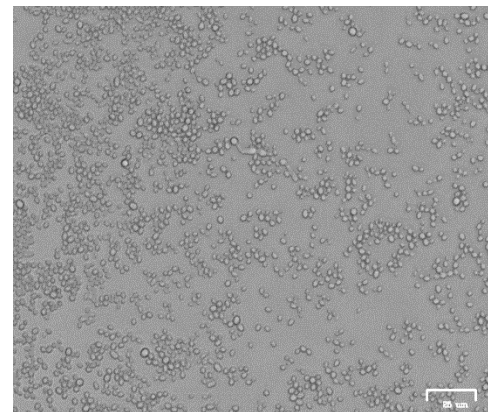
8977 (FBS 40min precoating)



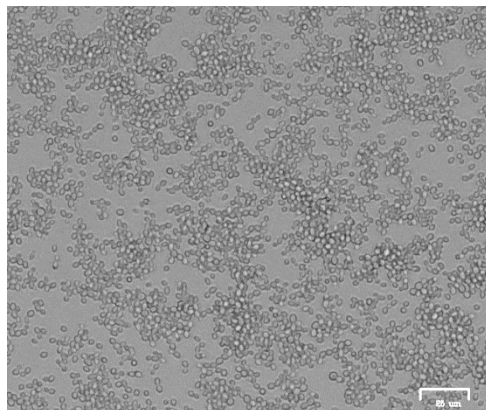
*med2* $\Delta$  8977



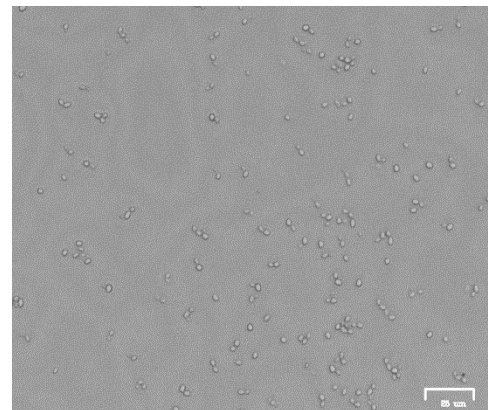
*med2* $\Delta$  8977 (FBS 40min precoating)



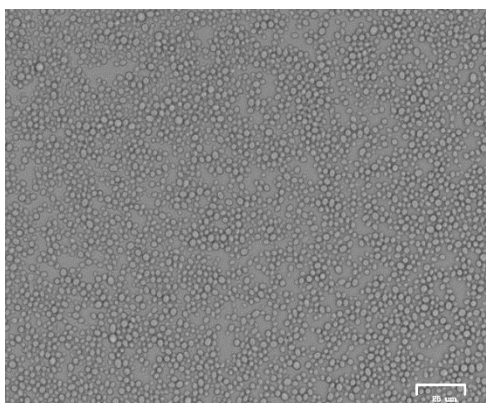
*med15* $\Delta$  8977



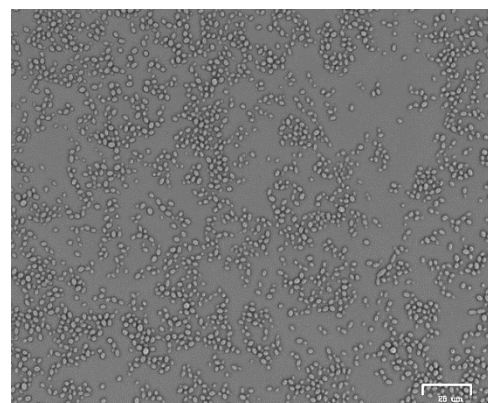
*med15* $\Delta$  8977 (FBS 40min precoating)



*med15* $\Delta$  8977 com



*med15* $\Delta$  8977 com (FBS 40min precoating)



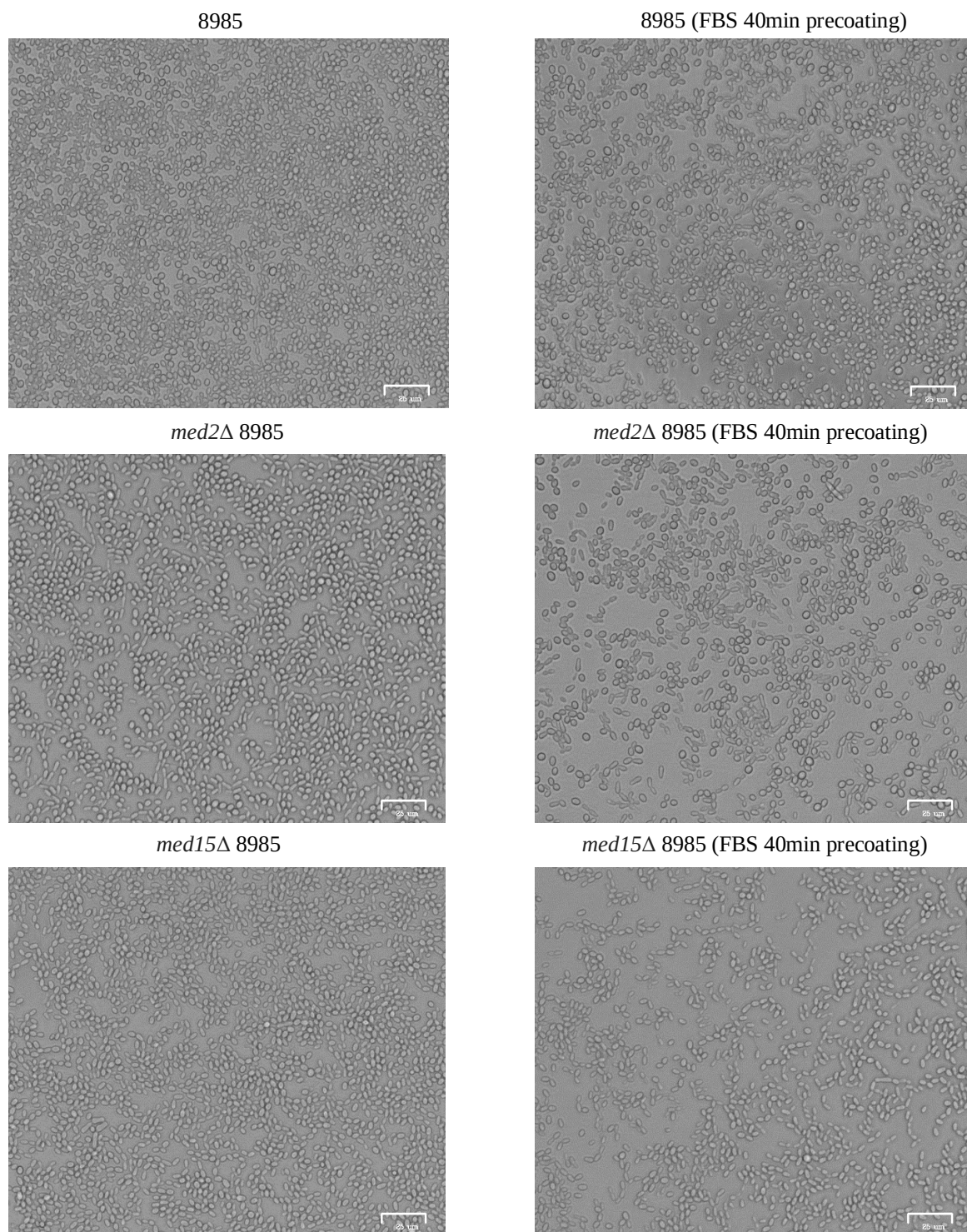


**Figure 5.3.8. Biofilm morphology of *C. auris* strains on hydrophobic or FBS precoated surfaces**

*C. auris* biofilms were formed after growth in RPMI (37°C) for 24h on hydrophobic or surfaces precoated by FBS for 40 min (labelled as FBS 40min precoating) in 96 well plates. Wells with biofilms were washed twice in PBS, visualized, and imaged using ZOE™ Fluorescent Cell Imager.

### 5.3.9 Colony morphology switching assays showing white-opaque switching of aggregative 8977

Colony morphology switching assays were carried out as described in Section 5.2.4 to find out the white and opaque switching capabilities of *C. auris* strains (Figure 5.3.9). Prior to this investigation, the cell morphology of all *C. auris* strains on phloxine B YEPD (PB) plates was first examined (Figure 5.3.9A). Strain 8977 and Mediator tail mutants of 8977 formed light-pink colonies with a glossy surface on PB plates after incubation at 25°C for 6 days. Cultures of 8985 and *med2Δ* 8985 formed light-pink colonies with a matt surface on PB plates whereas *med15Δ* 8977 formed light pink colonies with a glossy surface on PB plates after incubation at 25°C for 6 days.

White-opaque switching was also observed in *C. auris* colonies grown on PB plates after incubation at 30°C for 2 days (Figure 5.3.9B). Opaque cell derivatives were identified as intensely pink-stained colonies or sectors within white colonies on PB plates. All colonies of strain 8985 and its Mediator mutants were found to be white on phloxine B plates with no evidence of white-opaque switching (data not shown). However, 8977 and *med2Δ* 8977 formed smooth pink colonies at observable frequencies ( $10^{-2}$  to  $10^{-3}$ ) on PB plates at 30°C for 2 days (Figure 5.3.9B). These opaque variants were not observed with *med15Δ* 8977. Smooth pink colonies of 8977 and *med2Δ* 8977 could be maintained by culture on phloxine B YPG plates following incubation at 25°C for 3 days, whereas white colonies were seen to arise from *med2Δ* 8977 pink cells after a longer period of incubation (7 days at 25°C).

A)

8985 (YEPD, 6 days, 30°C)



*med2Δ* 8985 (YEPD, 6 days, 30°C)



*med15Δ* 8985 (YEPD, 6 days, 30°C)



8977 (YEPD, 6 days, 30°C)



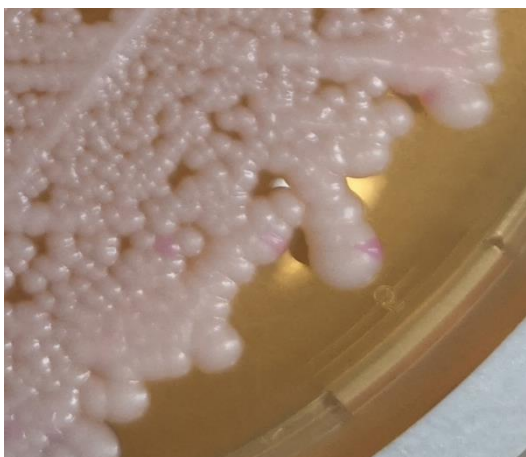
*med2Δ* 8977 (YEPD, 6 days, 30°C)



*med15Δ* 8977 (YEPD, 6 days, 30°C)



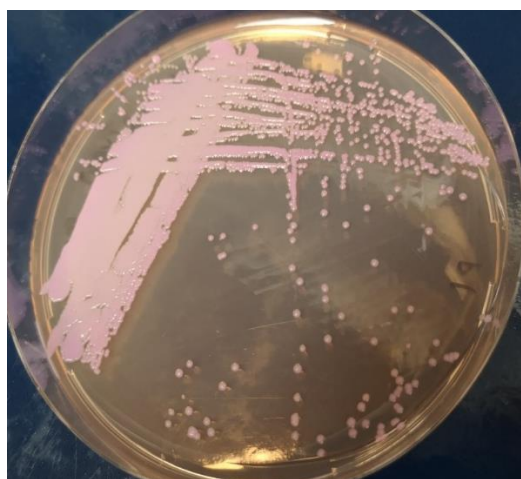
B) 8977 (YEPD, 2 days, 30°C)



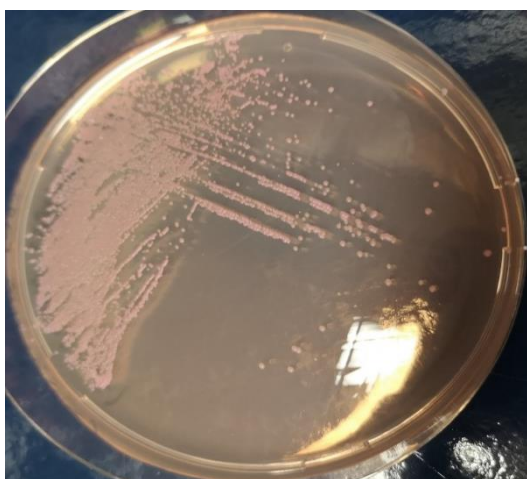
*med2Δ* 8977 (YEPD, 2 days, 30°C)



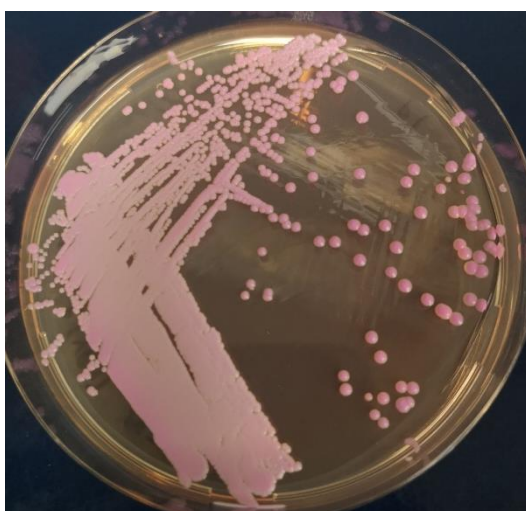
8977 pink cells (YPG, 3 days, 25°C)



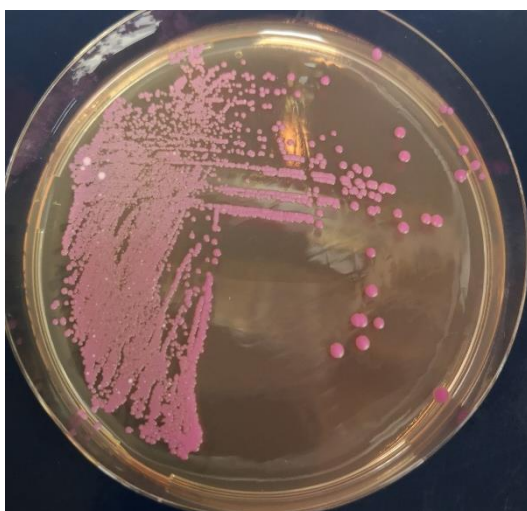
*med2Δ* 8977 pink cells (YPG, 3 days, 25°C)



8977 pink cells (YPG, 7 days, 25°C)



*med2Δ* 8977 pink cells (YPG, 7 days, 25°C)



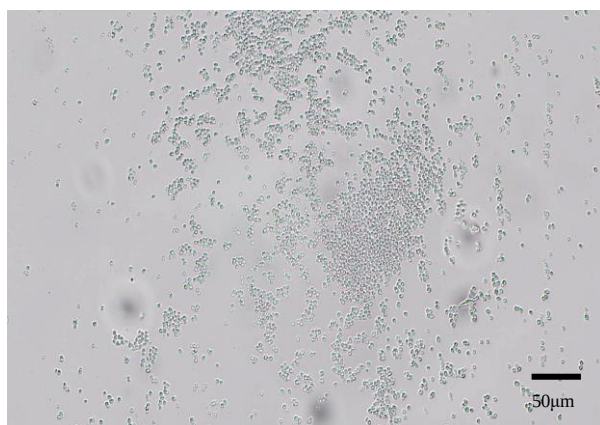
### **Figure 5.3.9. Morphology switching assays of *C. auris* on phloxine B plates**

*C. auris* strains were grown on phloxine B (PB; 5 µg/ml) plates with YEPD or YPG (labelled on top) at lower temperature (30°C or 25°C) for several days (labelled on top). A) Overnight cultures grown in YEPD media at 37°C were diluted to 100 cfu/ml and plated on YPG plates with phloxine B to grow for 6 days at 25°C. B) Normal cells from YEPD plates (24-48 h) were streaked onto phloxine B YEPD plates to grow for 2 days at 30°C and pink cells were re-streaked onto YPG plates with phloxine B for a prolonged period (3 - 7 days).

#### 5.3.10 Cell morphology of white and opaque *C. auris* cells in PBS buffer

Cell morphology of white and opaque (pink) *C. auris* cells was assessed to determine if the cells are aggregative or weakly-aggregative (Figure 5.3.10). The white cells of 8977 showed high level of aggregation as described previously, whereas the white cells of *med2Δ* 8977 showed intermediate level of aggregation. Interestingly, the pink cells of both 8977 and *med2Δ* 8977 showed low levels of aggregation.

8977 (white cells from YEPD + Phloxine B)



8977 (pink cells from YEPD + Phloxine B)



*med2Δ* 8977 (white cells from YPG + Phloxine B)



*med2Δ* 8977 (pink cells from YPG + Phloxine B)

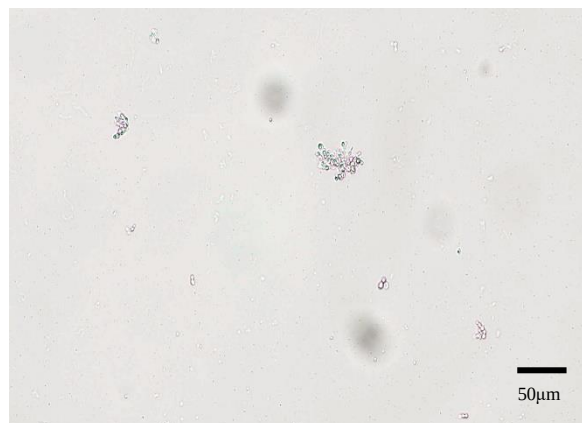


**Figure 5.3.10. Cell morphology of white-opaque *C. auris* cells from phloxine B plates**

White and pink cells from YEPD/YPG with phloxine B (5 μg/ml) plates re-streaked on new YEPD/YPG plates (25°C) resuspended in PBS buffer were vortexed for 1 min at 2200 rpm and visualized at 10x under Zeiss Axio microscope (light).

## 5.4 Discussion

This chapter focused on investigating the role of two Mediator tail subunit encoding genes, *MED2* and *MED15*, in mediating the aggregation and other virulence phenotypes of *C. auris*.

### 5.4.1 *MED2* and *MED15* does not affect *C. auris* azole or echinocandins susceptibility but affects its stress resistance

The initial antifungal susceptibility testing showed no fluconazole and caspofungin susceptibility differences between Mediator tail mutants and their parental strains 8985 and 8977. The results suggest that both *MED2/MED15* of *C. auris* might not be involved in the azole resistance pathway like *C. albicans* *TLO* genes (*C. auris* *MED2* ortholog). However, there is a possibility that both multidrug resistant *C. auris* strain 8985 and 8977 have various antifungal resistant mechanism which could provide them high azole/caspofungin resistance despite one of the antifungal mechanisms being impaired by *MED2/MED15* deletion.

Growth rate measuring experiments in YEPD media revealed that the deletion of Mediator tail components in 8977 and 8985 did not significantly affect the growth rate of either strain, suggesting that Mediator tail components are not essential for the function of pathways required for cell growth. However, the growth rate experiment in YEPD media with 10% NaCl revealed that the absence of *MED15* reduces the salt tolerance of aggregative strain 8977. The 10% NaCl spot plate assays further confirmed the reduction of salt tolerance due to the deletion of *MED15* in 8977. This suggest that a possible sodium ion transporter like Ena21 in *C. albicans* (Enjalbert *et al.*, 2009) was being downregulated due to *MED15* deletion in aggregative strain 8977. The fluconazole spot assays did not reveal any fluconazole resistance differences between Mediator tail mutants and their parental strains 8985 and 8977. This suggests that aggregation (discussed below) might not contribute to the fluconazole resistance of *C. auris* or the presence of multiple,

redundant fluconazole resistance mechanisms in these strains. Susceptibility to cell wall stressors like calcofluor white and Congo red that bind to chitin and  $\beta$ -1,3-glucan, respectively, were tested via spot plate assays revealing an unexpected result. The deletion of both *MED2* and *MED15* did not affect the resistance of 8977 to calcofluor white and Congo red. However, the deletion of *MED15* greatly increased the resistance of 8985 to calcofluor white and Congo red, indicating the presence of cell wall changes following deletion of *MED15*. Although 8985 was inherently resistant to caspofungin (MIC 32  $\mu$ g/ml), these cell wall changes did not effect caspofungin resistance. All these results suggest that Mediator tail components, in particularly Med15, regulate the biosynthesis of cell wall components in the weakly-aggregating strain 8985, which might contribute to the echinocandin-induced aggregation observed in strain 8985 shown in Figure 5.3.2. These results also suggest Mediator tail components, especially Med15, likely regulate the adhesins or ECM of aggregative 8977 strain without changing its cell wall composition.

#### 5.4.2 *MED2* and *MED15*, in particularly *MED15*, are required for *C. auris* aggregation

The initial investigations of the PBS-induced aggregative cell morphology in *C. auris* strains revealed differences between strain 8985 and strain 8977. Both Mediator tail mutants of naturally aggregative 8977 showed reduced aggregation (<30%; low aggregation level) compared to the parental strain 8977 (> 40%; high aggregation level) in PBS after a 1 min vortex. The two Mediator tail mutants of weakly-aggregative strain 8985 did not show any changes, exhibiting low levels of aggregation similar to the parental strain 8985 in the same assay. This suggests that Mediator tail components, Med2 and Med15, might be crucial in mediating PBS-induced aggregation phenotypes in naturally aggregative strains of *C. auris*. The PBS-induced aggregation defect might be due to reduced expression of adhesins or extracellular matrix (ECM) components as no obvious cell separation defects were observed in

both *med2*Δ8977 and *med15*Δ8977 after calcofluor white staining. The complementation of *MED15* in *med15*Δ 8977 restored its PBS-induced aggregation showing high aggregation levels similar to 8977. Conversely, the echinocandin-induced aggregation phenotypes of *C. auris* strains 8985 and 8977 showed a slightly different pattern compared to the PBS-induced phenotype. In this assay strain 8977 showed a slight reduction in aggregation (moderate level) compared the previous assay and this level was similar in the *med2*Δ8977 and *med15*Δ8977 derivatives. The greatest level of echinocandin induced aggregation was observed in the normally non-aggregating strain 8985, which suggests that echinocandins can induce the formation of aggregates in both aggregative and weakly-aggregative strains, possibly due to cell separation defects. This result matches the echinocandin-induced aggregation observed in the study by Pelletier *et al.* (2024). In our study the deletion of *MED2* in 8985 reduced its aggregation ability to a moderate level of aggregation whereas the deletion of *MED15* in 8985 strongly reduced its aggregation ability to a low aggregation level. The result showed that both Mediator tail components, in particularly *MED15*, are required to mediate echinocandin-induced aggregation in weakly-aggregative strain but not in the aggregative strain. The aggregation kinetic assays quantified media-induced aggregation and further confirmed the absence of both Mediator tail components, Med2 and Med15, impairs the PBS-induced aggregation of 8977, having low aggregation level even after long periods of incubation (3 hours) at 37°C. The absence of Med2 and Med15 did not affect the aggregation capability of 8985 as all strains showed low aggregation level over time. The complementation of *MED15* in *med15*Δ 8977 restored its PBS-induced aggregation showing a high aggregation level similar to parental 8977. Overall, the involvement of Mediator in these phenotypes indicate that regulation of gene expression plays a role in aggregation and that echinocandin induced aggregation may require Mediator induced gene expression.

#### 5.4.3 *MED2* and *MED15*, in particularly *MED15*, are required for the biofilm formation of aggregative strain 8977

The biofilm formation assays were then carried out to find out if the Mediator tail mutants and the clinical isolates of *C. auris* can form high biofilm mass on hydrophobic and fetal bovine serum (FBS) precoated surfaces. Strain 8985 was found to form intermediate levels of biofilm mass on both hydrophobic and FBS precoated surfaces whereas aggregating strain 8977 formed high biofilm mass (significantly higher than all other strains) on hydrophobic surfaces but formed significantly lower biofilm mass (slightly lower than 8985) on FBS precoated surfaces. The precoating of FBS on hydrophobic surfaces was shown to allow high biofilm formation of *C. albicans* (Krom *et al.*, 2007; Sadanandan *et al.*, 2022). However, in this biofilm formation assay, the precoating of FBS significantly reduced the biofilm formation of 8977, suggesting that FBS serum might have components that can inhibit the biofilm formation of aggregative *C. auris* strains or block hydrophobic interactions. Besides this, the biofilm assay showed *C. auris* aggregative strains can form higher biomass than weakly-aggregative strains on hydrophobic surfaces at 24 h, which is not always the same case observed by other studies (Sherry *et al.*, 2017; Brown *et al.*, 2020). The deletion of *MED2* did not significantly reduce the biofilm formation on hydrophobic and FBS precoated surfaces but the deletion of *MED15* did significantly reduce the biofilm formation on both FBS precoated and hydrophobic surfaces. The complementation of *MED15* restored the biofilm mass of 8977 on both hydrophobic and FBS-precoated surfaces. These results suggest that both *MED2* and *MED15* are important for the biofilm formation of aggregative strains on hydrophobic surface, possibly due to the downregulation of adhesins like Scf1 (Santana *et al.*, 2023) and *ALS4112* (Bing *et al.*, 2023) as both could enhance the biofilm formation of aggregative *C. auris* strains. Furthermore, the deletion of *MED15* impairs the biofilm forming capability of both 8985 and 8977 on FBS precoated surfaces, especially 8977, suggesting that *MED15* is the main Mediator tail

component that mediates the biofilm formation on FBS precoated surface. The biofilm morphology images of all *C. auris* strains revealed that all strains shared the same round yeast cell morphology, suggesting that the deletion of Mediator tail components (Med2 and Med15) in both weakly-aggregative and aggregative *C. auris* strains did not alter biofilm formation by changing cellular morphology.

#### 5.4.4 *MED2* and *MED15* affects the weakly-aggregative to aggregative switching of *C. auris*

Colony morphology switching assays revealed white-opaque switching phenotypes that might correlate with aggregation. The colony morphology of all *C. auris* strains on PB plates was examined and slight differences between *C. auris* strains was observed. Strain 8977 formed white glossy colonies whereas 8985 formed white matt colonies, suggesting cell wall or nutrient pathway differences between them. The Mediator tail mutants of 8977 formed the same white glossy colonies as 8977. However, the deletion of *MED15* in 8985 changed the formation of matt colonies to pink glossy colonies, further confirming the existence of cell wall changes that could be associated with resistance to calcofluor white and Congo red (Figure 5.3.7).

The formation of intense-pink stained colonies on PB, referred to as white-opaque switching, was only observed in 8977 and *med2*Δ 8977 and not *med15*Δ 8977, suggesting that the deletion of *MED15* might cause a defect in white-opaque switching. Due to time limitations, the analysis carried out here was purely qualitative and exact switching rates were not determined. The opaque (pink) cells were re-streaked on YPG plates containing phloxine B to test if they would change into a filamentous phenotype like its close relative *C. haemulonii* (Deng *et al.*, 2021). However, although they largely maintained the pink ‘opaque cell’ phenotype, they did not form filaments, suggesting that *C. auris* has a different filamentation regulation pathway compared to its close relative *C. haemulonii*. The white and opaque (pink) cells of 8977 were also

observed under the microscope. White cells seemed to form aggregates in PBS whereas pink cells seemed to exhibit reduced aggregate formation in PBS. This result is the opposite of the colony morphology observed by the study investigating the function of Als4 in an aggregative *C. auris* strain (Bing *et al.*, 2023), revealing that pink cells were aggregative cells and that white cells were weakly-aggregative cells. However, the same study (Bing *et al.*, 2023) showed that aggregative cells are the majority (as in this study) and they are not always stained pink when grown on PB plates suggesting other underlying variables that might affect PB dye intake by *C. auris* cells.

#### 5.4.5 Summary and possible future work

Combining all the results shown in this Chapter, Mediator tail components, Med2 and Med15, are involved in regulating the PBS-induced aggregation of aggregative strain 8977 and the regulation of echinocandin-induced aggregation of weakly-aggregative strain 8985. Among the two Mediator tail components, Med15 seemed to be the main component regulating various aggregation and stress resistance related phenotypes in both 8977 and 8985. Med15 contributes to media-induced aggregation and the biofilm formation of 8977. Besides, Med15 contributes to echinocandin-induced aggregation and the resistance to cell wall stressors of 8985, suggesting a difference in gene regulation by Med15 between aggregative and weakly-aggregative strains. Med2 is another important component that mediates the PBS-induced aggregation and biofilm formation of 8977 and is partially involved in regulating echinocandin-induced aggregation and the cell wall components of 8985. There are several future investigations that could provide a better insight on how Mediator tail components mediate aggregation related phenotypes. Calcofluor white staining and electron microscopy can be carried out to identify the presence of cell wall separation defects or ECM production defects due to the deletion of Mediator tail components. Virulence assays using *Galleria mellonella*

larvae and the skin infection assays using a mouse model would be useful to reveal any defects in virulence and in vivo skin adhesion of the two Mediator tail mutants. Cell wall component related assays can be done to reveal the cell wall component changes due to the absence of Mediator tail components. It would be interesting to delete the other Mediator tail component *MED3* or to delete two Mediator tail component encoding genes in a same aggregative strain to observe any virulence-related phenotypic changes. Gene deletion of *MED2* and *MED15* could be done on other *C. auris* strains from other clades (II, IV and V) to reveal its function on various clades and find out other possible underlying antifungal mechanisms that interact with these Mediator tail components.

To find out which genes that are responsible for aggregation and other virulence phenotypes and are regulated by the two Mediator Tail components, Med2 and Med15, RNA sequencing experiments and analysis were carried out and will be discussed in Chapter 6.

## Chapter 6

RNA sequencing reveals that *SCF1* and other putative adhesin encoding genes are regulated by *MED2* and *MED15* in aggregative *C. auris*

## 6.1 Introduction

### 6.1.1 RNA-sequencing

The core of molecular biology is to decipher the flow of information that is stored in genes as DNA, then transcribed into RNA, and finally translated into proteins (Crick, 1970). Individual organisms express a subset of unique genes that differs when exposed to various environmental factors. The expression of these genes characterizes the phenotype of an organism. Since DNA must be transcribed into RNA to facilitate protein production, the whole set of transcribed RNA molecules within a cell, also known as the transcriptome, is important for interpreting the functional elements of the genome and understanding development and disease (Kukurba and Montgomery, 2015).

Initial gene expression studies relied on low-throughput methods like Northern blots and quantitative polymerase chain reaction (qPCR) that can only measure single transcripts (Kukurba and Montgomery, 2015). After a few decades, the low-throughput methods evolved into genome-wide quantification of gene expression known as transcriptomics. The first transcriptomics study was carried out using hybridization-based microarray methods that provided high-throughput gene expression analysis at a low cost (Schena *et al.*, 1995). However, the accuracy of this method was low as it could not separate RNA with high similarities and quantify lowly expressed genes (Shendure, 2008). Sanger sequencing was later being developed for gene expression studies, but it is relatively low throughput thus not ideal for quantifying transcripts (Adams *et al.*, 1991; Itoh *et al.*, 1994). Tag based methods like serial analysis of gene expression (SAGE) and cap analysis gene expression (CAGE) that quantifies the number of tagged sequences were used to replace array-based methods with a higher sensitivity (Shiraki *et al.*, 2003). Still, these assays are expensive and require a large amount of input RNA which greatly limited its use.

In recent years, the introduction of high-throughput next generation sequencing (NGS) completely revolutionized transcriptomics by allowing RNA analysis through the sequencing of complementary DNA (cDNA) (Wang *et al.*, 2009). This method is known as RNA-sequencing, a method that has distinct advantages over previous approaches as it provides a more detailed and quantitative view of gene expression, alternative splicing, and allele specific expression at a low cost.

A typical RNA sequencing workflow consists of RNA isolation, the conversion of RNA into complementary DNA (cDNA), the preparation of the sequencing library, and cDNA sequencing on an NGS platform. The growth conditions of cells for RNA isolation can affect the genes they express and thus, provides an opportunity to discover different physiological and pathological conditions of an organism. Apart from that, there are multiple variables that could affect RNA-sequencing experiments such as the need for high quality RNA, biological and technical replicates, and the depth of sequencing must be considered prior to RNA sequencing (Kukurba and Montgomery, 2015).

#### 6.1.1 The aim of this study

RNA sequencing is a widely used tool to unravel gene expression in cells under a specific condition and can be paired with CRISPR-Cas9 knockout methods to study how a specific gene regulates/interacts with all other genes and ultimately contributes to pathogenesis/survival related phenotypes. Thus, RNA-sequencing was carried out to investigate the effect of *MED2* and *MED15* deletion (via CRISPR-Cas9) on various genes that contribute to aggregation/cell wall changes or other important virulence phenotypes of the two aggregative and weakly aggregative *C. auris* strains.

## 6.2 Materials and Methods

*C. auris* strains 8977 and 8985 and their respective Mediator tail mutants (*med2Δ* or *med15Δ*) were subjected to RNA-sequencing. The list of strains can be found in Table 2.2.

### 6.2.1 Growth conditions

*C. auris* cultures were grown overnight in YEPD medium at 37°C in a shaking incubator set at 200 rpm. On the following day, the OD<sub>600</sub> value of the overnight culture was measured, and a calculated amount of culture was transferred to a flask containing 25 ml YEPD medium to establish a new culture (OD<sub>600</sub> 0.1) which was grown to mid-exponential phase (~0.8) in the shaking incubator set at 200 rpm, 37°C. A 15 ml volume of mid-log phase *C. auris* culture was then removed for RNA extraction.

### 6.2.2 RNA extraction and sample checking

A 15ml volume of *C. auris* culture was subjected to the same RNA extraction protocol optimised for *C. albicans* strains described in Section 2.4.1. The quantity of RNA was measured using a Nanodrop spectrophotometer as described in Section 2.3.3. After the quantification of RNA, the quality of RNA was assessed by running on a 1% agarose gel containing 1% household bleach [Musgrave Retail Partners Ireland, Lucan, Dublin]. High quality RNA samples exhibited 2 distinct bands that represented the 28S and 18S ribosomal subunits after gel electrophoresis, with 28S band having a higher intensity (double) than of the 18S band. High quality RNA samples were then stored at -80°C and RNA samples with a concentration of 400 ng were later sent to Novogene (Cambridge, United Kingdom) for RNA sequencing. RNA sequencing was performed by Novogene using the Illumina platform.

### 6.2.3 RNA sequencing data analysis

The quality scores of all the sequences were listed in Appendix 1. On average, ~7.5 G of data were obtained from each sample, from the average amount of 50,101,620 raw reads. No samples had an error rate higher than 0.03%. The average samples have 98% of reads with a quality score of Q20, and 94.2% of reads with a quality score of Q30. Novogene, quality checked and aligned the sequencing data of the Mediator mutants to the *C. auris* reference genome (<http://www.candidagenome.org/>). Individual gene expression was quantified in count and fragment per kilobase read format and differential transcript abundance was determined using DeSeq2. Functional analysis like Gene Ontology (GO) analysis and Gene Set Enrichment Analysis (GSEA) were also carried out by Novogene. Venn diagrams were generated from lists using the online tool provided by BioinfoGP (<https://bioinfogp.cnb.csic.es/tools/venny/>).

## 6.3 Results

### 6.3.1 Sequencing quality and mapping statistics

*C. auris* 8977 and 8985 with their Mediator tail mutants (*med2*Δ and *med15*Δ) were grown to mid-exponential phase in YEPD medium at 37°C and subjected to RNA isolation for sequencing. Principle component analysis (PCA) revealed that gene expression profiles of each strain were largely reproducible (Figure 6.3.1). The profiles of strain 8985 and 8977 could be separated across axis 1, showing that strain background was the greatest variable in the experiment. The Mediator tail mutants diverged from their respective parents across axis 2, with the Med15 mutants showing the greatest difference from the parental strains (Fig. 6.3.1).

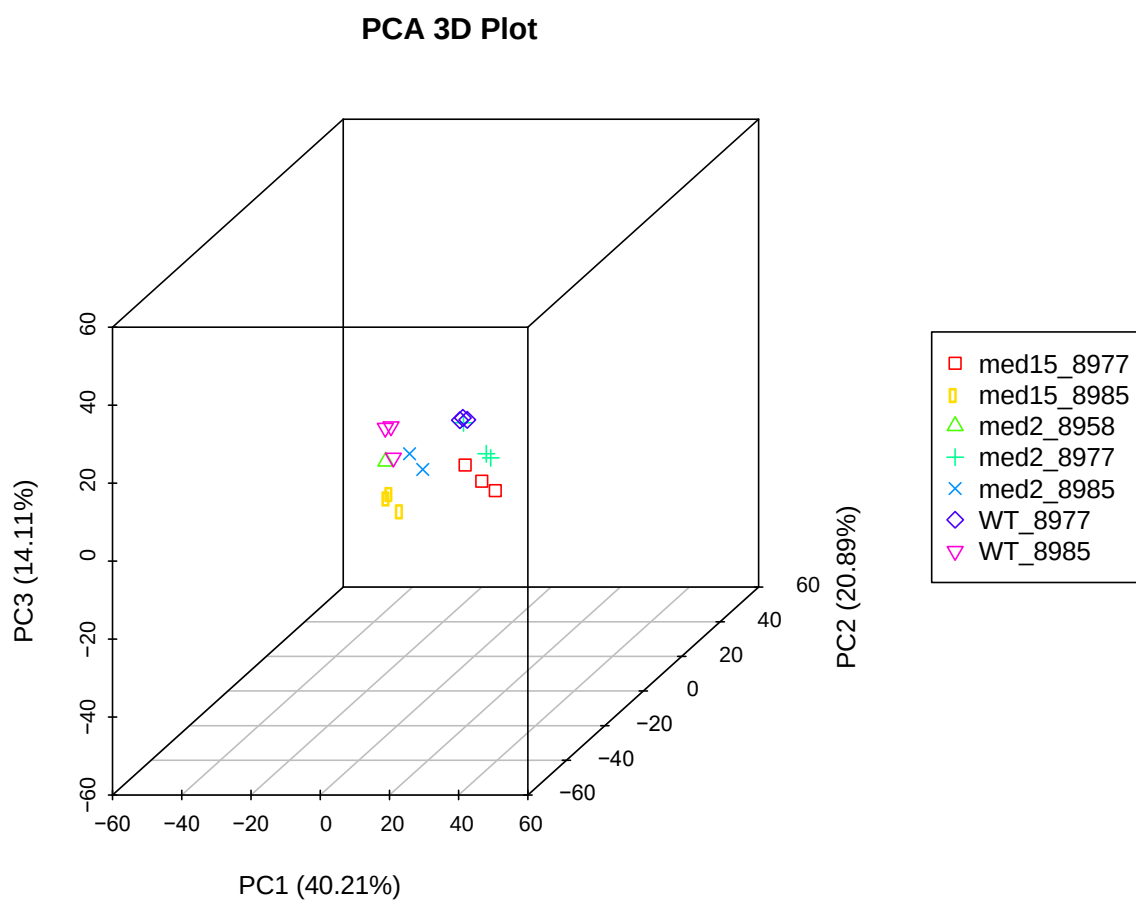


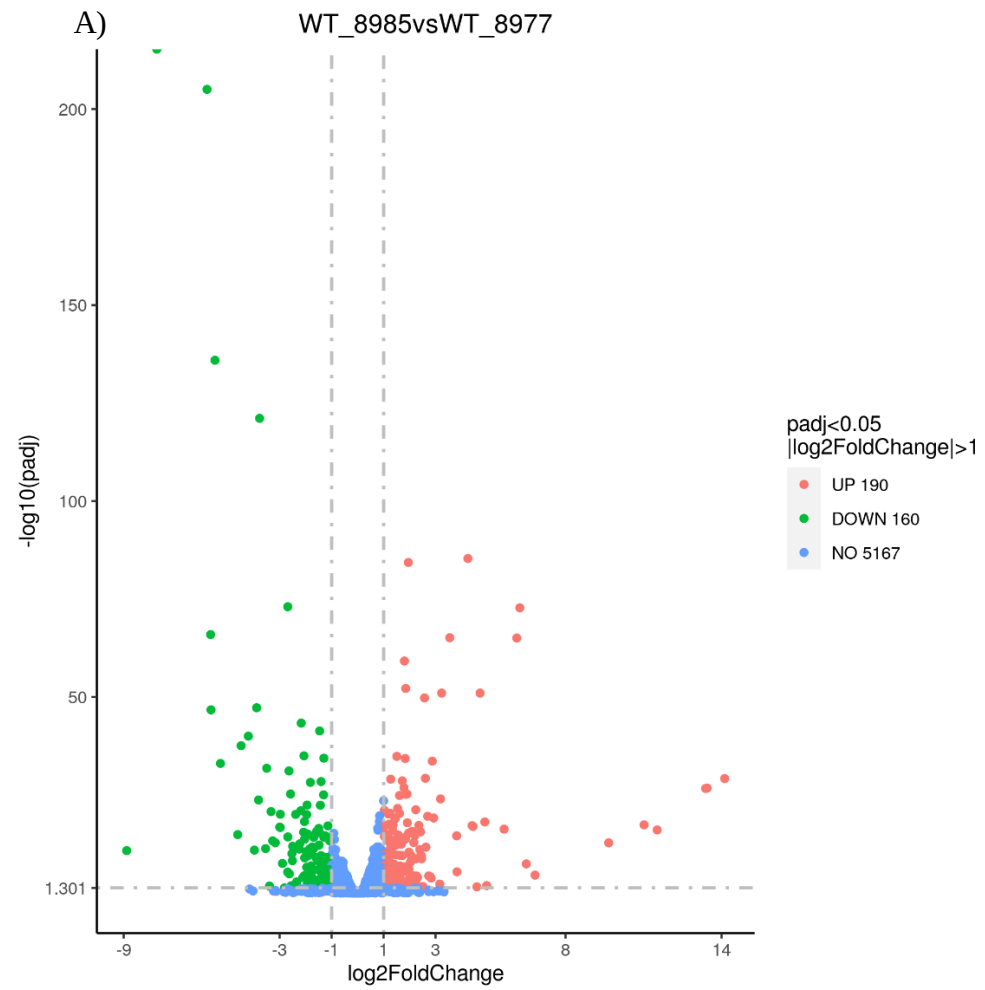
**Figure 6.3.1. Principle component analysis of total gene expression correlation between *C. auris* strains**

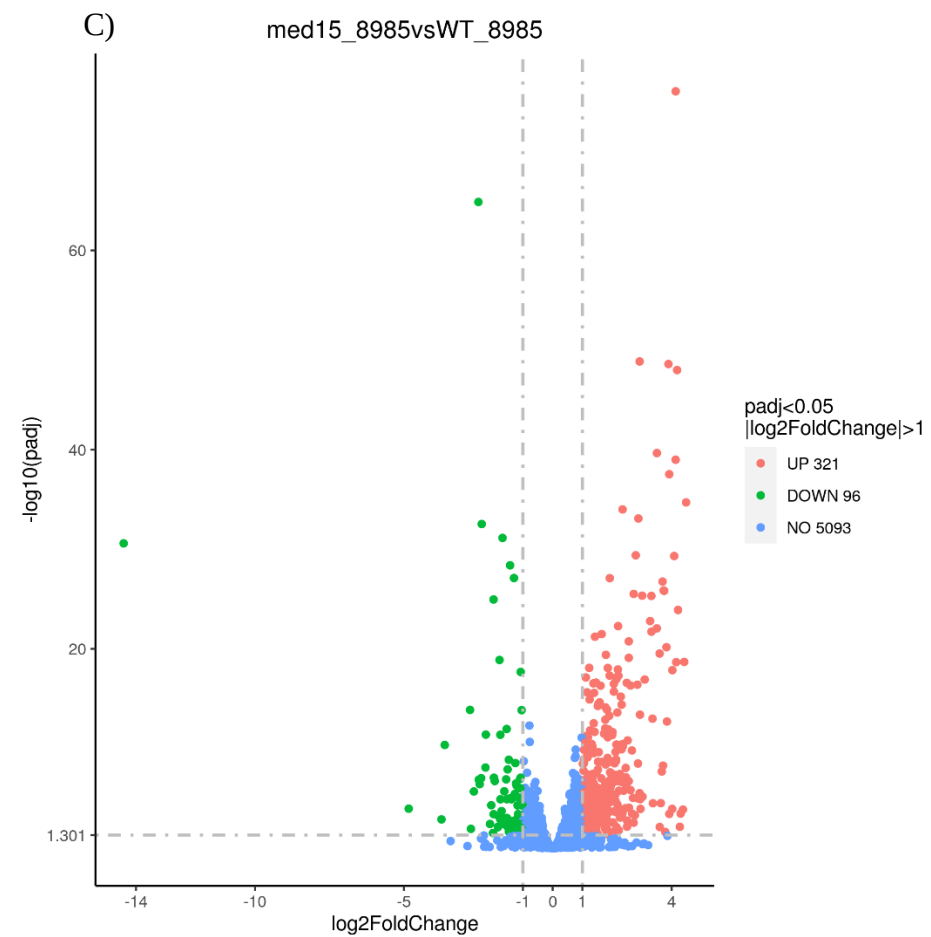
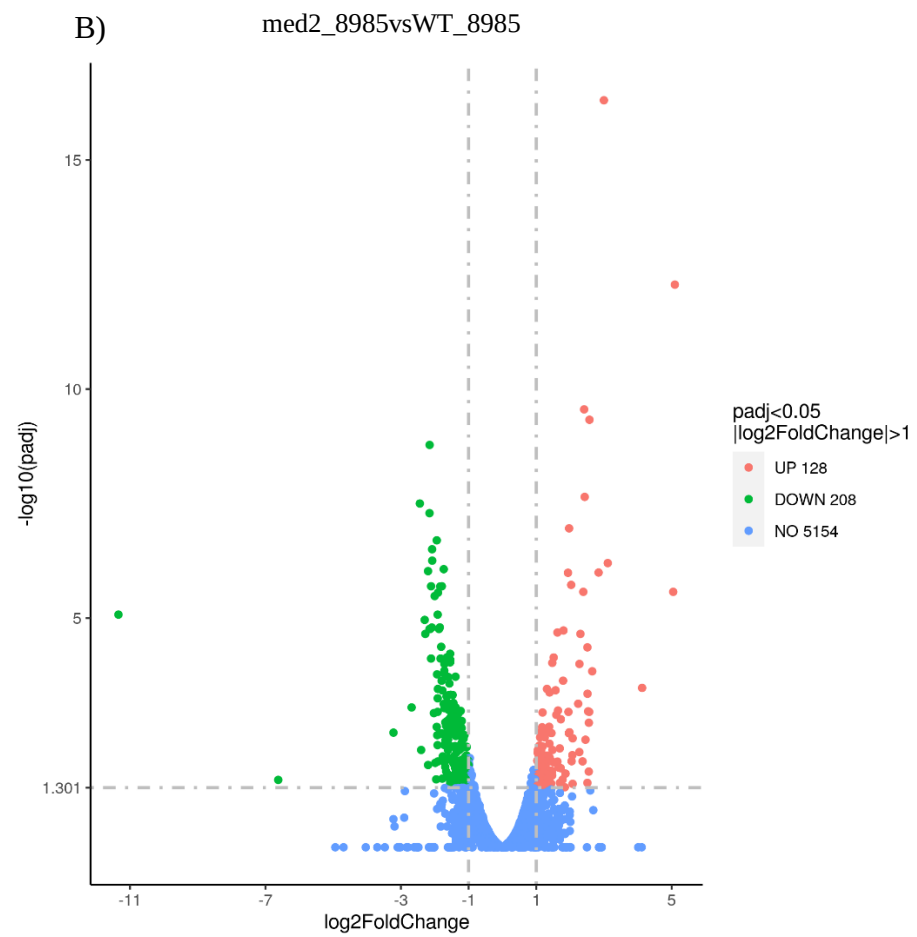
The PCA plot is based on the gene expression similarity between samples (fragments per kilobase million). The distance between the samples can be used to indicate how similar or distinct the samples are.

### 6.3.2 Comparative transcriptomics of the Mediator tail mutants and their parental strains in nutrient rich YEPD

Phenotypic analysis revealed multiple differences between the weakly-aggregative strain 8985, the high-aggregative strain 8977 and their corresponding Mediator tail mutants (Chapter 5). To identify differences in transcriptional profile that might explain the molecular basis that contributes to these phenotypic differences, differential gene expression analysis was performed.

The differential gene expression analysis data were plotted into volcano plots by Novogene showing differences in gene expression between pairs of strains (Figure 6.3.2). The comparison between strains 8985 and 8977 showed a total of 350 genes were being differentially expressed, with 190 genes being significantly ( $p < 0.05$ ) upregulated and 160 genes being significantly downregulated in 8985 compared to 8977, with a log<sub>2</sub> fold change larger than 1 (Figure 6.3.2A). The comparison between *med2Δ* 8985 and WT 8985 showed a total of 336 differentially expressed genes, with 128 genes being significantly upregulated and 208 genes being significantly downregulated in *med2Δ* 8985 compared to WT 8985 (Figure 6.3.2B). The comparison between *med15Δ* 8985 and WT 8985 identified a total of 417 differentially expressed genes, with 321 genes being significantly upregulated and 96 genes being significantly downregulated (Figure 6.3.2C). The comparison between *med2Δ* 8977 and WT 8977 identified 208 differentially expressed genes, with 199 genes being significantly upregulated and only 9 genes being significantly downregulated (Figure 6.3.2D). Finally, the comparison between *med15Δ* 8977 and WT 8977 identified a total of 384 differentially expressed genes, with 333 genes significantly upregulated and 51 genes significantly downregulated (Figure 6.3.2E).





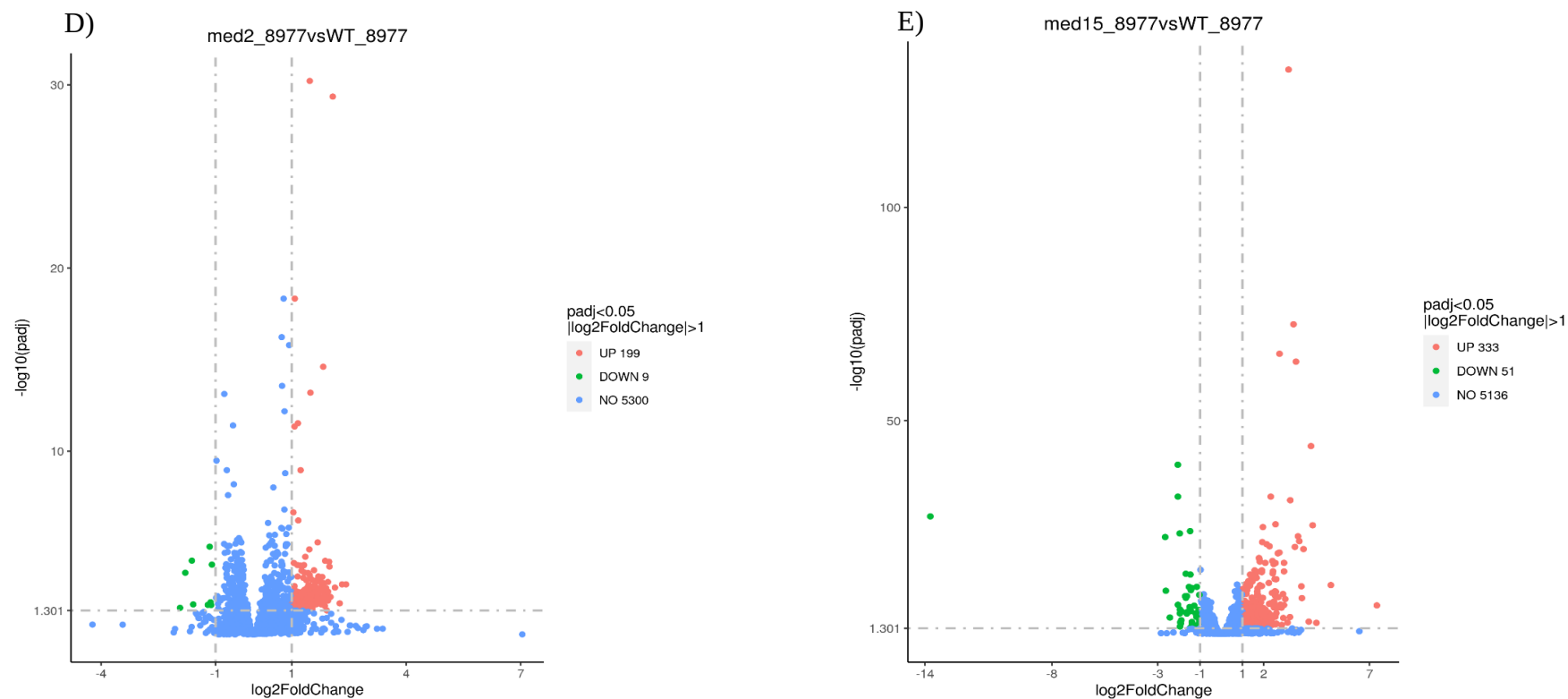


**Figure 6.3.2. Differential gene expression in 8985 compared with 8977 and their corresponding Mediator tail mutants grown to mid-exponential phase in YEPD at 37°C.**

The  $\log_2$  fold change of the expression of each gene is plotted on the x-axis and the  $-\log_{10}$  of the adjusted p value on the y-axis. The red dots indicate significantly higher gene expression whereas the green dots indicate significantly lower gene expression in the first strain compared to another strain labelled on top. The blue dots indicate no significant gene expression between compared strains.

### 6.3.3 Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

KEGG analysis was performed to reveal differences in expression in genes involved in biochemical pathways that were significantly upregulated/downregulated by the absence of Mediator Tail subunits in strains 8985 and 8977 *C. auris* strains (Figure 6.3.3). Since *C. auris* is a relatively new organism with a poorly characterised genome, functional annotations are commonly derived based on homology to known genes in *C. albicans*.

Comparing the total gene expression of 8985 with 8977, we observed that genes responsible in the category “Biosynthesis of secondary metabolites” had significantly higher expression in strain 8985 (Figure 6.3.3A). This included subcategories of “Glycolysis” (including *PFK1* and *PFK2* orthologs) and multiple “Porphyrin Metabolism” genes (*HEM4*, *HEM13*, *HEM15*) were also highly expressed in strain 8985. Other genes that were involved in the production of amino acids (*LEU2*, *ARG1*, *ARG3*, *PST3*) were also more highly expressed in strain 8985. Multiple ergosterol biosynthetic genes (*ERG1*, *ERG6*, *ERG10*, and *ERG25*) were found to be more highly expressed in 8985. Fewer significantly expressed categories of genes were identified in 8977 compared to 8985, with “Purine metabolism” and “ABC transporters” exhibiting higher expression in strain 8977 (Figure 6.3.3A).

The comparison of *med2Δ* 8985 and *med2Δ* 8977 to their respective parental isolates revealed very few significant associations in KEGG gene categories. Analysis of the *med2Δ* 8985 gene set only revealed significant downregulation the category “Ribosome”, including genes like *RPS6A*, *RPS14B*, *RPL29*, *RPL32*, *RPL37B*, and *MRPL19* (Figure 6.3.3B). Conversely, the comparison of *med2Δ* 8977 and WT 8977 (Figure 6.3.3D) revealed significant upregulation of genes in the category “Ribosome Biogenesis in Eukaryotes” (*NOG1*, *NOG2*, *NOP4*, *NOP5*, *RIX7*,

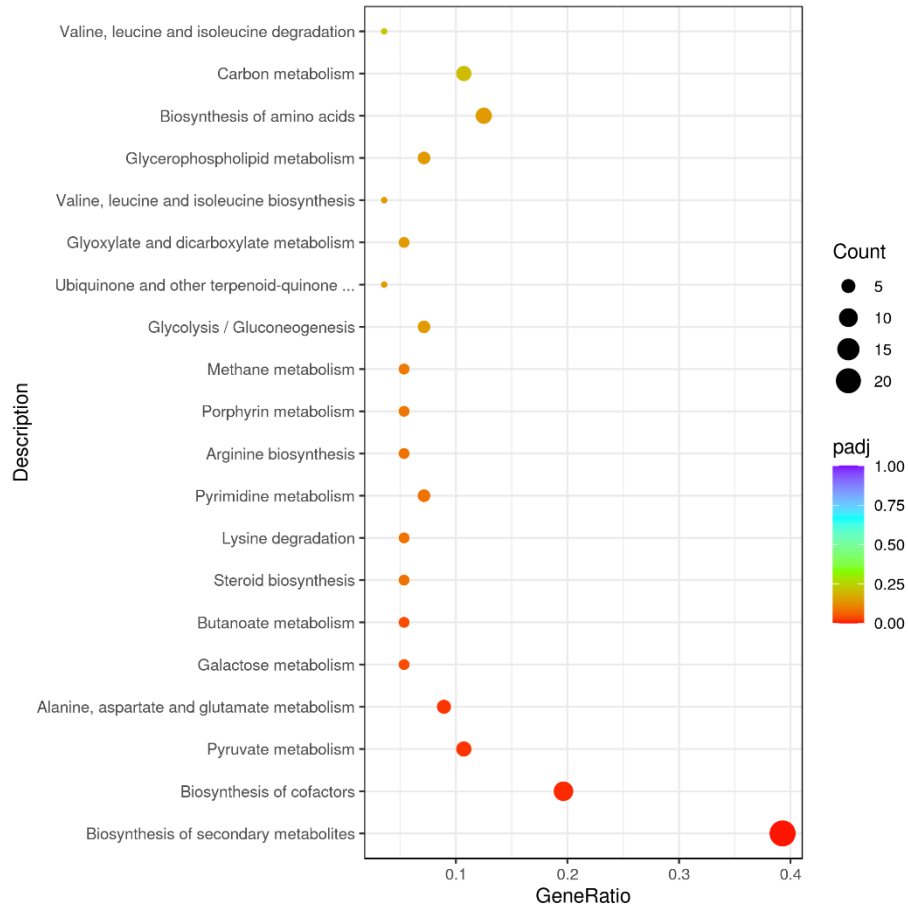
*RCL1*, *RIO2*, *DIP2*, *IMP4*, *HBR3* etc.) and “Nucleotide metabolism” (*UTP5*, *UTP6*, *UTP8*, *UTP9*, *UTP13* and *UTP21*). The category “RNA polymerase” was also upregulated (*RET1*, *RPC19*, *RPC53*, *RPA12*, *RPA34*, *RPA49*, *RPA135* and *RPA190*). Only 9 genes were downregulated in *med2Δ* 8977 which made KEGG analysis meaningless (Figure 6.3.3D).

On the contrary, the comparison of *med15Δ* 8985 and *med15Δ* 8977 to their respective parental isolates revealed a greater number of significant KEGG categories in common. Both *med15Δ* mutants exhibited deregulation of amino acid metabolism, carbohydrate metabolism and fatty acid metabolism (Figure 6.3.3C). This included down regulation of “Glycolysis/Gluconeogenesis”, “Galactose Metabolism”, “Biosynthesis of Amino Acids” and “Biosynthesis of nucleotide Sugars”. In both mutants, amino acid metabolism categories were also upregulated including “Valine, Leucine and Isoleucine degradation” in addition to “Starch and Sucrose Metabolism”. Both mutants exhibited upregulation of *FKS2* (target of echinocandins) and *BGL22* (glucanase, involved in cell wall regeneration) in the category “Starch Metabolism”.

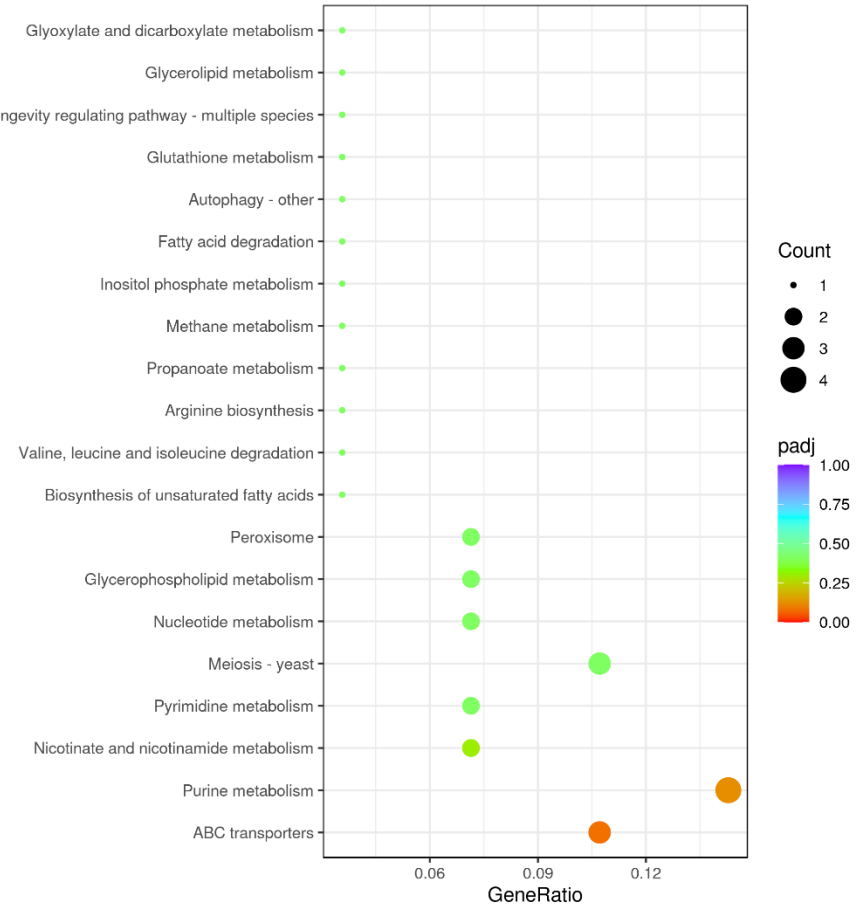
The *med15Δ* derivative of strain 8985 also exhibited upregulation of “ABC transporters” including orthologues of known genes such as *STE6* and *CDR4*. Downregulation of “Steroid biosynthesis” was observed in *med15Δ* 8977 and both *med15Δ* mutants exhibited down-regulation of *ERG3* and *ERG11*.

A)

Increased expression 8985 vs 8977

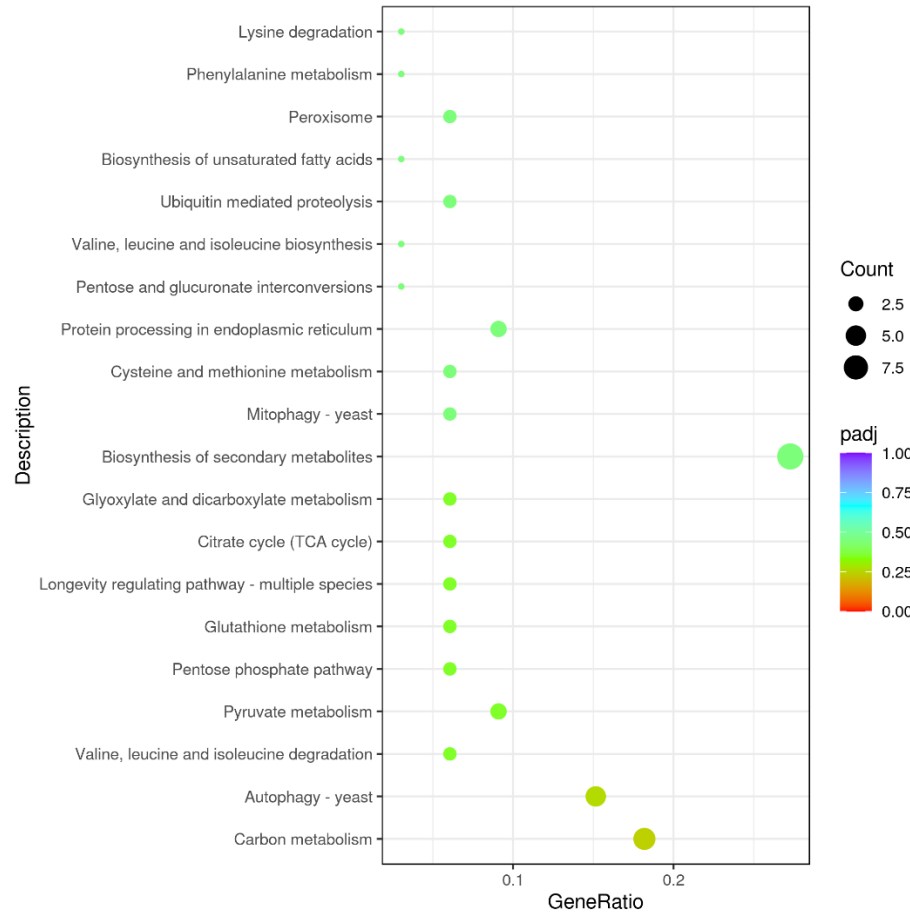


Increased expression 8977 vs 8985

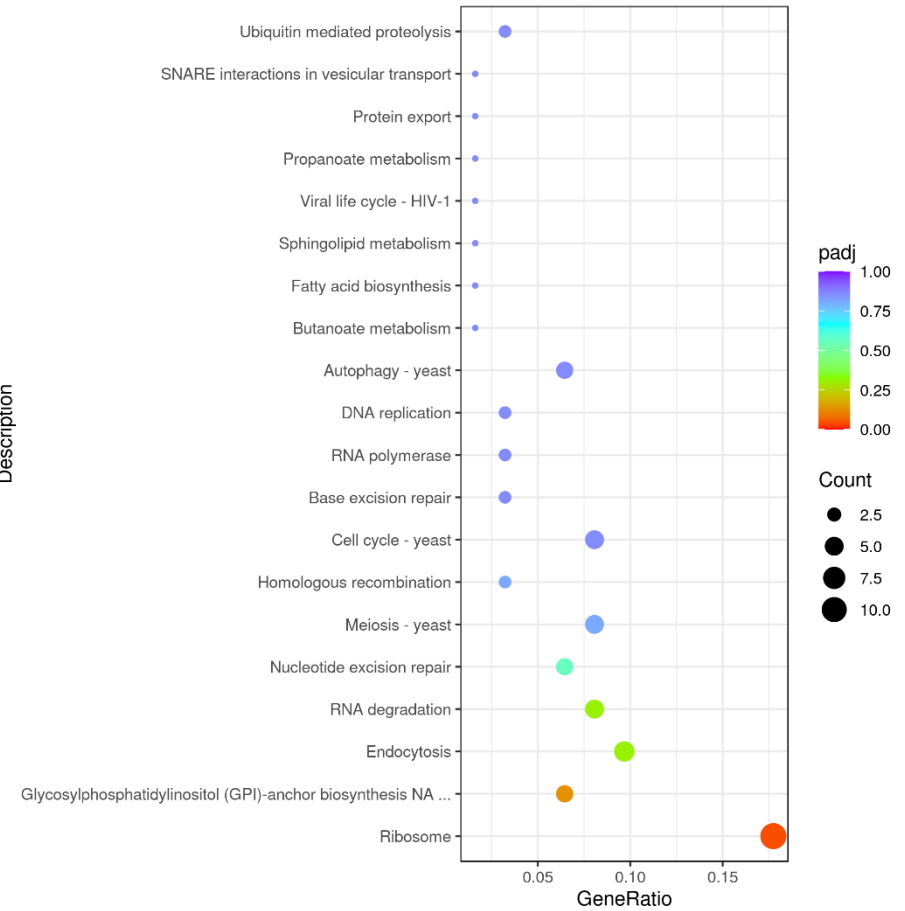


B)

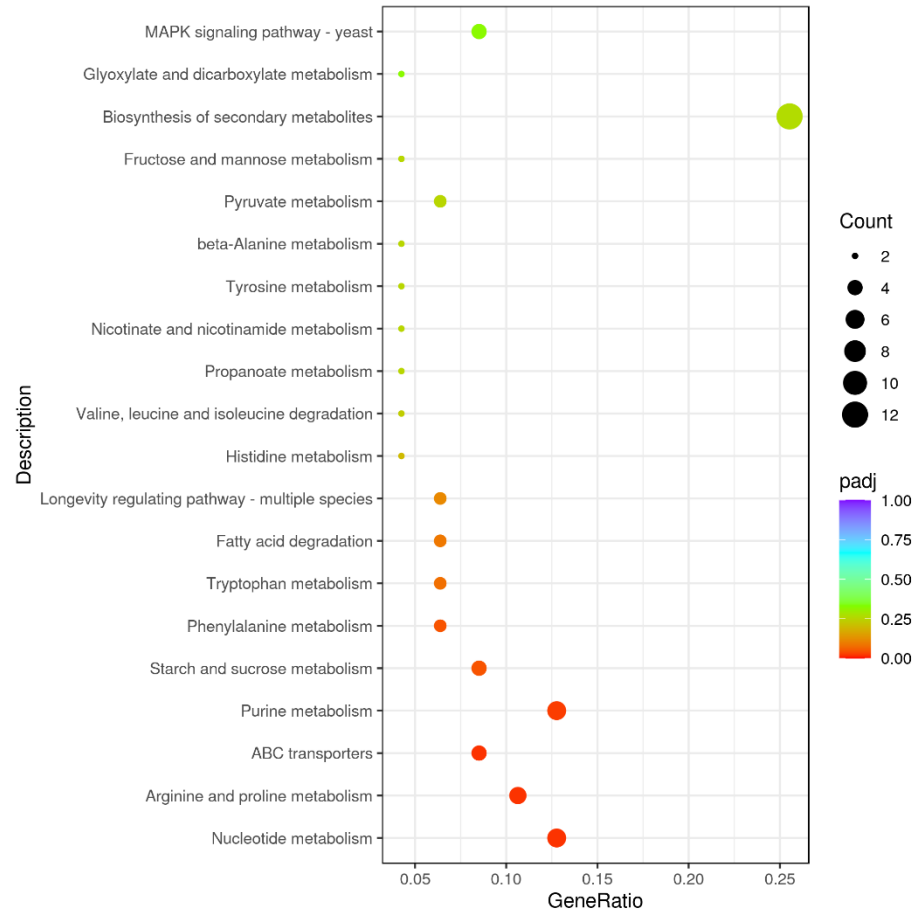
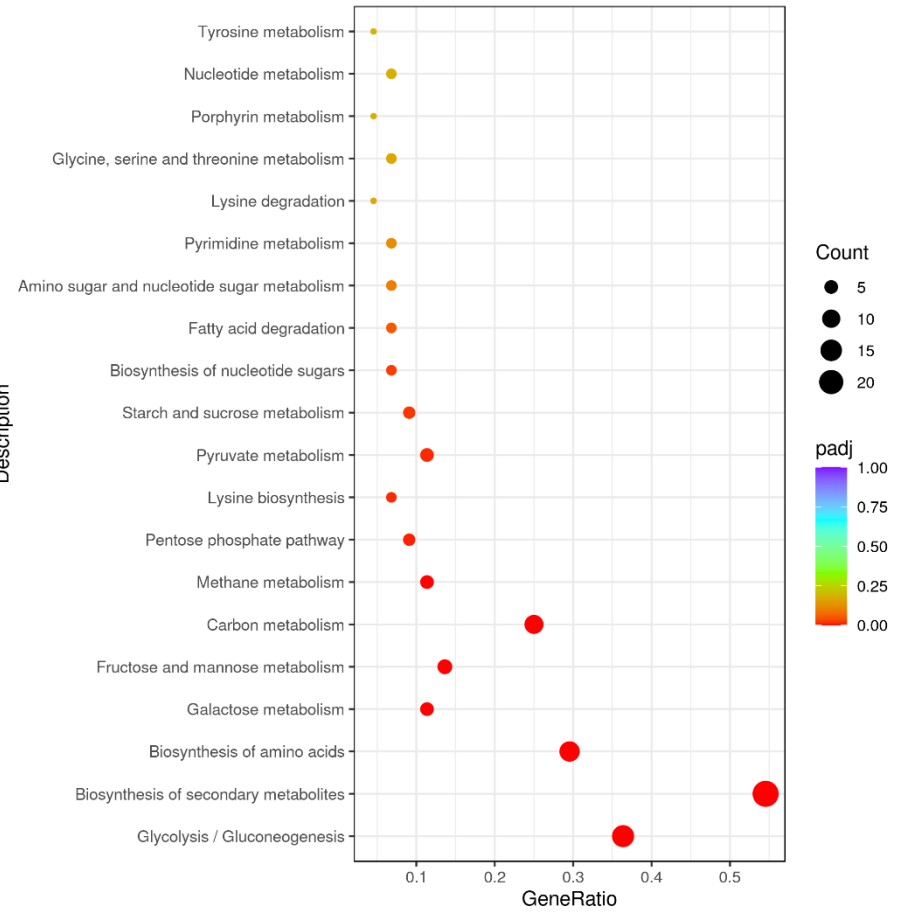
*med2Δ* 8985 vs 8985 (upregulated)



*med2Δ* 8985 vs 8985 (downregulated)

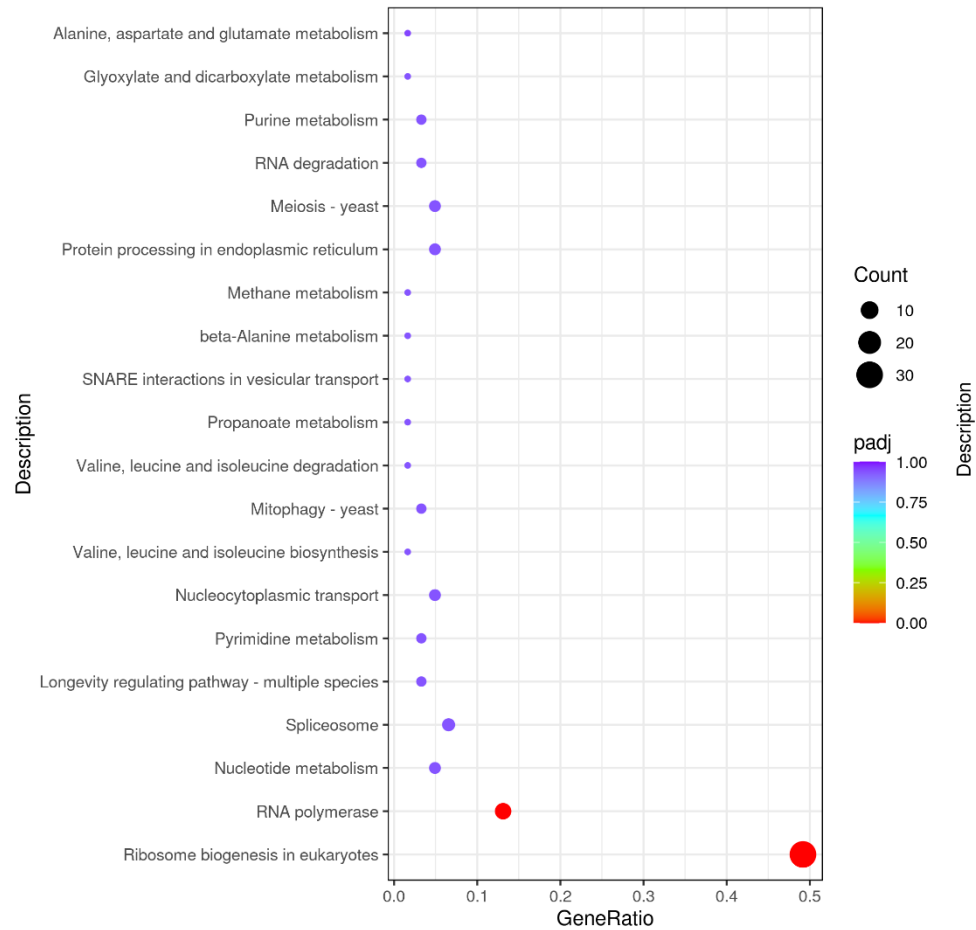


C)

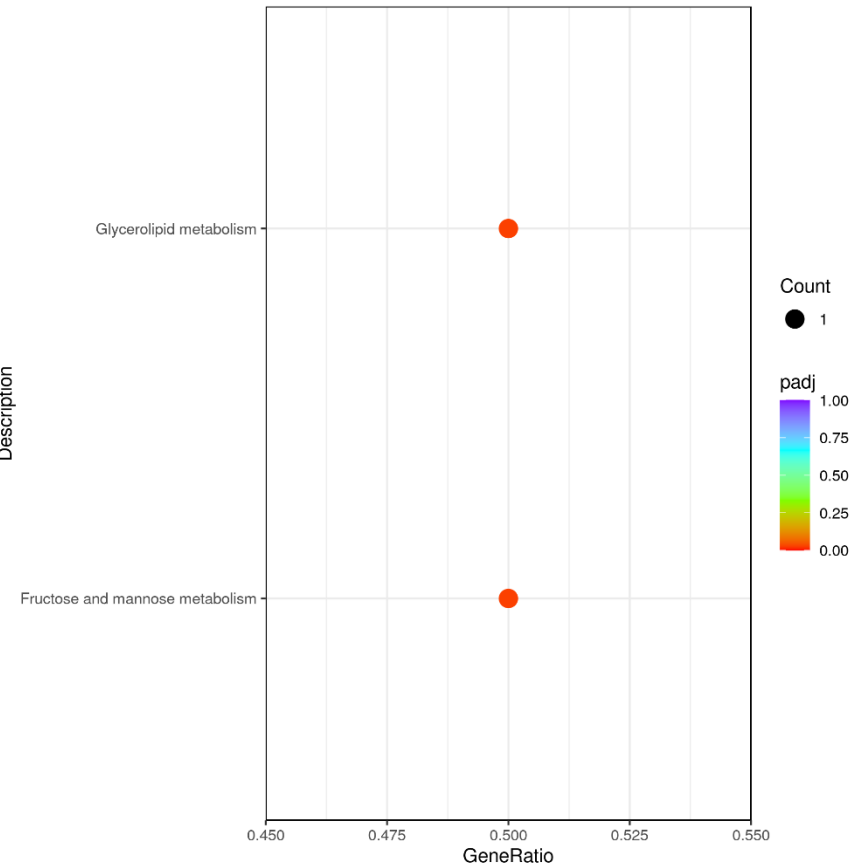
*med15*Δ 8985 vs 8985 (upregulated)*med15*Δ 8985 vs 8985 (downregulated)

D)

*med2Δ* 8977 vs 8977 (upregulated)



*med2Δ* 8977 vs 8977 (downregulated)



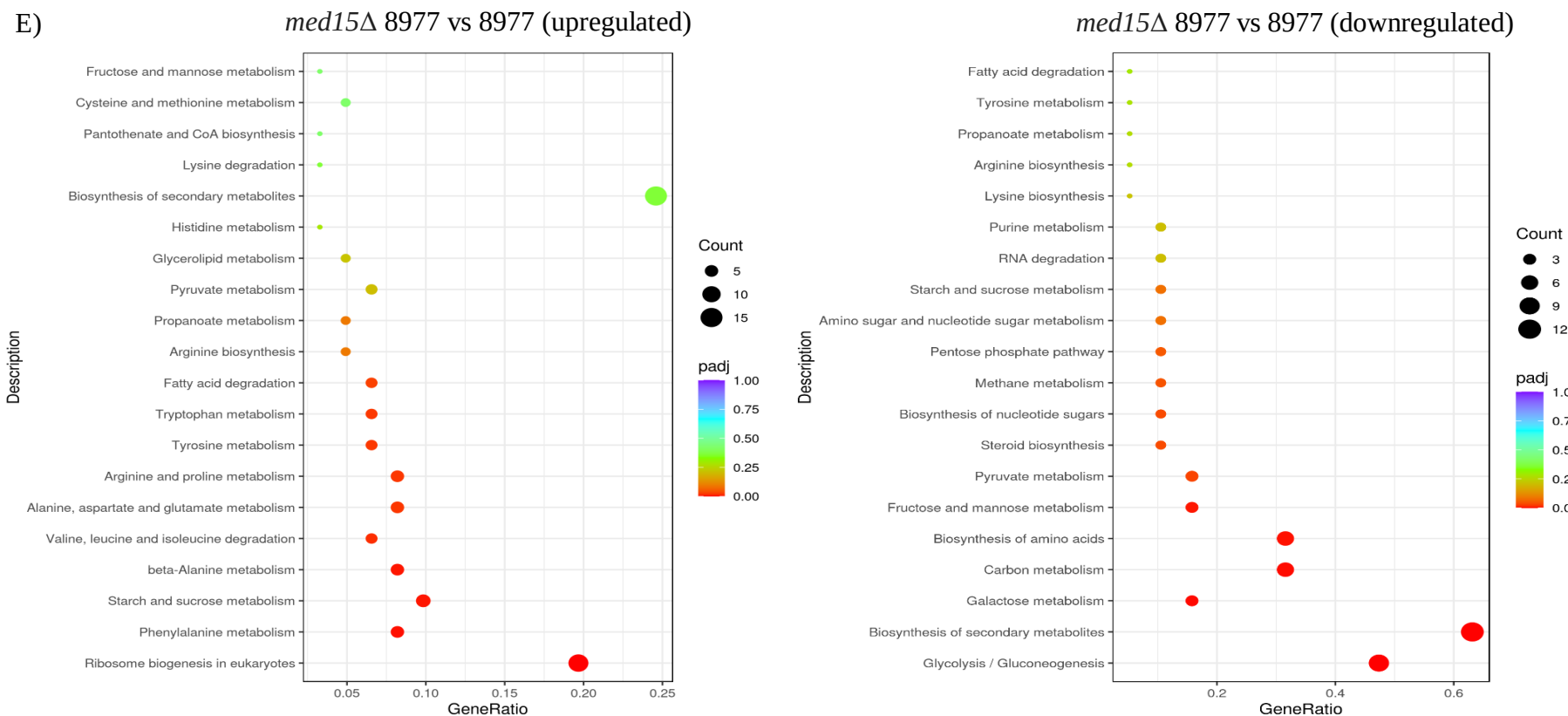


**Figure 6.3.3. KEGG pathway enrichment analysis between Mediator tail mutants and its parental *C. auris* strains**

The x-axis represents the gene ratio (the percentage of significant genes over the total genes in a given pathway) and the y-axis represents the enriched pathway name. The number of genes correlates with the size of the dots and padj value (adjusted p-value) were labelled in different colours that indicates different values (red indicates padj = 0). The gene expression of the strain compared to its counterpart were shown in each plot and the strains are A) 8985, B) *med2Δ* 8985, C) *med15Δ* 8985, D) *med2Δ* 8977 and E) *med15Δ* 8977.

#### 6.3.4 Analysis of differentially expressed genes in 8985 and 8977

We investigated the functions of the most differentially expressed genes between the weakly-aggregative 8985 compared to aggregative 8977. The Log<sub>2</sub> fold change of the top 20 genes were used to rank genes and the Candida Genome Database (CGD) was used to infer functions based on homology to known genes (Tables 6.1A & 6.1B). Most *C. auris* genes are not experimentally validated and often are of unknown function.

The most highly expressed genes in strain 8985 compared to strain 8977 (Table 6.1A) are genes *OBPA*, *PIKA* and *PAP1* which constitute the MTL $\alpha$  mating-type locus, suggesting that these genes are absent in 8977 (i.e. 8977 is MTL $\alpha$ ). Strain 8985 also exhibited higher expression of B9J08\_004498, a putative cell-surface adhesin that might be similar to *C. albicans* Als3p. We also observed higher expression of B9J08\_001932, an ortholog of the heat-shock protein encoding gene *C. albicans* *ASR1*.

Analysis of the genes more highly expressed by 8977 identified orthologues of the Mrr1-regulated genes *MGD1* (methylglyoxal reductase) and *MDR1* (fluconazole efflux pump) which were both shown to be specifically highly expressed in *C. auris* clade III strains (Biermann and Hogan, 2022) (Table 6.1B). Strain 8977 also exhibited greater expression of orthologues of the heme acquisition protein encoding genes *RBT5* (GPI-anchored protein) and *FRP1* (ferric reductase) relative to 8985. Other cell wall related genes were also more highly expressed in 8977 including the chitin-deacetylase encoding gene *CDA1* and the ortholog of *C. albicans* *PHR1* (Cell surface glycosidase). An orthologue of *WOR1*, the master regulator of white-opaque switching in *C. albicans* was also more highly expressed in strain 8977. Several aggregation related genes were also more highly expressed in strain 8977 including *C. auris*

**Table 6.1. Transcriptomics comparison of 8985 and 8977**

**Table 6.1A. Top 20 genes more highly expressed in 8985 compared to 8977.**

| <i>C. auris</i> gene names | Gene functions                                                                                                                                                     | Log2 fold change |
|----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|
| <b>B9J08_001454</b>        | ( <i>C. albicans</i> OBPA) Putative oxysterol binding protein; non-sex gene located within the MTL $\alpha$ mating-type-like locus                                 | 14.11844698      |
| <b>B9J08_001455</b>        | ( <i>C. albicans</i> PIKA) Phosphatidylinositol 4-kinase; controls levels of phosphatidylinositol-4-phosphate (PI(4)P) in the Golgi, located in MTL $\alpha$ locus | 13.44467687      |
| <b>B9J08_001453</b>        | ( <i>C. albicans</i> PAP1) Poly(A) polymerase, likely involved in mRNA polyadenylation; non-sex gene located within the MTL $\alpha$ mating-type-like locus        | 13.3864566       |
| <b>B9J08_004828</b>        | ( <i>C. auris</i> MGD2) Putative methylglyoxal reductase; expression does not appear induced by methylglyoxal, unlike MGD1                                         | 11.52094927      |
| <b>B9J08_005590</b>        | -                                                                                                                                                                  | 11.01918189      |
| <b>B9J08_005589</b>        | -                                                                                                                                                                  | 9.656986812      |
| <b>B9J08_001529</b>        | ORF, uncharacterized, has domain(s) with predicted hydrolase activity, hydrolyzing O-glycosyl compounds activity and role in organic substance metabolic process)  | 6.82377176       |
| <b>B9J08_004498</b>        | Putative cell-surface adhesin, similar to <i>C. albicans</i> Als3p                                                                                                 | 6.487570719      |
| <b>B9J08_000799</b>        | Orthologue of <i>C. albicans</i> CR_06140W, protein of unknown function; regulated by Tsa1.                                                                        | 6.23826832       |
| <b>B9J08_004451</b>        | ORF, uncharacterized                                                                                                                                               | 6.12189464       |
| <b>B9J08_005553</b>        | ORF, uncharacterized, has domain(s) with predicted membrane localization                                                                                           | 5.633384752      |
| <b>B9J08_004582</b>        | ORF, uncharacterized                                                                                                                                               | 4.962845159      |
| <b>B9J08_000266</b>        | ORF, uncharacterized                                                                                                                                               | 4.892421872      |
| <b>B9J08_005565</b>        | ORF, uncharacterized, has domain(s) with predicted metalloendopeptidase activity, metallopeptidase activity and role in proteolysis                                | 4.709416449      |
| <b>B9J08_004194</b>        | ORF, uncharacterized                                                                                                                                               | 4.587785086      |
| <b>B9J08_000389</b>        | ORF, uncharacterized, has domain(s) with predicted catalytic activity                                                                                              | 4.440544961      |
| <b>B9J08_000685</b>        | ORF, uncharacterized                                                                                                                                               | 4.403175788      |
| <b>B9J08_003911</b>        | ORF, uncharacterized, has domain(s) with predicted aspartic-type endopeptidase activity and role in proteolysis                                                    | 4.240780722      |
| <b>B9J08_002288</b>        | ORF, uncharacterized, Ortholog of <i>C. albicans</i> SC5314 : C1_07150W_A                                                                                          | 3.820593743      |
| <b>B9J08_001932</b>        | ( <i>C. albicans</i> ASR1) Heat shock protein; In <i>C. albicans</i> transcript regulated by cAMP, osmotic stress, ciclopirox olamine and ketoconazole.            | 3.80806638       |

**Table 6.1B. Top 20 genes more highly expressed in 8977 compared to 8985.**

| <i>C. auris</i> gene names | Gene functions                                                                                                                                                                | Log2 fold change |
|----------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|
| <b>B9J08_001061</b>        | rRNA, uncharacterized                                                                                                                                                         | 8.88321768       |
| <b>B9J08_000656</b>        | ( <i>C. auris</i> <i>MGD1</i> ) Putative methylglyoxal reductase; expression appears to be induced by the transcription factor Mrr1p in response to methylglyoxal and benomyl | 7.725140399      |
| <b>B9J08_003981</b>        | ( <i>C. auris</i> <i>MDR1</i> ) Putative efflux transporter of the major facilitator superfamily; expression is increased in some fluconazole-resistant isolates              | 5.794197809      |
| <b>B9J08_002584</b>        | ORF, uncharacterized                                                                                                                                                          | 5.655190489      |
| <b>B9J08_005545</b>        | ( <i>C. albicans</i> <i>RBT5</i> ) GPI-linked cell wall protein; involved in hemoglobin utilization.                                                                          | 5.64106557       |
| <b>B9J08_004841</b>        | ( <i>C. albicans</i> <i>CDA2</i> ) Putative chitin deacetylase.                                                                                                               | 5.487108066      |
| <b>B9J08_002250</b>        | ( <i>C. albicans</i> <i>HGT12</i> ) Glucose, fructose, mannose transporter; major facilitator superfamily.                                                                    | 5.275336817      |
| <b>B9J08_001487</b>        | ORF, uncharacterized, has domain(s) with predicted transmembrane transporter activity and role in transmembrane transport                                                     | 4.612948245      |
| <b>B9J08_000001</b>        | ORF, uncharacterized                                                                                                                                                          | 4.48220245       |
| <b>B9J08_001951</b>        | ORF, uncharacterized                                                                                                                                                          | 4.204602615      |
| <b>B9J08_004468</b>        | ( <i>C. albicans</i> <i>FRP1</i> ) Ferric reductase-related protein involved in heme acquisition.                                                                             | 3.970657531      |
| <b>B9J08_001479</b>        | ORF, uncharacterized                                                                                                                                                          | 3.882985655      |
| <b>B9J08_005063</b>        | ORF, uncharacterized                                                                                                                                                          | 3.811236108      |
| <b>B9J08_000918</b>        | ( <i>C. albicans</i> <i>PHR1</i> ) Cell surface glycosidase; may act on cell-wall beta-1,3-glucan prior to beta-1,6-glucan linkage.                                           | 3.769775214      |
| <b>B9J08_004027</b>        | ( <i>C. albicans</i> <i>WOR1</i> ) Transcription factor ("master switch") of white-opaque phenotypic switching in <i>C. albicans</i> .                                        | 3.540040326      |
| <b>B9J08_004101</b>        | ORF, uncharacterized                                                                                                                                                          | 3.497081558      |
| <b>B9J08_001548</b>        | ORF, uncharacterized                                                                                                                                                          | 3.392000974      |
| <b>B9J08_005563</b>        | ORF, uncharacterized                                                                                                                                                          | 3.331016313      |
| <b>B9J08_004287</b>        | ORF, uncharacterized                                                                                                                                                          | 3.26864898       |
| <b>B9J08_000030</b>        | ( <i>C. albicans</i> <i>RNT1</i> ) Protein of unknown function.                                                                                                               | 3.165706311      |

*SCF1* (3 Log2-fold increment, placed at 22<sup>nd</sup>) and *AAF1* (2.89 Log2-fold increment, 25<sup>th</sup> placed), a possible regulatory protein/adhesin like protein that might contribute to aggregation.

#### 6.3.5 Analysis of differentially expressed genes in *med2Δ* mutants of 8985 and 8977

Deletion of *MED2* in weakly-aggregative 8985 resulted in >Log2-fold increased expression of a number of genes (Table 6.2B). Unexpectedly, many of the genes that were weakly expressed relative to strain 8977 (Table 6.1B) exhibited increased expression upon deletion of *MED2*, including *RBT5*, *FRP1*, *WOR1* and *MGD1*, suggesting that Med2 may play an important role in shaping the differential expression between the two strains. Two genes involved in iron metabolism, *FTH1* and *CFL4* along with two oxidoreductase encoding genes, *CIP1* and *ZTA1*, were also upregulated in *med2Δ* 8985. Interestingly, the important adhesin encoding gene *C. auris SCF1*, which was weakly expressed in WT 8985 compared to aggregative 8977, exhibited increased expression in *med2Δ* 8985. Examination of the top 20 downregulated genes in *med2Δ* 8985 showed that *MED2* (B9J08\_001249) was the most affected gene (Table 6.2B). Again, genes that were differentially expressed between 8985 and 8977 were among the most affected genes in *med2Δ* 8985, with reduced expression of the 8985 highly expressed B9J08\_004498 (*ALS3*-like gene) and B9J08\_004828 (*MGD2*). Other cell surface protein encoding genes that may contribute to cell adherence were also downregulated (*MSB1* and *MSB2*).

The deletion of *MED2* in aggregative 8977 caused the upregulation (Table 6.2C) of many genes involved in ribosome biogenesis (*CR\_10260W*, *NOP14*, *DRS1*, *CR\_02420W*, *DBP8*, *RRP9*, *C3\_02040C*, *MPP10*, *SRP40*, *CR\_10470C*, *C1\_07960W* and *UTP21*). The most highly induced gene was an orthologue of *FCR1* (transcription factor) which regulates antifungal susceptibility in *C. albicans*. The deletion of *MED2* in aggregative 8977 only caused 9 genes

**Table 6.2. Transcriptomics comparison of *MED2* mutants and their parental strains**

**Table 6.2A. Top 20 Upregulated genes in *med2Δ* 8985 compared to 8985.**

| <i>C. auris</i> gene names | Gene functions                                                                                                                                                                                | Log2 fold change |
|----------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|
| <b>B9J08_005545</b>        | ( <i>C. albicans RBT5</i> ) GPI-linked cell wall protein; involved in hemoglobin utilization                                                                                                  | 5.093021182      |
| <b>B9J08_004468</b>        | ( <i>C. albicans FRP1</i> ) Ferric reductase-related protein involved in heme acquisition.                                                                                                    | 5.043856515      |
| <b>B9J08_004450</b>        | Ortholog(s) have role in thiamine biosynthetic process                                                                                                                                        | 4.126088081      |
| <b>B9J08_003024</b>        | ( <i>C. albicans CIP1</i> ) Possible oxidoreductase.                                                                                                                                          | 3.110943811      |
| <b>B9J08_000483</b>        | ( <i>C. albicans HSP70</i> ) Putative hsp70 chaperone.                                                                                                                                        | 2.997267705      |
| <b>B9J08_000170</b>        | ( <i>C. albicans FTH1</i> ) Putative iron permease.                                                                                                                                           | 2.840004435      |
| <b>B9J08_001951</b>        | Ortholog of <i>Candida lusitanae</i> ATCC 42720 : CLUG_04093                                                                                                                                  | 2.652141925      |
| <b>B9J08_004366</b>        | Protein of unknown function                                                                                                                                                                   | 2.570520198      |
| <b>B9J08_005568</b>        | ( <i>C. albicans HGT2</i> ) Putative MFS glucose transporter.                                                                                                                                 | 2.56178088       |
| <b>B9J08_002408</b>        | Has domain(s) with predicted FMN binding, oxidoreductase activity                                                                                                                             | 2.55306266       |
| <b>B9J08_004027</b>        | ( <i>C. albicans WOR1</i> ) Transcription factor ("master switch") of white-opaque phenotypic switching in <i>C. albicans</i> .                                                               | 2.550872056      |
| <b>B9J08_001450</b>        | ( <i>C. albicans CFL4</i> ) C-terminus similar to ferric reductases.                                                                                                                          | 2.53150863       |
| <b>B9J08_001458</b>        | <i>SCF1</i> (Ortholog of <i>Candida lusitanae</i> ATCC 42720: CLUG_05295) Surface Colonization Factor                                                                                         | 2.512827141      |
| <b>B9J08_000169</b>        | ( <i>C. albicans C2_05060C</i> ) Putative sulfiredoxin.                                                                                                                                       | 2.509752712      |
| <b>B9J08_004449</b>        | Protein of unknown function                                                                                                                                                                   | 2.509412516      |
| <b>B9J08_000562</b>        | Protein of unknown function                                                                                                                                                                   | 2.452789996      |
| <b>B9J08_000656</b>        | ( <i>C. auris MGD1</i> ) Putative methylglyoxal reductase; expression appears to be induced by the transcription factor Mrr1p in response to methylglyoxal and benomyl                        | 2.429465451      |
| <b>B9J08_002479</b>        | ( <i>C. albicans ZTA1</i> ) Probable quinone oxidoreductase; involved in protection from oxidative stress.                                                                                    | 2.413369888      |
| <b>B9J08_002295</b>        | Protein of unknown function                                                                                                                                                                   | 2.388591702      |
| <b>B9J08_001153</b>        | ( <i>C. albicans C2_07630C</i> ) Possible stress protein; in <i>C. albicans</i> increased transcription associated with <i>CDR1</i> and <i>CDR2</i> overexpression or fluphenazine treatment. | 2.366893513      |

**Table 6.2B. Top 20 Downregulated genes in *med2Δ* 8985 compared to 8985.**

| <i>C. auris</i> gene names | Gene functions                                                                                                                                                                                                                     | Log2 fold change |
|----------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|
| <b>B9J08_001249</b>        | <i>C. albicans</i> TLO2                                                                                                                                                                                                            | 11.34123093      |
| <b>B9J08_004498</b>        | Putative cell-surface adhesin, similar to <i>C. albicans</i> Als3p                                                                                                                                                                 | 6.621976006      |
| <b>B9J08_004828</b>        | ( <i>C. albicans</i> MGD2) Putative methylglyoxal reductase; expression does not appear induced by methylglyoxal, unlike MGD1                                                                                                      | 3.218331817      |
| <b>B9J08_002660</b>        | ( <i>C. albicans</i> HGT17) Putative MFS family glucose transporter.                                                                                                                                                               | 2.682186449      |
| <b>B9J08_003607</b>        | Protein of unknown function                                                                                                                                                                                                        | 2.439548558      |
| <b>B9J08_004493</b>        | ( <i>C. albicans</i> C1_04970W) Ortholog(s) have RNA binding activity                                                                                                                                                              | 2.400204404      |
| <b>B9J08_003605</b>        | ( <i>C. albicans</i> MRPL19) Putative ribosomal protein; induced upon adherence to polystyrene; Spider biofilm repressed                                                                                                           | 2.295955419      |
| <b>B9J08_003608</b>        | ( <i>C. albicans</i> C1_08390C) Protein involved in regulation of filamentation.                                                                                                                                                   | 2.278216735      |
| <b>B9J08_005263</b>        | ( <i>C. albicans</i> C1_11930W) Protein involved in regulation of ergosterol biosynthesis genes.                                                                                                                                   | 2.196825774      |
| <b>B9J08_001476</b>        | ( <i>C. albicans</i> PRP22) Putative RNA-dependent ATPase; induced upon adherence to polystyrene; induced by Mnl1p under weak acid stress                                                                                          | 2.195997462      |
| <b>B9J08_003925</b>        | Ortholog of <i>Candida lusitanae</i> ATCC 42720 : CLUG_04719                                                                                                                                                                       | 2.153630601      |
| <b>B9J08_005334</b>        | ( <i>C. albicans</i> STE50) Protein with sterile alpha motif (SAM) and Ras-associated domain (RAD); similar to <i>S. cerevisiae</i> Rad50p, which is involved in signal transduction via interaction with and regulation of MAPKKK | 2.153430331      |
| <b>B9J08_003230</b>        | ( <i>C. albicans</i> MSB1) Putative regulator of transcription; expression in <i>S. cerevisiae</i> flo8 and flo11 mutants suggests a role in regulation of adhesion factors                                                        | 2.150605818      |
| <b>B9J08_003390</b>        | ( <i>C. albicans</i> PCL7) Putative cyclin-like protein; possible Pho85 cyclin.                                                                                                                                                    | 2.13357055       |
| <b>B9J08_000159</b>        | ( <i>C. albicans</i> MSB2) Mucin family adhesin-like protein.                                                                                                                                                                      | 2.108243952      |
| <b>B9J08_004166</b>        | ( <i>C. albicans</i> RAD50) Putative DNA double-strand break repair factor; involved in response to oxidative stress and drug resistance.                                                                                          | 2.107624427      |
| <b>B9J08_004190</b>        | ( <i>C. albicans</i> VRP1) Verprolin-related protein involved in actin cytoskeleton organization and polarized morphogenesis.                                                                                                      | 2.084967058      |
| <b>B9J08_000402</b>        | Protein of unknown function                                                                                                                                                                                                        | 2.07765174       |
| <b>B9J08_000597</b>        | Ortholog of <i>C. albicans</i> SC5314 : C1_06380C_A                                                                                                                                                                                | 2.074186967      |
| <b>B9J08_001880</b>        | ( <i>C. albicans</i> C3_07390C) Ortholog(s) have structural constituent of ribosome activity and mitochondrial small ribosomal subunit localization                                                                                | 2.024424976      |

**Table 6.2C. Top 20 Upregulated genes in *med2Δ* 8977 compared to 8977.**

| <i>C. auris</i> gene names | Gene functions                                                                                                                                                                                                           | Log2 fold change |
|----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|
| <b>B9J08_001232</b>        | ( <i>C. albicans FCR1</i> ) Transcription factor; repressor of fluconazole/ketoconazole/brefeldin A resistance in <i>C. albicans</i>                                                                                     | 2.422275395      |
| <b>B9J08_003905</b>        | ( <i>C. albicans CR_10260W</i> ) Putative mitochondrial 2' O-ribose methyltransferase; predicted role in methylation of U(2791) in 21S rRNA.                                                                             | 2.318752153      |
| <b>B9J08_004918</b>        | ( <i>C. auris HSP90</i> ) Essential chaperone protein; involved in tolerance to azoles and temperature-induced signal transduction in <i>C. albicans</i> .                                                               | 2.256323697      |
| <b>B9J08_004826</b>        | ( <i>C. albicans NOP14</i> ) Putative nucleolar protein.                                                                                                                                                                 | 2.131769775      |
| <b>B9J08_000229</b>        | ( <i>C. albicans LEU2</i> ) Isopropyl malate dehydrogenase; leucine biosynthesis.                                                                                                                                        | 2.074535496      |
| <b>B9J08_001512</b>        | ( <i>C. albicans DRS1</i> ) Putative nucleolar DEAD-box protein.                                                                                                                                                         | 2.014250368      |
| <b>B9J08_004775</b>        | ( <i>C. auris TPO3</i> ) Putative polyamine transporter of the MFS-MDR family.                                                                                                                                           | 1.988063419      |
| <b>B9J08_000799</b>        | ( <i>C. albicans CR_06140W</i> ) Protein of unknown function.                                                                                                                                                            | 1.977409044      |
| <b>B9J08_001133</b>        | ( <i>C. albicans CR_02420W</i> ) Ortholog(s) have small ribosomal subunit rRNA binding activity                                                                                                                          | 1.949795537      |
| <b>B9J08_004323</b>        | Has domain(s) with predicted tRNA (adenine(58)-N1)-methyltransferase activity, role in tRNA methylation and tRNA (m1A) methyltransferase complex localization                                                            | 1.946877057      |
| <b>B9J08_005116</b>        | ( <i>C. albicans DBP8</i> ) Protein similar to <i>S. cerevisiae</i> Dbp8p, an ATP-dependent helicase involved in rRNA processing.                                                                                        | 1.946866646      |
| <b>B9J08_004080</b>        | ( <i>C. albicans RRP9</i> ) Ribosomal protein; mutation confers resistance to 5-fluorocytosine (5-FC), 5-fluorouracil (5-FU), and tubercidin (7-deazaadenosine).                                                         | 1.919782033      |
| <b>B9J08_004113</b>        | Has domain(s) with predicted transmembrane transporter activity and role in transmembrane transport                                                                                                                      | 1.915768013      |
| <b>B9J08_000207</b>        | ( <i>C. albicans C3_02040C</i> ) Ortholog of <i>S. cerevisiae</i> Loc1, a nuclear protein involved in asymmetric localization of ASH1 mRNA in <i>S. cerevisiae</i> .                                                     | 1.899372448      |
| <b>B9J08_003906</b>        | ( <i>C. albicans MPP10</i> ) Putative SSU processome and 90S preribosome component; repressed in core stress response; repressed by prostaglandins                                                                       | 1.896875804      |
| <b>B9J08_003014</b>        | ( <i>C. albicans SRP40</i> ) Putative chaperone of small nucleolar ribonucleoprotein particles.                                                                                                                          | 1.876464901      |
| <b>B9J08_000928</b>        | ( <i>C. albicans AQY1</i> ) Aquaporin water channel; osmotic shock resistance, WT freeze tolerance.                                                                                                                      | 1.875752427      |
| <b>B9J08_002604</b>        | ( <i>C. albicans CR_10470C</i> ) Ortholog(s) have role in maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA), rRNA processing and nucleolus, small-subunit processome localization | 1.867328311      |
| <b>B9J08_001610</b>        | ( <i>C. albicans C1_07960W</i> ) Predicted nuclear exosome-associated nucleic acid binding protein.                                                                                                                      | 1.865295655      |
| <b>B9J08_003640</b>        | ( <i>C. albicans UTP21</i> ) Putative U3 snoRNP protein; Hap43-induce; physically interacts with TAP-tagged Nop1; Spider biofilm induced                                                                                 | 1.830185921      |

**Table 6.2D. Top 20 Downregulated genes in *med2Δ* 8977 compared to 8977.**

| <i>C. auris</i> gene names | Gene functions                                                                                                                                                                                   | Log2 fold change |
|----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|
| <b>B9J08_004378</b>        | ( <i>C. albicans</i> <i>RHR2</i> ) Glycerol 3-phosphatase; In <i>C. albicans</i> has roles in osmotic tolerance, glycerol accumulation in response to salt.                                      | 1.930695322      |
| <b>B9J08_003002</b>        | Putative iron ion transmembrane transporter activity                                                                                                                                             | 1.792889532      |
| <b>B9J08_004831</b>        | ( <i>C. albicans</i> <i>AAF1</i> ) Possible regulatory protein; possible adhesin-like; Glu-rich domain; production in <i>S. cerevisiae</i> increases endothelial cell adherence and flocculence. | 1.620043902      |
| <b>B9J08_001458</b>        | <i>SCF1</i> (Ortholog of <i>Candida lusitanae</i> ATCC 42720 : CLUG_05295) Surface Colonization Factor                                                                                           | 1.584823872      |
| <b>B9J08_003867</b>        | Ortholog(s) have alpha-ketoglutarate-dependent xanthine dioxygenase activity and role in xanthine catabolic process                                                                              | 1.202036363      |
| <b>B9J08_005300</b>        | ( <i>C. albicans</i> C1_11080W) Putative 6-phosphofructo-2-kinase; catalyzes synthesis of fructose-2,6-bisphosphate.                                                                             | 1.153710441      |
| <b>B9J08_001313</b>        | <i>C. albicans</i> SC5314 Ortholog(s) : <a href="#">C5_00390C_A</a>                                                                                                                              | 1.129576604      |
| <b>B9J08_005276</b>        | tRNA, Uncharacterized                                                                                                                                                                            | 1.115378699      |
| <b>B9J08_002691</b>        | ( <i>C. albicans</i> <i>YHB1</i> ) nitric oxide reductase activity                                                                                                                               | 1.094314313      |

to be significantly downregulated in 8977 (Table 6.2D). Importantly, two genes that were highly expressed in aggregative 8977 relative to strains 8985 and might contribute to the aggregation phenotype, namely *SCF1* and *AAF1*, were downregulated following deletion of *MED2* in 8977.

### 6.3.6 Analysis of differentially expressed genes in *med15*Δ mutants of 8985 and 8977

The deletion of *MED15* in strain 8985 had similar effects as the *MED2* deletion (Table 6.3A & 6.3B). Again, genes that were weakly expressed in 8985 relative to 8977 exhibited increased expression when *MED15* was deleted (*RBT5*, *WOR1*, *SCF1*) and genes that were more highly expressed in the 8985 background exhibited reduced expression (*MGD2*, *ALS3*-like gene). Among the top 20 highly expressed genes in *med15*Δ 8985 (Table 6.3A), more than half of the genes have unknown functions. Genes with various functions like *PTR22* (oligopeptide transporter), *BIO32* (putative class III aminotransferase), *EFH1* (transcription factor), and *MUM2* (protein essential for meiotic DNA replication and sporulation) were highly induced in *med15*Δ 8985. Various important genes were downregulated due to the absence of *MED15* in 8985 (Table 6.3B) including genes involved in glycolysis/gluconeogenesis (*HXK2*, *PGK1*, *PDC11* and *CDC19*), *PHO84* (high-affinity phosphate transporter), *ADH1* (alcohol dehydrogenase), *YHB1* (nitric oxide reductase) and *RHR2* (Glycerol 3-phosphatase, roles in osmotic tolerance).

The deletion of *MED15* in aggregative 8977 caused the upregulation/downregulation of a similar set of genes (Tables 6.5C, 6.5D & 6.5E) including down regulation of glycolysis (*HXK2*, *PGK1*, *PDC11*, *CDC19*, *ADH1*), *RHR2* (glycerol 3-phosphatase) and *FGR41* (putative GPI-anchored adhesin-like protein). Multiple *HGT* genes that normally encode for glucose transporters (*HGT2*, *HGT10*, *HGT12*, *HGT16*) and various transporter encoding genes (*XUT1*,

*STE6*, *JEN2*, *GPT1*, *MDR2* and *PTR22*) were significantly upregulated in *med15Δ* 8977. Several permease encoding genes (*DAL9*, *UGA4*, *AGP2* and *PUT4*) and an aquaporin water channel encoding gene *AQY1* were significantly upregulated as well. *FKS2*, encoding a putative cell wall glucan biosynthetic enzyme targeted by echinocandins was downregulated in *med15Δ* 8977 and the putative GPI-anchored adhesin-like proteins homologous to *C. albicans* *PGA38* and *FGR41*, were downregulated in *med15Δ* 8977. Interestingly, several aggregation related genes that were highly expressed in strain 8977 like *C. auris* *SCF1* (1.384 Log2-fold reduction, placed at 32<sup>nd</sup>) and *AAF1* (-1.376 Log2-fold reduction, 33<sup>rd</sup> placed) were downregulated in *med15Δ* 8977.

#### 6.3.7 Analysis of differentially expressed genes in both Mediator tail mutants of 8985 and 8977

We compared the list of significantly up- and downregulated genes in both the *MED15* mutants of 8977 and 8985 and the *MED2* mutants of 8977 and 8985 (Figure 6.3.4). A significant number of the upregulated/downregulated genes were shared between the *MED15* null mutants (174 significantly upregulated genes and 51 significantly downregulated genes in common) whereas only 7 shared genes were significantly upregulated in both *MED2* null mutants. *MDR1* and *MGD1*, which are both regulated by the Mrr1 transcription factor were differently expressed between *MED15* null mutants as both genes were upregulated in *med15Δ* 8985 but downregulated in *med15Δ* 8977. A putative cell surface adhesin *ALS4498* (by B9J08\_4498) and a putative transmembrane transporter encoding gene B9J08\_4243 were both found to be downregulated in *med15Δ* 8985 but upregulated in *med15Δ* 8977. *CCC1*, a putative manganese transporter encoding gene, was upregulated in *med2Δ* 8977 but downregulated in *med2Δ* 8985. Interestingly, *SCF1* (encoding an adhesin) was upregulated in both Mediator tail mutants of 8985 and downregulated in both Mediator tail mutants of 8977.

**Table 6.3. Transcriptomics comparison of *MED15* mutants and their parental strains**

**Table 6.3A. Top 20 Upregulated genes in *med15Δ* 8985 compared to 8985.**

| <i>C. auris</i> gene names | Gene functions                                                                                                                                                                          | Log2 fold change |
|----------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|
| <b>B9J08_002407</b>        | Protein of unknown function                                                                                                                                                             | 4.484321036      |
| <b>B9J08_004481</b>        | Ortholog of <i>C. albicans</i> SC5314 : C2_04090W_A                                                                                                                                     | 4.422043892      |
| <b>B9J08_001548</b>        | Protein of unknown function                                                                                                                                                             | 4.379674565      |
| <b>B9J08_004175</b>        | Has domain(s) with predicted triglyceride lipase activity and role in lipid catabolic process                                                                                           | 4.301361131      |
| <b>B9J08_005551</b>        | Protein of unknown function                                                                                                                                                             | 4.274494109      |
| <b>B9J08_004188</b>        | ( <i>C. albicans</i> PTR22) Oligopeptide transporter involved in uptake of di-/tripeptides.                                                                                             | 4.214543491      |
| <b>B9J08_000990</b>        | ( <i>C. albicans</i> BIO32) Putative class III aminotransferase with a predicted role in biotin biosynthesis.                                                                           | 4.182526018      |
| <b>B9J08_000079</b>        | ( <i>C. albicans</i> EFH1) APSES transcription factor; regulates filamentous growth in <i>C. albicans</i> .                                                                             | 4.152220994      |
| <b>B9J08_004098</b>        | Protein of unknown function                                                                                                                                                             | 4.134880818      |
| <b>B9J08_004284</b>        | Protein of unknown function                                                                                                                                                             | 4.133384358      |
| <b>B9J08_004097</b>        | Has domain(s) with predicted transmembrane transporter activity and role in transmembrane transport                                                                                     | 4.084884765      |
| <b>B9J08_001517</b>        | Protein of unknown function                                                                                                                                                             | 4.023716638      |
| <b>B9J08_004102</b>        | Protein of unknown function                                                                                                                                                             | 4.017149949      |
| <b>B9J08_002247</b>        | Protein of unknown function                                                                                                                                                             | 3.919743744      |
| <b>B9J08_004765</b>        | Protein of unknown function                                                                                                                                                             | 3.91880184       |
| <b>B9J08_000784</b>        | ( <i>C. albicans</i> MUM2) Protein similar to <i>S. cerevisiae</i> Mum2, a protein essential for meiotic DNA replication and sporulation; induced by alpha pheromone in SpiderM medium. | 3.891807859      |
| <b>B9J08_004027</b>        | ( <i>C. albicans</i> WOR1) Transcription factor ("master switch") of white-opaque phenotypic switching in <i>C. albicans</i> .                                                          | 3.842287128      |
| <b>B9J08_005545</b>        | ( <i>C. albicans</i> RBT5) GPI-linked cell wall protein; involved in hemoglobin utilization.                                                                                            | 3.825404731      |
| <b>B9J08_001527</b>        | Has domain(s) with predicted transmembrane transporter activity, role in transmembrane transport and membrane localization                                                              | 3.782931737      |
| <b>B9J08_001458</b>        | SCF1 (Ortholog of <i>Candida lusitanae</i> ATCC 42720 : CLUG_05295) Surface Colonization Factor                                                                                         | 3.748013604      |

**Table 6.3B. Top 20 Downregulated genes in *med15A* 8985 compared to 8985.**

| <i>C. auris</i> gene names | Gene functions                                                                                                                                                                                         | Log2 fold change |
|----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|
| <b>B9J08_003619</b>        | <i>C. albicans MED15</i>                                                                                                                                                                               | 14.41558585      |
| <b>B9J08_004498</b>        | Putative cell-surface adhesin, similar to <i>C. albicans</i> Als3p                                                                                                                                     | 4.836709296      |
| <b>B9J08_001464</b>        | ( <i>C. albicans PHO84</i> ) High-affinity phosphate transporter.                                                                                                                                      | 3.735514308      |
| <b>B9J08_004828</b>        | ( <i>C. auris MGD2</i> ) Putative methylglyoxal reductase; expression does not appear induced by methylglyoxal, unlike MGD1                                                                            | 3.624053113      |
| <b>B9J08_004331</b>        | ( <i>C. albicans ADH1</i> ) Alcohol dehydrogenase; oxidizes ethanol to acetaldehyde.                                                                                                                   | 2.775035883      |
| <b>B9J08_002480</b>        | Has domain(s) with predicted transmembrane transporter activity, role in transmembrane transport and membrane localization                                                                             | 2.745233865      |
| <b>B9J08_002481</b>        | Has domain(s) with predicted transmembrane transporter activity, role in transmembrane transport and membrane localization.                                                                            | 2.647083592      |
| <b>B9J08_002691</b>        | ( <i>C. albicans YHB1</i> ) nitric oxide reductase activity                                                                                                                                            | 2.494264952      |
| <b>B9J08_002566</b>        | ( <i>C. albicans HXK2</i> ) Hexokinase II.                                                                                                                                                             | 2.481954412      |
| <b>B9J08_001860</b>        | ( <i>C. albicans PGK1</i> ) Phosphoglycerate kinase.                                                                                                                                                   | 2.452262843      |
| <b>B9J08_003306</b>        | Has domain(s) with predicted NAD binding, NADP binding, oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor activity and role in glucose metabolic process | 2.401438437      |
| <b>B9J08_002231</b>        | ( <i>C. albicans PDC11</i> ) Pyruvate decarboxylase.                                                                                                                                                   | 2.382399328      |
| <b>B9J08_000274</b>        | ( <i>C. albicans ENO1</i> ) Enolase, involved in glycolysis and gluconeogenesis; also has transglutaminase activity involved in assembly of cell wall polysaccharides.                                 | 2.256395122      |
| <b>B9J08_000824</b>        | ( <i>C. albicans FGR41</i> ) Putative GPI-anchored adhesin-like protein involved in regulation of covering (masking) cell wall glucan.                                                                 | 2.244481184      |
| <b>B9J08_002900</b>        | ( <i>C. albicans C3_06700C</i> ) Putative mitochondrial protein with a predicted role in respiratory growth.                                                                                           | 2.103647474      |
| <b>B9J08_003632</b>        | ( <i>C. albicans CDC19</i> ) Pyruvate kinase.                                                                                                                                                          | 2.062763423      |
| <b>B9J08_002873</b>        | tRNA, Uncharacterized                                                                                                                                                                                  | 2.003498544      |
| <b>B9J08_002141</b>        | <i>C. albicans C7_01380W</i> , Uncharacterized                                                                                                                                                         | 1.984737183      |
| <b>B9J08_001171</b>        | Has domain(s) with predicted oxidoreductase activity, zinc ion binding activity                                                                                                                        | 1.981872923      |
| <b>B9J08_004378</b>        | ( <i>C. albicans RHR2</i> ) Glycerol 3-phosphatase; roles in osmotic tolerance, glycerol accumulation in response to salt.                                                                             | 1.972866908      |

**Table 6.3C. Top 20 Upregulated genes in *med15Δ* 8977 compared to 8977.**

| <i>C. auris</i> gene names | Gene functions                                                                                                                       | Log2 fold change |
|----------------------------|--------------------------------------------------------------------------------------------------------------------------------------|------------------|
| <b>B9J08_004194</b>        | Protein of unknown function                                                                                                          | 7.350112708      |
| <b>B9J08_002695</b>        | Protein of unknown function                                                                                                          | 5.177904533      |
| <b>B9J08_005551</b>        | Protein of unknown function                                                                                                          | 4.493867728      |
| <b>B9J08_004284</b>        | Protein of unknown function                                                                                                          | 4.318431838      |
| <b>B9J08_000990</b>        | ( <i>C. albicans</i> BIO32) Putative class III aminotransferase with a predicted role in biotin biosynthesis; Spider biofilm induced | 4.239115588      |
| <b>B9J08_001526</b>        | Has domain(s) with predicted transmembrane transporter activity, role in transmembrane transport and membrane localization           | 4.133914916      |
| <b>B9J08_004755</b>        | Protein of unknown function                                                                                                          | 3.885018996      |
| <b>B9J08_004193</b>        | Protein of unknown function                                                                                                          | 3.809621237      |
| <b>B9J08_004195</b>        | Protein of unknown function                                                                                                          | 3.785432055      |
| <b>B9J08_004897</b>        | Protein of unknown function                                                                                                          | 3.685590617      |
| <b>B9J08_004097</b>        | Has domain(s) with predicted transmembrane transporter activity and role in transmembrane transport                                  | 3.625573409      |
| <b>B9J08_000562</b>        | Protein of unknown function                                                                                                          | 3.530026562      |
| <b>B9J08_002407</b>        | Protein of unknown function                                                                                                          | 3.478522254      |
| <b>B9J08_004098</b>        | Protein of unknown function                                                                                                          | 3.413001679      |
| <b>B9J08_004584</b>        | Has domain(s) with predicted transmembrane transporter activity and role in transmembrane transport                                  | 3.259843445      |
| <b>B9J08_005557</b>        | Protein of unknown function                                                                                                          | 3.243695843      |
| <b>B9J08_002688</b>        | ( <i>C. albicans</i> FDH1) Formate dehydrogenase; oxidizes formate to CO <sub>2</sub> .                                              | 3.178818636      |
| <b>B9J08_004174</b>        | Protein of unknown function                                                                                                          | 3.131657297      |
| <b>B9J08_004634</b>        | Protein of unknown function                                                                                                          | 3.02194523       |
| <b>B9J08_004884</b>        | Has domain(s) with predicted hydrolase activity, hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds activity      | 2.964021866      |

**Table 6.3D. 20 significantly upregulated genes in *med15Δ* 8977 compared to 8977.**

| <i>C. auris</i> gene names | Gene functions                                                                                                                                                                            |
|----------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>B9J08_000990</b>        | ( <i>C. albicans</i> BIO32) Putative class III aminotransferase with a predicted role in biotin biosynthesis; Spider biofilm induced                                                      |
| <b>B9J08_004099</b>        | ( <i>C. albicans</i> DAL9) Putative allantoate permease; fungal-specific (no human or murine homolog)                                                                                     |
| <b>B9J08_000928</b>        | ( <i>C. albicans</i> AQY1) Aquaporin water channel; osmotic shock resistance.                                                                                                             |
| <b>B9J08_005568</b>        | ( <i>C. albicans</i> HGT2) Putative MFS glucose transporter.                                                                                                                              |
| <b>B9J08_000938</b>        | ( <i>C. albicans</i> HGT10) Glycerol permease involved in glycerol uptake; member of the major facilitator superfamily.                                                                   |
| <b>B9J08_002250</b>        | ( <i>C. albicans</i> HGT12) Glucose, fructose, mannose transporter; major facilitator superfamily.                                                                                        |
| <b>B9J08_000582</b>        | ( <i>C. albicans</i> HGT16) Putative glucose transporter of the major facilitator superfamily.                                                                                            |
| <b>B9J08_004775</b>        | ( <i>C. albicans</i> TPO3) Putative polyamine transporter; MFS-MDR family.                                                                                                                |
| <b>B9J08_004960</b>        | ( <i>C. albicans</i> XUT1) Putative high-affinity, high-capacity xanthine-uric acid/H <sup>+</sup> symporter; similar to <i>A. nidulans</i> UapA.                                         |
| <b>B9J08_002389</b>        | ( <i>C. auris</i> STE6) Ortholog(s) have ABC-type peptide transporter activity, role in peptide pheromone export and Golgi apparatus, mating projection tip, plasma membrane localization |
| <b>B9J08_004204</b>        | ( <i>C. albicans</i> JEN2) Dicarboxylic acid transporter.                                                                                                                                 |
| <b>B9J08_005491</b>        | ( <i>C. albicans</i> UGA4) Putative gamma-aminobutyric acid/polyamine permease; nitrogen catabolite repressed gene, induced in absence of preferred N sources.                            |
| <b>B9J08_000853</b>        | ( <i>C. albicans</i> GPT1) GABA/polyamine transporter.                                                                                                                                    |
| <b>B9J08_002688</b>        | ( <i>C. albicans</i> FDH1) Formate dehydrogenase; oxidizes formate to CO <sub>2</sub> .                                                                                                   |
| <b>B9J08_000368</b>        | ( <i>C. albicans</i> MDR2) Putative efflux transporter of the major facilitator superfamily; expression level does not appear correlated to fluconazole resistance                        |
| <b>B9J08_004188</b>        | ( <i>C. albicans</i> PTR22) Oligopeptide transporter involved in uptake of di-/tripeptides.                                                                                               |
| <b>B9J08_002666</b>        | ( <i>C. albicans</i> AGP2) Amino acid permease.                                                                                                                                           |
| <b>B9J08_003690</b>        | ( <i>C. albicans</i> PUT4) Putative proline permease.                                                                                                                                     |
| <b>B9J08_001020</b>        | ( <i>C. auris</i> FKS2) Putative 1,3-beta-D-glucan synthase involved in cell wall glucan biosynthesis; target of echinocandin                                                             |
| <b>B9J08_004010</b>        | ( <i>C. albicans</i> PLB4.5) Phospholipase B.                                                                                                                                             |

**Table 6.3E. Top 20 Downregulated genes in *med15Δ* 8977 compared to 8977.**

| <i>C. auris</i> gene names | Gene functions                                                                                                                                                                           | Log2 fold change |
|----------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|
| <b>B9J08_003619</b>        | <i>C. albicans MED15</i>                                                                                                                                                                 | 13.74366627      |
| <b>B9J08_002465</b>        | -                                                                                                                                                                                        | 2.647556686      |
| <b>B9J08_002691</b>        | ( <i>C. albicans YHB1</i> ) nitric oxide reductase activity                                                                                                                              | 2.617470298      |
| <b>B9J08_004544</b>        | -                                                                                                                                                                                        | 2.426212389      |
| <b>B9J08_000824</b>        | ( <i>C. albicans FGR41</i> ) Putative GPI-anchored adhesin-like protein involved in regulation of covering (masking) cell wall glucan.                                                   | 2.052066061      |
| <b>B9J08_001409</b>        | ( <i>C. albicans PGA38</i> ) Putative adhesin-like GPI-anchored protein.                                                                                                                 | 2.048195013      |
| <b>B9J08_001313</b>        | <i>C. albicans SC5314</i> Ortholog(s) : <a href="#">C5_00390C_A</a>                                                                                                                      | 2.045296035      |
| <b>B9J08_003002</b>        | Has domain(s) with predicted iron ion transmembrane transporter activity.                                                                                                                | 1.962259113      |
| <b>B9J08_004378</b>        | ( <i>C. albicans RHR2</i> ) Glycerol 3-phosphatase; roles in osmotic tolerance, glycerol accumulation in response to salt.                                                               | 1.95231832       |
| <b>B9J08_002566</b>        | ( <i>C. albicans HXK2</i> ) Hexokinase II.                                                                                                                                               | 1.952055192      |
| <b>B9J08_003306</b>        | Predicted NAD binding, NADP binding, oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor activity and role in glucose metabolic process      | 1.949150164      |
| <b>B9J08_004331</b>        | ( <i>C. albicans ADH1</i> ) Alcohol dehydrogenase; oxidizes ethanol to acetaldehyde.                                                                                                     | 1.934134685      |
| <b>B9J08_000740</b>        | ( <i>C. albicans GDH3</i> ) NADP-glutamate dehydrogenase.                                                                                                                                | 1.911696133      |
| <b>B9J08_002231</b>        | ( <i>C. albicans PDC11</i> ) Pyruvate decarboxylase.                                                                                                                                     | 1.888704961      |
| <b>B9J08_002481</b>        | Has domain(s) with predicted transmembrane transporter activity.                                                                                                                         | 1.873663855      |
| <b>B9J08_001860</b>        | ( <i>C. albicans PGK1</i> ) Phosphoglycerate kinase.                                                                                                                                     | 1.703963207      |
| <b>B9J08_003314</b>        | ( <i>C. albicans RBE1</i> ) Pry family cell wall protein; Ortholog(s) have sterol binding activity, role in fatty acid transport, sterol transport and extracellular region localization | 1.674901823      |
| <b>B9J08_003867</b>        | Ortholog(s) have alpha-ketoglutarate-dependent xanthine dioxygenase activity and role in xanthine catabolic process                                                                      | 1.671071269      |
| <b>B9J08_005567</b>        | ( <i>C. albicans SHA3</i> ) Putative ser/thr kinase involved in glucose transport.                                                                                                       | 1.640872478      |
| <b>B9J08_004602</b>        | ( <i>C. albicans OPT1</i> ) Oligopeptide transporter; transports 3-to-5-residue peptides.                                                                                                | 1.625997621      |

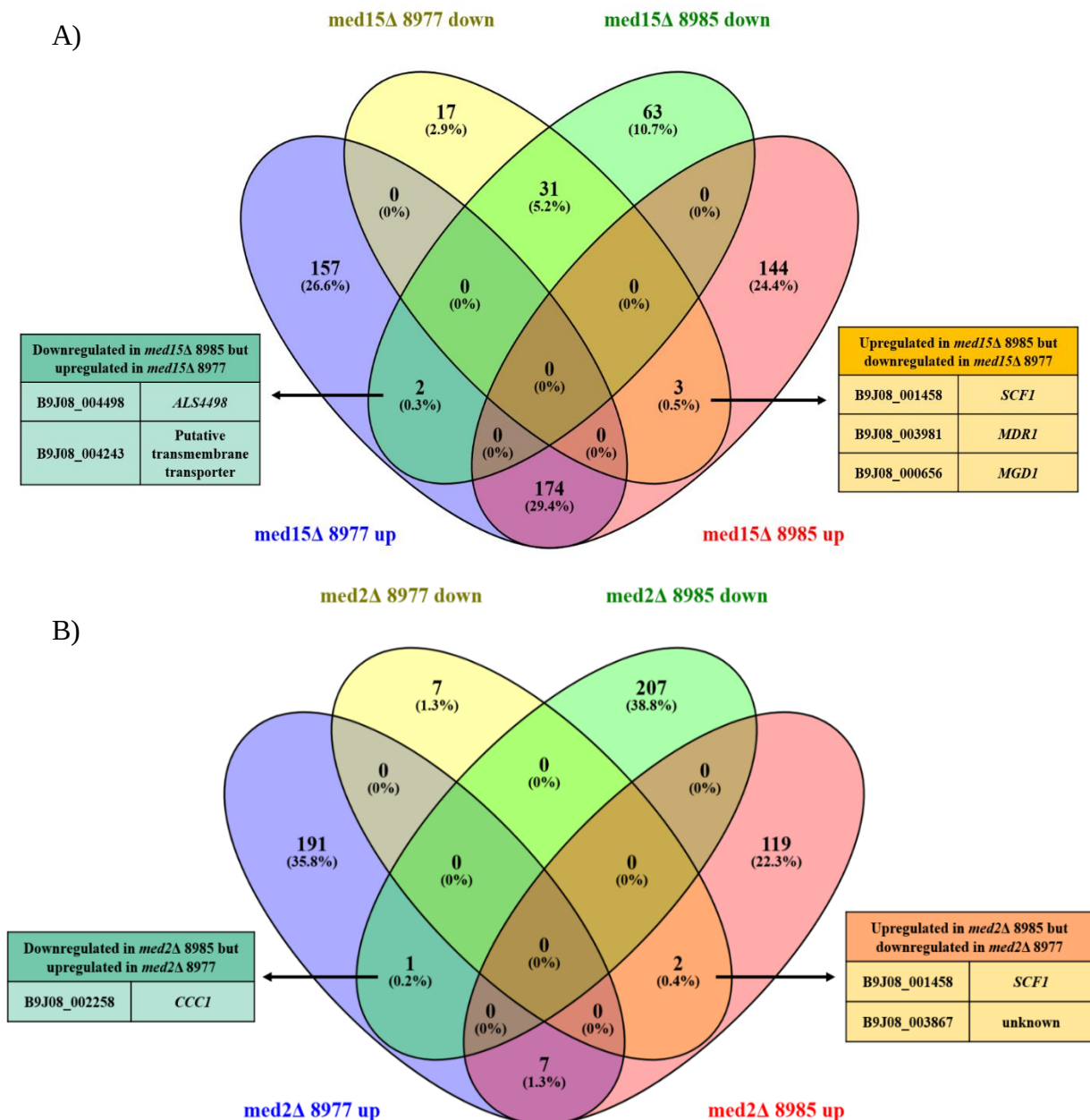


**Figure 6.3.4. Comparison of up- and downregulated genes in Mediator tail mutants (8985 and 8977) versus their corresponding parental strain**

List of genes differently expressed (up/down) in the Mediator tail mutants (*MED15* and *MED2*) versus their corresponding parental strain (8985 or 8977) were compared using Venn diagrams. A) Genes significantly up- and downregulated in *MED15* null mutants of 8985 and 8977. B) Genes significantly up- and downregulated in *MED2* null mutants of 8985 and 8977. Differently expressed genes between Mediator tail mutants of 8985 and 8977 were shown in tables. All significantly up- or downregulated genes has a Log2 fold change > 1 and a p value < 0.05.

### 6.3.8 Regulation of adhesins by *MED2* and *MED15*

All differentially-expressed genes that might contribute to aggregation are listed in Table 6.4A and a heatmap illustrating their relative expression in parental *C. auris* strains and Mediator tail mutants is shown in Figure 6.3.5. *SCF1* was highly expressed in aggregative 8977 compared to non-aggregative 8985. Deletion of Mediator tail components resulted in decreased expression of *SCF1* in strain 8977, however deletion of Mediator tail in 8985 resulted in increased expression of *SCF1*. *AAF1*, a possible adhesin-like protein, was also downregulated in the Mediator tail mutants of 8977 and was highly expressed in WT 8977. Two GPI-anchored protein encoding genes, *FGR41* and *PGA38*, along with *ERG11*, were downregulated in both *med15Δ* 8977 and *med15Δ* 8985. *GPI15* was downregulated *med2Δ* 8985, *med15Δ* 8985 and *med15Δ* 8977. B9J08\_004498, a gene that might encode for an Als3p ortholog, was highly expressed in 8985 and downregulated due to the deletion of *MED2* and *MED15* in 8985. GPI-linked cell wall protein encoding gene *RBT5* and *WOR1*, a gene that might encode for transcription factor that regulate white-opaque switching, were expressed at lower levels in 8985, but were upregulated following the deletion of *MED2* and *MED15* in 8985.

Various heat shock proteins (HSP) were also upregulated due to the absence of Mediator tail components in 8985 and 8977 (Table 6.4B). *HSP70*, *HSP21* and *HSP104* were upregulated in *med2Δ* 8985 and *HSP70*, *HSP90*, *HSP104* and *SSA2* were upregulated in *med15Δ* 8985. *HSP12*, *HSP90*, and *SSA2* were upregulated in *med2Δ* 8977. Finally, *HSP12* and *HSP70* were upregulated in *med15Δ* 8977.

## RNA-seq based heatmap of expression of aggregation-related genes

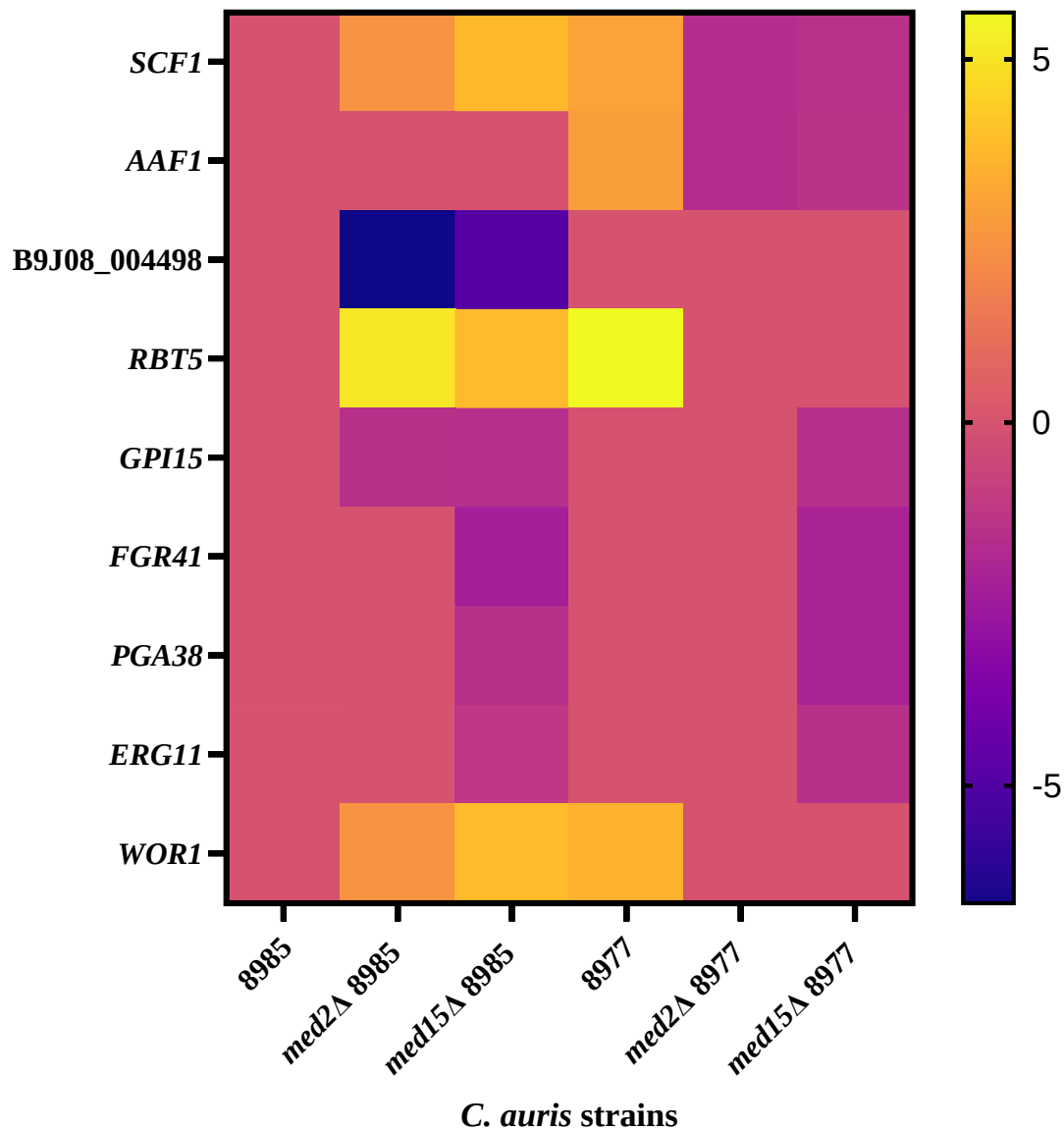


**Figure 6.3.5.** RNA-seq based heatmap revealing the expression of aggregation-related genes in *C. auris* strains

The differential expression of various genes that encodes adhesins or regulate cell wall components (log2 fold change) is displayed as a heatmap, in which 8977 was compared with 8985 and all Mediator tail mutants were compared to their corresponding parental strain. Basal gene expression was labelled in red. Bright yellow and orange colour indicate the upregulation of a specific gene whereas purple and dark blue indicate the downregulation of a specific gene.

Table 6.4. Important genes that are upregulated/downregulated in multiple *C. auris* strains

Table 6.4A. Genes that might contribute to *C. auris* aggregation phenotype

| <i>C. auris</i> gene names | Gene functions                                                                                                                                                                                   |
|----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                            | <b>Downregulated in <i>med2Δ</i> 8977, <i>med15Δ</i> 8977 but highly expressed in 8977</b>                                                                                                       |
| <b>B9J08_001458</b>        | <i>SCF1</i> (Ortholog of <i>Candida lusitanae</i> ATCC 42720 : CLUG_05295) Surface Colonization Factor                                                                                           |
| <b>B9J08_004831</b>        | ( <i>C. albicans</i> <i>AAF1</i> ) Possible regulatory protein; possible adhesin-like; Glu-rich domain; production in <i>S. cerevisiae</i> increases endothelial cell adherence and flocculence. |
|                            | <b>Highly downregulated in <i>med2Δ</i> 8985 and <i>med15Δ</i> 8985 but highly expressed in 8985</b>                                                                                             |
| <b>B9J08_004498</b>        | Putative cell-surface adhesin, similar to <i>C. albicans</i> Als3p                                                                                                                               |
|                            | <b>Highly upregulated in <i>med2Δ</i> 8985 and <i>med15Δ</i> 8985 but weakly expressed in 8985</b>                                                                                               |
| <b>B9J08_001458</b>        | <i>SCF1</i> (Ortholog of <i>Candida lusitanae</i> ATCC 42720 : CLUG_05295) Surface Colonization Factor                                                                                           |
| <b>B9J08_004027</b>        | ( <i>C. albicans</i> <i>WOR1</i> ) Transcription factor ("master switch") of white-opaque phenotypic switching in <i>C. albicans</i>                                                             |
| <b>B9J08_005545</b>        | ( <i>C. albicans</i> <i>RBT5</i> ) GPI-linked cell wall protein involved in hemoglobin utilization.                                                                                              |
|                            | <b>Downregulated in <i>med2Δ</i> 8985, <i>med15Δ</i> 8985 and <i>med15Δ</i> 8977</b>                                                                                                             |
| <b>B9J08_000749</b>        | ( <i>C. albicans</i> <i>GPI15</i> ) Ortholog of <i>S. cerevisiae</i> Gpa15: involved in the synthesis of glycosylphosphatidylinositol (GPI) anchors.                                             |
|                            | <b>Downregulated in <i>med15Δ</i> 8977 and <i>med15Δ</i> 8985</b>                                                                                                                                |
| <b>B9J08_000824</b>        | ( <i>C. albicans</i> <i>FGR41</i> ) Putative GPI-anchored adhesin-like protein involved in regulation of covering (masking) cell wall glucan.                                                    |
| <b>B9J08_001409</b>        | ( <i>C. albicans</i> <i>PGA38</i> ) Putative adhesin-like GPI-anchored protein.                                                                                                                  |
| <b>B9J08_001448</b>        | ( <i>C. albicans</i> <i>ERG11</i> ) Lanosterol 14- $\alpha$ -demethylase; cytochrome P450 family; role in ergosterol biosynthesis; target of azole antifungals.                                  |

**Table 6.4B. Heat shock protein encoding genes highly expressed in *C. auris* Mediator tail mutants.**

| <i>C. auris</i> strains   | Gene names                                                                                                                                                              |
|---------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b><i>med2Δ</i> 8985</b>  | B9J08_000483 ( <i>C. albicans HSP70</i> ), B9J08_000476 ( <i>C. albicans HSP104</i> ), B9J08_003627 ( <i>C. albicans HSP21</i> )                                        |
| <b><i>med15Δ</i> 8985</b> | B9J08_004918 ( <i>C. auris HSP90</i> ), B9J08_000483 ( <i>C. albicans HSP70</i> ), B9J08_000476 ( <i>C. albicans HSP104</i> ), B9J08_003364 ( <i>C. albicans SSA2</i> ) |
| <b><i>med2Δ</i> 8977</b>  | B9J08_004918 ( <i>C. auris HSP90</i> ), B9J08_001940 ( <i>C. albicans HSP12</i> ), B9J08_003364 ( <i>C. albicans SSA2</i> )                                             |
| <b><i>med15Δ</i> 8977</b> | B9J08_000483 ( <i>C. albicans HSP70</i> ), B9J08_001940 ( <i>C. albicans HSP12</i> )                                                                                    |

## 6.4 Discussion

This chapter focused on discovering if the Mediator tail components Med2 and Med15 in both weakly-aggregative and aggregative strains of *C. auris* strains could regulate genes that contribute to important virulence phenotypes, in particular aggregation, via RNA-sequencing.

### 6.4.1 Transcriptomics comparison of 8985 and 8977

The comparison between weakly-aggregative 8985 and aggregative 8977 revealed multiple differences in transcriptomes. The glycolytic genes *PFK1* and *PFK2* and multiple *HEM* genes involved in heme biosynthesis were more highly expressed in 8985, suggesting differences in glycolysis and heme biosynthetic process between the two strains. Multiple *ERG* genes (*ERG1*, *ERG6*, *ERG10*, *ERG25*) involved in the ergosterol biosynthesis pathway were highly expressed in 8985, suggesting possible membrane differences between 8985 and 8977. It is difficult to infer whether this could contribute to azole resistance in strain 8985 as both strains are highly resistant to azole drugs. Differences in susceptibility to calcofluor white and congo red were observed between strains 8985 and 8977, with strain 8977 exhibiting greater resistance to these cell wall damaging agents. Strain 8977 exhibited increased expression of the chitin deacetylase *CDA1* and the cell wall glucanase *PHR1* which could play a role in increasing cell wall strength. Two major genes that contribute to aggregation and biofilm formation in *C. auris*, namely *SCF1* (a recently discovered gene encoding for an adhesin that contributes to aggregation), and *AAF1* (a possible adhesin encoding gene), were highly expressed in 8977 relative to 8985. A homologue of *RBT5*, encoding a putative GPI-linked cell wall protein that might also contribute to cell wall stress resistance, aggregation, and biofilm formation, was also highly expressed in 8977. Conversely, another possible adhesin encoding gene, B9J08\_004498 (encoding an Als3p-like protein) was highly expressed in non-aggregative 8985 and could possibly play a role in adhesion to host cells.

As the Illumina reads generated here were aligned to the reference genome of *Candida auris* B8441, we could detect the ‘a’ type mating locus in strain 8985, but not strain 8977, suggesting that the latter strain is an “alpha” mating-type strain. Interestingly, *WOR1*, a gene encoding for transcriptional factor that regulates mating competence and white-opaque switching of *C. albicans*, was highly expressed only in 8977. In chapter 5, we observed a switching phenotype in strain 8977 on phloxine B plates, but further work would be required to determine if this is related to mating competence or Wor1 expression.

Finally, our expression data also confirms the finding of Biermann and Hogan (2022) which show that Clade III *C. auris* strains have a hyperactive copy of the transcription factor Mrr1 (Mrr1<sup>N647T</sup>), resulting in increased expression of *MDR1*, *MGD1* and *MGD2*. Strain 8977 partly shares this transcriptional pattern, as it exhibited increased expression of *MDR1* and *MGD1*. However, unlike the previous study, expression of the second methylglyoxal reductase encoded by *MGD2* was more highly expressed in 8985. This will be discussed further, below.

#### 6.4.2 Transcriptomic comparison of *med2Δ* and *med15Δ* Mediator tail mutants and their parental strains

The deletion of both *MED2* and *MED15* hugely affected the expression of various genes in *C. auris*. The deletion of *MED2* had a less severe impact compared to deletion of *MED15*. In both backgrounds, deletion of *MED2* impacted the expression of various ribosome-related encoding genes. We did not identify any other highly significant KEGG categories in the *MED2* mutants which suggests that *MED2* is less important than *MED2/TLO* in *C. albicans*. Deletion of *MED15* in both *C. auris* backgrounds affected a large number of KEGG categories which were similar in both strains. This suggests that deletion of *MED15* has a far greater impact on Mediator tail function in *C. auris*. Unlike *MED2*, the deletion of *MED15* caused the

downregulation of various genes involved in glycolysis/gluconeogenesis like *PFK1* and *PFK2*, suggesting *MED15* might play a major role in carbohydrate metabolism. Amino acid metabolism, nucleotide metabolism and fatty-acid metabolism were all perturbed in the *MED15* mutants and this is similar to the impact of *MED2/TLO* deletion in *C. albicans* (Fletcher *et al.*, 2023).

Both *MED15* mutants also exhibited decreased expression of *YHB1* and *RHR2*, genes involved in regulating nitric oxide sensitivity and sensitivity to osmotic stressors, respectively, indicating a role for *C. auris* Mediator tail in regulating stress responses, similar to *C. albicans*.

Deletion of *MED2* or *MED15* in the aggregative strain 8977 resulted in decreased expression of the gene *SCF1* which has been shown to be required for cellular aggregation in *C. auris*. A second putative adhesin, related to *C. albicans* *AAF1*, also required Mediator tail for expression in *C. auris* 8977. These results suggest that Mediator tail could ultimately regulate the aggregation and the biofilm formation capabilities of naturally aggregative *C. auris* strains. Conversely, deletion of *MED2* or *MED15* in the non-aggregating strain 8985 resulted in increased expression of *SCF1*. However, phenotypically, Mediator tail mutants of 8985 did not exhibit increased aggregation, as was observed in a recent study on *Scf1* by Santana *et al.* (2023) who demonstrated that the overexpression of *SCF1* in a weakly-aggregative clade I *C. auris* strain could provide enhanced aggregation and biofilm formation capability to that strain. In addition to the upregulation of *SCF1* in the 8985 mutants, several putative adhesin encoding genes were strongly downregulated as well, including B9J08\_004498, which could counteract the enhanced aggregation and biofilm formation capability provided by the high expression of *SCF1*. Several additional GPI-anchored proteins were specifically down-regulated in both *MED15* mutants including orthologues of *FGR41* and *PGA38* which could also compromise

the aggregative phenotypes of the mutants. Moreover, in *med2Δ* 8985, we observed decreased expression of the putative adhesin regulator *MSB1* and the mucin-like adhesin, *MSB2*, which could both compromise the aggregation of *med2Δ* 8985.

We previously noted how the Mrr1 regulated genes *MGD1* and *MDR1* were more highly expressed in 8977. Reduced expression of both genes was observed in *med15Δ* 8977 (although *MDR1* was not in the top 20 downregulated genes). Conversely, expression of *MGD2*, which was highly expressed in 8985, was reduced in strains *med2Δ* 8985 and *med15Δ* 8985 and this was associated with upregulation of *MGD1*. These data suggest the Mediator tail is required for clade-specific activity of the *MGD/MDR* gene regulator Mrr1. In this situation, it appears that Mediator tail is acting as a co-activator of the Mrr1 regulated genes *MDR1* and *MGD1* in 8977, whereas in 8985 it appears to repress *MGD1* and is required for expression of the second gene *MGD2*.

The deletion of the two Mediator tail components in both 8985 and 8977 activates the expression of various heat shock protein encoding genes, suggesting that these Mediator tail mutants were under stress when Mediator tail components were absent. However, no obvious growth defect was observed in all Mediator tail mutants likely due to the nature of *C. auris* being highly resistance towards various stressors.

The expression of *WOR1* is not affected by the deletion of the two Mediator tail components in 8977. However, upregulation of *WOR1* was observed following the deletion of both Mediator tail components in 8985, although no obvious cell morphology changes were observed. There is still a possibility that the regulation of *WOR1* might affect the switching rate of 8985 from yeast cells to filamentous cells after passaging through mice, or other underlying phenotypic

switching events as *WOR1* was shown to be downregulated in *C. auris* filamentous cells compared to yeast cells (Yue *et al.*, 2018).

#### 6.4.3 Summary and possible future work

In conclusion, both Med2 and Med15 are required for the gene expression of *SCF1* that confers the aggregation ability and biofilm formation on hydrophobic surface of *C. auris*. Mediator tail components could also mediate the expression of another putative adhesin encoding gene *AAF1* that might contribute to the cell-cell adhesion of aggregative *C. auris* strains as well. The deletion of Mediator tail components, especially *MED15*, drastically changed the cell wall components of both 8985 and 8977, but in a different manner. Interestingly, *SCF1* and another putative GPI-linked cell wall protein encoding gene *RBT5* were upregulated in weakly-aggregative 8985 without Med2 and Med15 but did not show enhanced aggregation and biofilm formation possibly due to the downregulation of other putative adhesins (e.g. Als3p encoding gene B9J08\_004498). It appears that many of the strain-specific patterns of gene expression observed in strains 8985 and 8977 requires Mediator to be maintained (Figure 6.4.1). These genes may play central roles in the virulence attributes of *C. auris*, making Mediator a possible antifungal target.

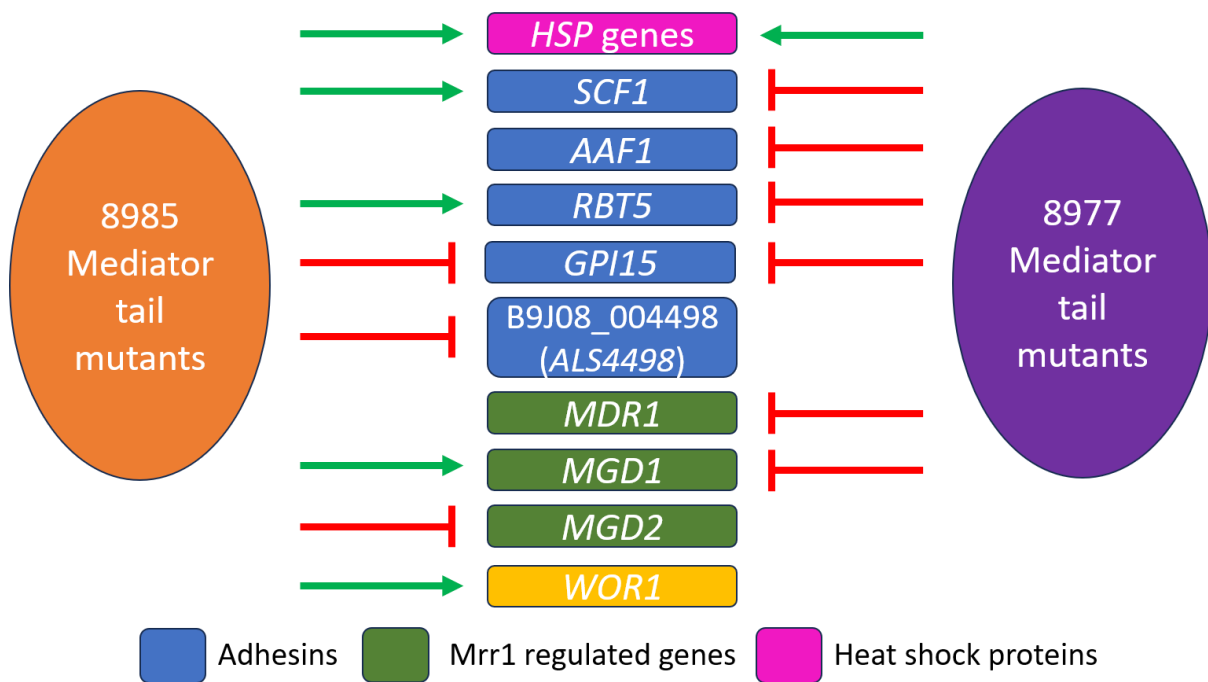


**Figure 6.4.1. Summary of important genes that are highly upregulated/downregulated due to the deletion of Mediator tail components (*MED2* and *MED15*) in 8985 and 8977**

A summary of important virulence-related genes that were highly upregulated (green arrows) or downregulated (red bars) due to the deletion of Mediator tail components in both weakly-aggregative 8985 and high-aggregative 8977.

## **Chapter 7**

### General discussion

## 7.1 General discussion

Mediator plays a central role in the activation of gene expression in Eukaryotes. The complex can facilitate the activity of DNA-bound transcriptional activators and can activate or repress gene expression, particularly when the repressive CDK8 module is present. This study aimed to investigate the role of this complex in the regulation of drug susceptibility in *Candida* species, in particular *C. albicans* and *C. auris*.

The role of Mediator in *C. albicans* is of particular interest due to the presence of the large *TLO* gene family, encoding for a Med2 ortholog. Previously, Tlos of the  $\alpha$  (*TLO $\alpha$ 1*) and  $\beta$  (*TLO $\beta$ 2*) subfamilies were shown to be involved in various virulence-related phenotypes of *C. albicans*. Both *TLO $\alpha$ 1* and *TLO $\beta$ 2* significantly restore the growth, virulence, and stress tolerance defects in a *C. albicans tlo $\Delta$*  mutant (Fletcher *et al.*, 2023). The RNA-sequencing analysis of *C. albicans* further revealed the downregulation of *CDR1* in *tlo $\Delta$*  (Fletcher *et al.*, 2023). Based on all these findings, one of the major goals of this study is to find out if *TLO $\alpha$ 1* and/or *TLO $\beta$ 2* are required for hyperactivated Tac1 activated *CDR1* expression and azole resistance, as shown for the other Mediator tail components, Med3 and Med15 (Liu and Myers, 2017b). The terbinafine resistance activated by Tac1 was also investigated. To conduct this study, CRISPR-Cas9 mutagenesis was used to introduce *TAC1-5* point mutations into WT, *tlo $\Delta$*  and *med3 $\Delta$*  null mutants and later *TLO $\alpha$ 1* and *TLO $\beta$ 2* were reintroduced into *tlo $\Delta$*  with the *TAC1-5* point mutation.

Our study shows that the absence of *TLO* genes compromises the activity of the hyperactive *TAC1-5* allele resulting in lower *CDR1* expression and a 2-fold decrease in fluconazole and terbinafine MIC. The *TAC1-5* allele still imparts some azole resistance to the *tlo $\Delta$*  mutant, suggesting that it may act to reduce fluconazole susceptibility using a mechanism that does not

involve regulation of *CDR1* mRNA. It also suggests that Cdr1 overexpression could be under translational control by a mechanism dependent on *TAC1*<sup>GOF</sup> or that other genes are regulated by Tac1 in a Mediator independent mechanism. RNAseq analysis of the impact of *TAC1-5* on gene expression in the *tloΔ* mutant could reveal this mechanism. The result further revealed that an antifungal targeting the Med2 protein encoded by *TLOs* might have lower potency in *C. albicans* compared to the inhibitor that targets *C. glabrata* Pdr1-Med15 interactions (Nishikawa *et al.*, 2016).

The reintroduction of *TLOα1* and *TLOβ2* into *tloΔ-TAC1-5* was carried out to find out which Tlo interacts with hyperactive Tac1 to overexpress *CDR1* and ultimately confer fluconazole and terbinafine resistance in *C. albicans*. qRT-PCR analysis showed that the mRNA expression of *TLOα1* is normal after being reintroduced into *tloΔ-TAC1-5* and fluconazole E-test assays revealed that *TLOα1* almost fully restored the Tac1-activated fluconazole resistance of *tloΔ TAC1-5*. Chip-qPCR revealed that Tloα1 interacts with hyperactive Tac1 at two promoter regions of *CDR1*, *CDR1* 1-3 and *CDR1* DRE, of which *CDR1* DRE is known to be bound by Tac1. This confirmed that *TLOα1* is required for Tac1-activated *CDR1* expression and ultimately confers fluconazole and terbinafine resistance. However, it remains unclear if *TLOα1* directly binds to these two *CDR1* promoter sites or binds indirectly to them via Tac1. As *TLOα1* is only 1 of 5 alpha *TLO* genes in *C. albicans*, it would be interesting to determine if other alpha Tlo proteins have this activity or whether deletion of individual alpha Tlos could affect the azole susceptibility of azole resistant *C. albicans* strains.

The most unexpected finding of this study was the potentially antagonistic relationship between *TAC1-5* and *TLOβ2*. The mRNA expression of *TLOβ2* was low after being reintroduced into the *tloΔ TAC1-5* background and Western blotting analysis further showed

that Tlo $\beta$ 2 protein was weakly expressed in *tlo* $\Delta$ -*TAC1-5* + *TLO* $\beta$ 2. This suggests that *TAC1-5* downregulates *TLO* $\beta$ 2 mRNA expression but not *TLO* $\alpha$ 1 in *C. albicans*, suggesting that hyperactive Tac1 isoforms interact differently with individual *TLO* genes and could downregulate specific *TLO* genes like *TLO* $\beta$ 2 directly/indirectly. As *TLO* $\alpha$ 1 and *TLO* $\beta$ 2 are expressed from the same pTET promoter in these experiments, it is unlikely that *TAC1-5* interacts with the promoter, as both genes would be affected. Other *TLO* $\beta$ 2-associated phenotypes were also defective, as expression of *TLO* $\beta$ 2 in *tlo* $\Delta$  *TAC1-5* did not induce significant hypha formation in YEPD as was observed when *TLO* $\beta$ 2 is introduced into the normal *tlo* $\Delta$  mutant. Despite having low mRNA and protein expression, the reintroduction of *TLO* $\beta$ 2 partially restored fluconazole resistance in *tlo* $\Delta$  *TAC1-5*. This suggests that Tlo $\beta$ 2 might interact with other underlying antifungal mechanisms to restore fluconazole resistance. An antagonistic relationship between *TLO* $\beta$ 2 and *TAC1-5* was further confirmed as the presence of *TLO* $\beta$ 2 could reverse *TAC1-5* activated terbinafine resistance in the *tlo* $\Delta$  mutant. It is possible that Tlo $\beta$ 2 could antagonise the function or repress the expression of *TAC1-5* which may explain the effects on terbinafine susceptibility. It would be interesting to investigate the expression of *TAC1-5* in these strains by qRT-PCR or investigate Tlo $\beta$ 2-Tac1-5 protein-protein interactions by co-IP. Further investigations of *TLO* $\beta$ 2 expression in wild-type fluconazole susceptible and resistant clinical strains would also be interesting to determine if *TLO* $\beta$ 2 is expressed at lower levels in resistant isolates to facilitate hyperactive Tac1 activity.

Overall, this study revealed that Tlo $\alpha$ 1 is the major Med2 component that interacts with Tac1 at the *CDR1* promoter site to initiate *CDR1* overexpression and confers fluconazole and terbinafine resistance to *C. albicans*. Future co-IP work or tagging of Tac1p could be carried out to gain more understanding on how Tlo $\alpha$ 1 interacts with Tac1 to overexpress *CDR1*.

Furthermore, small molecule screening studies, similar to those that have been carried out in *C. glabrata* (Waseem *et al.*, 2023), to identify novel inhibitors of Tlo $\alpha$ 1 should be performed to discover novel Tlo $\alpha$ 1 inhibitors that synergize with azole drugs to treat patients infected by azole resistant *C. albicans*.

As Mediator has been shown to play a major role in azole susceptibility in *C. albicans* and *C. glabrata*, the other major aim of this study was to determine the function of *MED2* and *MED15* in *C. auris*. To conduct this study, CRISPR-Cas9 mutagenesis was used to delete *MED2* and *MED15* in strain 8985 (Clade I) and strain 8977 (Clade III). Both strains were highly resistant to azole drugs and deletion of *MED2* or *MED15* had little impact on azole susceptibility and minor effects on caspofungin susceptibility in 8977. Despite little to no changes in azole and caspofungin susceptibility in 8977 and 8985, *MED2* and *MED15* still might have a role in the azole/caspofungin resistance pathway as both multidrug resistance strain 8977 and 8985 might have various azole/caspofungin resistance pathways that stops them from being susceptible to both antifungals when *MED2/MED15* was absent.

Further investigations showed that *MED2* and *MED15* had a major role in regulating another major phenotype in *C. auris*, namely cellular aggregation. Microscopic imaging revealed that both the Mediator tail mutants of the aggregative strain 8977 seemed to have a defect in PBS-induced aggregation whereas the Mediator tail mutants of 8985 did not show any significant changes. However, analysis revealed that *med2* $\Delta$  8985 and *med15* $\Delta$  8985 exhibited lower aggregation compared to WT 8985 after exposure to echinocandin drugs. All these findings suggest that Mediator tail components were required for the PBS-induced aggregation of aggregative strain 8977 and the echinocandin-induced aggregation of strain 8985. Biofilm formation assays showed that 8977 can form significantly higher levels of biofilm mass on

hydrophobic surface compared to 8985 and that this also required *MED2* and *MED15*. We also observed differences in susceptibility to cell wall damaging agents, with strain 8985 exhibiting increased susceptibility to calcofluor white and congo red compared to 8977. However, the deletion of Mediator Tail components, especially *MED15* in 8985, decreased its susceptibility to both cell wall stressors (Fig. 5.3.3) and this was associated with a visible change in colony morphology on phloxine B plates (Fig. 5.3.9). All of these findings suggest that Mediator tail components regulate cell wall components differently in Clade I and Clade III *C. auris* strains and *MED15* seemed to have an important role in regulating cell wall component encoding genes. Interestingly, the deletion of Mediator tail components in 8985 did not affect its resistance towards both fluconazole and caspofungin. The deletion of Mediator tail components in 8977 did not affect the fluconazole resistance of 8977 but did slightly reduce the caspofungin susceptibility of 8977, suggesting the presence of cell wall changes contributing to this outcome.

Colony morphology switching assays revealed that the aggregative strain 8977 could switch between aggregative and weakly-aggregative forms when grown at a lower temperature (30°C or 25°C) for several days. On phloxine B plates, white colonies maintained the aggregative phenotype and pink (opaque) colonies were weakly-aggregative. Although *med2Δ* 8977 showed white-opaque switching like WT 8977, *med15Δ* 8977 did not, suggesting that the deletion of *MED15* in aggregative strains might inhibit/reduce the switching from aggregative to non-aggregative types. The ‘pink’ non-aggregative cells of both 8977 and *med2Δ* 8977 were able to switch back to ‘white’ aggregative forms at a visible rate when grown on phloxine B plates at 25°C for several days. The presence of glycerol did not induce the formation of filamentous cells by non-aggregative opaque cells of 8977, suggesting that changing from glucose to glycerol could not activate the morphological switching of *C. auris* like its close relative *C. haemulonii*.

Analysis of our RNA-Seq data suggests that the phenotypic differences between the Clade I strain 8985 and the Clade III strain 8977 are likely caused by transcriptional rewiring events that are facilitated by the Med2/Med15 complex in Mediator tail. Ergosterol biosynthetic genes were found to be highly expressed in 8985, suggesting differences in cell membrane composition between 8985 and 8977. *ERG3* and *ERG11* were also downregulated in *med15Δ* 8985 showing that Mediator was required for this pattern of gene expression. *WOR1* (white-opaque switching regulator of *C. albicans*) was highly expressed in the “switching” strain 8977 but was Mediator repressed in strain 8985. All mediator tail mutants of 8985 and 8977 showed high expression of *WOR1* which might correlate with the yeast cell morphology of all these strains as filamentous *C. auris* cells have low expression of *WOR1* (Yue *et al.*, 2018). There is a possibility that all Mediator tail mutants of 8985 and 8977 might be unable to form filamentous cells after passaging through mouse model.

We also observed rewiring of Mrr1 regulated genes in 8985 and 8977, with high-level *MDR1* and *MGD1* expression in strain 8977 which was dependent on Mediator tail. Conversely, in strain 8985, we observed expression of the alternative gene *MGD2* and repression of *MGD1*, both of which were Mediator-dependent expression patterns. This difference may be due to the presence of hyperactive forms of Mrr1 in Clade III strains. Although, fluconazole susceptibility was high in all Mediator tail mutants, it would be interesting to investigate susceptibility to methylglyoxal (MG) in the parents and mutants. MG has been shown to accumulate at sites of infection and MG resistance has been linked to resistance to neutrophil killing (Biermann and Hogan, 2022).

Apart from that, we observed significant rewiring of adhesins and putative-adhesins. Differences in aggregation phenotypes appears to be related to expression of adhesin encoding

genes like *SCF1* and the putative adhesin encoding gene *AAF1*, which were highly expressed in 8977. The deletion of *MED2* and *MED15* in 8977 significantly downregulated the expression of *SCF1* and *AAF1*, which likely explains why the Mediator tail mutants of 8977 showed low aggregation and biofilm formation capability on hydrophobic surfaces. However, the deletion of *MED2* and *MED15* in 8985 caused the upregulation of *SCF1* and another putative GPI-linked cell wall protein encoding gene *RBT5*. The putative Als3p adhesin encoding gene was highly downregulated in 8985 due to the deletion of Mediator tail components. Like the case of Mrr1, the difference in adhesin gene expression between 8977 and 8985 may be due to a transcriptional rewiring event involving a “master-regulator” of adhesin encoding genes, which may require Mediator for function. Overall, this study highlights the transcriptional flexibility of *C. auris* and the overarching requirement for Mediator to execute these Clade-specific patterns of gene expression. It would be interesting to determine the virulence of these Mediator mutants in *Galleria mellonella* or murine models of infection. If the *in vivo* virulence properties of these strains have been attenuated, it may make a case for the development of a Mediator inhibitor as a novel antifungal.

## 7.2 Conclusions and future directions

This work has revealed that Tlo $\alpha$ 1, one of the components of Mediator tail, is required for Tac1-activated *CDR1* expression in *C. albicans*. The Tlo proteins may regulate other genes required for antifungal susceptibility. Studies to investigate the relationship between *TLO* genes and *UPC2*, a transcription factor involved in ergosterol biosynthesis in *C. albicans*, would also be interesting as RNA-sequencing data showed that *UPC2* was downregulated in *tlo* $\Delta$  (Fletcher *et al.*, 2023). The behaviour and expression of different *TLO* genes in clinical isolates should also be investigated, in particular due to the antagonistic behaviour of Tlo $\beta$ 2 which could be a negative regulator of Tac1 activity. Due to the multiple phenotypes associated with *TLO*

deletion, a small molecule Tlo inhibitor could synergize with azole drugs to treat azole resistant *C. albicans*.

In addition to this, the study also showed that *MED2* and *MED15* mediates the expression of *Scf1*, which is the major contributor to the aggregation and biofilm formation capabilities of aggregative *C. auris* strains. This study highlights the importance of transcriptional rewiring in *C. auris* and identified many candidate adhesins for investigation in future studies (e.g. *AAF1*, *ALS3*-like gene). The suggestion that these adhesins have a “master-regulator” also warrants further studies. Perhaps ChIP of *Med15* or *Med2* could be used to identify adhesin genes and regulatory sequences required for the function of this putative regulator.

## Appendix 1 – Quality statistics for RNA sequencing

Appendix 1 Table 1. Quality statistics of RNA sequencing samples

| Sample name       | Raw reads | Raw bases | Error rate (%) | Q30 (%) | GC content (%) |
|-------------------|-----------|-----------|----------------|---------|----------------|
| <b>WT_8985_1</b>  | 50562702  | 7.58      | 0.03           | 94.24   | 48.02          |
| <b>WT_8985_2</b>  | 57205962  | 8.58      | 0.02           | 94.69   | 48.43          |
| <b>WT_8985_3</b>  | 50871326  | 7.63      | 0.02           | 94.64   | 48.47          |
| <b>M2_8985_1</b>  | 50007996  | 7.5       | 0.02           | 94.44   | 48.36          |
| <b>M2_8985_2</b>  | 50390346  | 7.56      | 0.02           | 94.43   | 47.95          |
| <b>M2_8985_3</b>  | 36245244  | 5.44      | 0.03           | 93.01   | 48.02          |
| <b>M15_8985_1</b> | 46147026  | 6.92      | 0.03           | 93.28   | 47.78          |
| <b>M15_8985_2</b> | 58174438  | 8.73      | 0.02           | 94.35   | 47.62          |
| <b>M15_8985_3</b> | 51799794  | 7.77      | 0.02           | 94.54   | 47.87          |
| <b>WT_8977_1</b>  | 53272528  | 7.99      | 0.02           | 94.63   | 48.01          |
| <b>WT_8977_2</b>  | 54625820  | 8.19      | 0.02           | 94.58   | 48.11          |
| <b>WT_8977_3</b>  | 45561344  | 6.83      | 0.02           | 94.25   | 48.13          |
| <b>M2_8977_1</b>  | 45789918  | 6.87      | 0.02           | 94.66   | 48.1           |
| <b>M2_8977_2</b>  | 51186984  | 7.68      | 0.02           | 94.54   | 47.8           |
| <b>M2_8977_3</b>  | 55967226  | 8.4       | 0.02           | 94.63   | 48.01          |
| <b>M15_8977_1</b> | 48046792  | 7.21      | 0.02           | 94.45   | 47.71          |
| <b>M15_8977_2</b> | 47565460  | 7.13      | 0.03           | 93.54   | 47.69          |
| <b>M15_8977_3</b> | 48408254  | 7.26      | 0.03           | 94.23   | 47.62          |

**Appendix 1 Table 2. Mapping statistics from RNA-seq read alignment**

| Sample name       | Total reads | Total mapped reads | Total mapping rate | Uniquely mapping rate | Multiple mapping rate |
|-------------------|-------------|--------------------|--------------------|-----------------------|-----------------------|
| <b>WT_8985_1</b>  | 50522040    | 45405786           | (89.87%)           | (88.73%)              | (1.14%)               |
| <b>WT_8985_2</b>  | 52802208    | 50636867           | (95.9%)            | (94.07%)              | (1.83%)               |
| <b>WT_8985_3</b>  | 47305840    | 45007081           | (95.14%)           | (92.59%)              | (2.55%)               |
| <b>M2_8985_1</b>  | 47892058    | 44191850           | (92.27%)           | (89.87%)              | (2.4%)                |
| <b>M2_8985_2</b>  | 47546314    | 43679718           | (91.87%)           | (86.02%)              | (5.85%)               |
| <b>M2_8985_3</b>  | 36216808    | 33090003           | (91.37%)           | (89.25%)              | (2.12%)               |
| <b>M15_8985_1</b> | 46110056    | 42484574           | (92.14%)           | (90.42%)              | (1.71%)               |
| <b>M15_8985_2</b> | 55323910    | 52050271           | (94.08%)           | (90.84%)              | (3.24%)               |
| <b>M15_8985_3</b> | 51768198    | 47448178           | (91.66%)           | (89.51%)              | (2.15%)               |
| <b>WT_8977_1</b>  | 48717152    | 44395549           | (91.13%)           | (87.5%)               | (3.63%)               |
| <b>WT_8977_2</b>  | 49516712    | 44963025           | (90.8%)            | (88.51%)              | (2.3%)                |
| <b>WT_8977_3</b>  | 45530224    | 41637529           | (91.45%)           | (89.28%)              | (2.17%)               |
| <b>M2_8977_1</b>  | 45764820    | 42198720           | (92.21%)           | (90.73%)              | (1.48%)               |
| <b>M2_8977_2</b>  | 51159856    | 46030484           | (89.97%)           | (88.44%)              | (1.54%)               |
| <b>M2_8977_3</b>  | 55938204    | 50877339           | (90.95%)           | (89.09%)              | (1.86%)               |
| <b>M15_8977_1</b> | 45095914    | 40723396           | (90.3%)            | (86.88%)              | (3.43%)               |
| <b>M15_8977_2</b> | 47531810    | 42638112           | (89.7%)            | (86.36%)              | (3.35%)               |
| <b>M15_8977_3</b> | 44112192    | 40033855           | (90.75%)           | (88.04%)              | (2.72%)               |

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