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TOKYO INSTITUTE OF TECHNOLOGY

Doctoral Thesis

Study on *Candida albicans* adhesion in human oral cavity

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ABSTRACT

Objectives: *Candida albicans* is an opportunistic human pathogen that causes oral candidiasis. The adhesion of this fungus to saliva-coated surfaces is an important early step in the infection process of human oral cavity. A previous study has shown that the *C. albicans* cell wall proteins Bgl2 and Ecm33 may mediate the interaction between the yeast and hydroxyapatite via saliva proteins. This interaction is an initial event in the colonization of the oral cavity, which leads to oral diseases such as candidiasis and denture stomatitis. The aim of this research was to investigate the roles of these two cell wall proteins in the adherence of *C. albicans* to saliva-coated hydroxyapatite as a model for the tooth surface.

Methods: *C albicans BGL2* and *ECM33* null mutants were generated from wild-type strain SC5314 by using the *SAT1*-flipper gene disruption method. A novel method for measuring fungal adherence, based on labeling the yeast with the fluorogenic compound Nile Red (NR), was used to investigate the adherence of *C. albicans*. Hydroxyapatite beads coated with various proteins were incubated with *C. albicans* cells to determine the adhesion of the yeast to the beads, and then the cells attached to beads were stained with NR. *C. albicans* gene expression was measured by qRT-PCR.

Results: The adhesion of the *bgl2*Δ and *ecm33*Δ null mutants to saliva-coated hydroxyapatite beads was 76.4 % and 64.8% of the wild-type strain, respectively. Interestingly, the adhesion of the *bgl2*Δ*, ecm33*Δ mutant (87.7 %) was higher than that of either single mutant. qRT PCR analysis indicated that the *ALS1* gene was over-expressed in the *bgl2*Δ*,ecm33*Δ strain. This result suggested that the double mutant had developed a compensatory response to reduced cell wall integrity. Therefore, I disrupted all three genes (*bgl2*Δ*, ecm33*Δ*, als1*Δ) in *C. albicans* and the triple null mutant shoId a significantly reduced adherence to the beads, (37.6 %) compared to the wild-type.

Conclusion: This study has demonstrated that the fluorescent compound NR can be used to measure *C. albicans* adherence and the two cell wall proteins Bgl2 and Ecm33 contribute to interaction between *C. albicans* and saliva-coated surfaces. In addition, I propose that *ALS1* gene is associated with the compensatory responses in the fungal cells due to the deletion of both *BGL2* and *ECM33*.

Keywords: *C. albicans*; cell wall protein, cell adhesion, *BGL2, ECM33, ALS1,* saliva, tooth enamel

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ABBREVIATION

Als	Agglutinin-Like-Sequence
AFR	Amyloid-Forming Region
CV	Crystal Violet
CWPs	Cell wall proteins
ECM	Extracellular Matrix
RT-qPCR	Reverse Transcriptase quantitative PCR
YPD	Yeast Extract–Peptone–Dextrose Medium
NT	N-terminal domain
NTC	Nourseothricin
TR	Tandem repeats
PBC	Peptide-Binding Cavity

FIGURES AND CHARTS

TABLES

CHAPTER 1. RESEARCH BACKGROUND

1.1 INTRODUCTION

Candida spp. are opportunistic pathogens and some of the most common causes of fungal infections of humans.[1] Although they are considered harmless commensal organisms in healthy people, in immunocompromised individuals such as those with cancer, or who have undergone repeated surgery or the introduction of intravenous catheters, these fungi can become pathogenic. They can cause a variety of mucosal and systemic infections, including pseudomembranous candidiasis, erythematous candidiasis, hyperplastic candidiasis, and candidemia, which can be fatal.[2–4] *Candida albicans* is the most frequent cause of oral candidiasis[5] and possesses several factors and specific abilities that contribute to its high pathogenicity. These include polymorphism, the yeast-to-hypha morphological transition and biofilm formation. Figure 1-1 shows *C. albicans* biofilms formation in a sequential process including adhesion to host surfaces, induction of hyphal growth, production of extracellular matrix material, and finally dispersion of yeast cells from the biofilm complex.[6,7] *Candida* cells in a biofilm matrix exhibit high resistance to antimicrobial drugs, and are hidden from the host immune system, and this contributes to the virulence of this fungus.

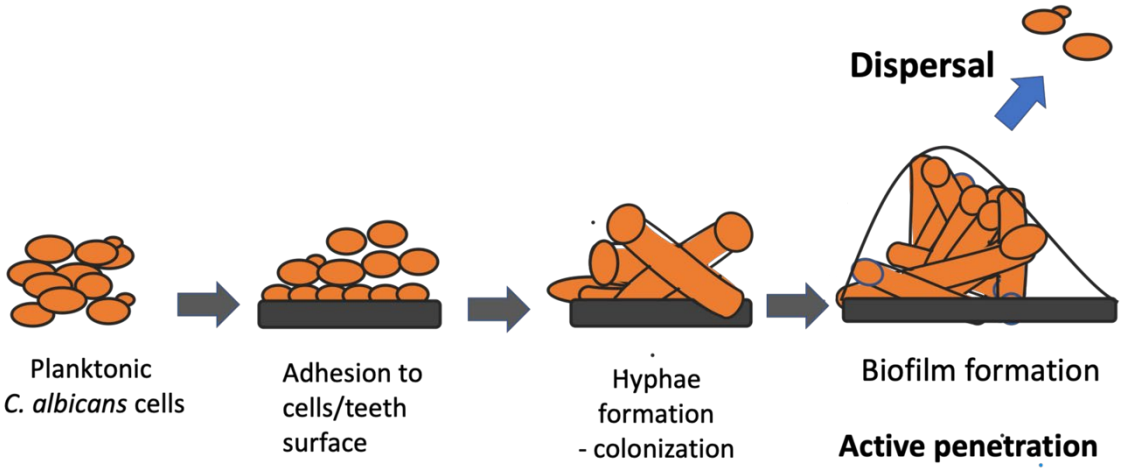

Figure 1-1: General steps in invasion process of *C. albicans*

C. albicans possesses many surface proteins involved in adhesion that bind to host receptors. These include the ALS protein family, the Sap protein family, Hwp1, Ywp1, Pra1, and Csh1 which have been well studied.[8–13] In addition, surface mannoproteins, which constitute the outer layer of the cell wall, are thought to be major candidates for putative adhesins. Therefore, it is important to investigate the functions of cell wall mannoproteins in order to understand the adhesion mechanisms, biofilm formation and virulence of *C. albicans*.[3,7,14,15]

1.2 Cell wall proteins research

The cell wall has two critical roles: (1) to maintain and protect the integrity of the cell and (2) to interact with the outer environment. The cell surface is also the contact point between *C. albicans* cell and the host surfaces including mucosal surfaces, or medical devices.[8] Because of this important role, for *C. albicans* only, the cell wall has attracted a consistent focus of attention over the last several decades. Keyword searches for "*C. albicans* cell wall", "*C. albicans* adhesion" in Pubmed (https://www.ncbi.nlm.nih.gov/pubmed/) showed that the number of publications on this topic increased by over 50% in the decade between 2000-2019 (Figure 1-2). One area that has consistent focus is research on *C. albicans* biofilm due to the availability of DNA sequences from Genomic Databases and the study of *C. albicans* cell wall proteins is increasing by utilizing a number of tools.[8]

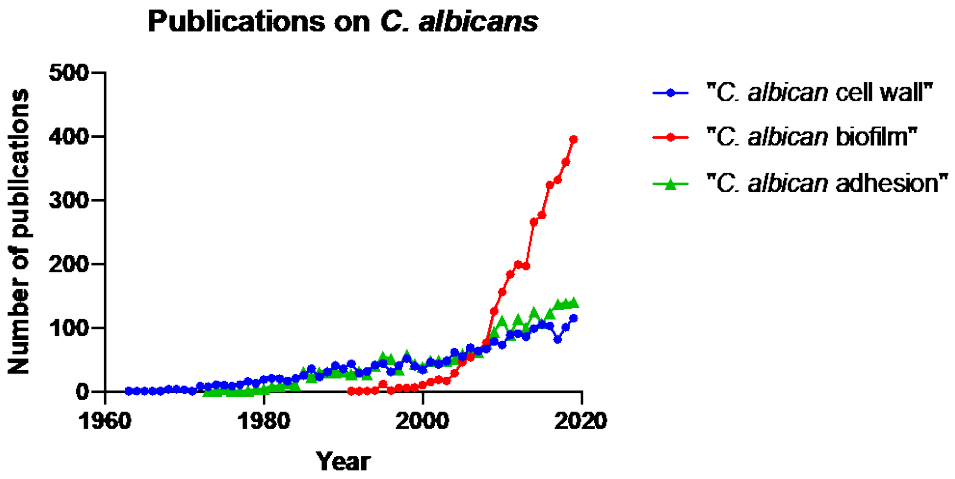

Figure 1-2: Publications on *C. albicans* from 1964-2019.

The cell wall is a specific and complex structure comprised of glucans, chitin, chitosan, and glycosylated proteins. These components contribute to the cell wall rigidity, and its relationship with virulence, pathogenicity and the interaction with the host's systems.[16] Therefore, in the following sections, the different components of the *C. albicans* cell wall will be discussed generally.

1.3 Cell wall structure

The fungal cell wall locates outside the plasma membrane and mediates all the relationships of the cell with the environment. Therefore, the cell wall allows interaction with the external environment since some of cell wall proteins are adhesins and receptors.[16]

The *C. albicans* cell wall contains 90% carbohydrate and 10% protein, Figure 1-3 showed electron microscopy of thin sections of the *C. albicans* cell wall which is comprised of differential abundances of cell wall constituents.[17] There are two main layers in *C. albicans* structure which has different characteristics: (i) outer layer which is comprised of mannan and cell wall proteins; and (ii) the inner layer which is mostly enriched of chitin and polysaccharide matrix.

The *C. albicans* inner cell wall consist of a network including primarily branches of β-1,3- and β-1,6-glucans, chitin and mannoproteins.

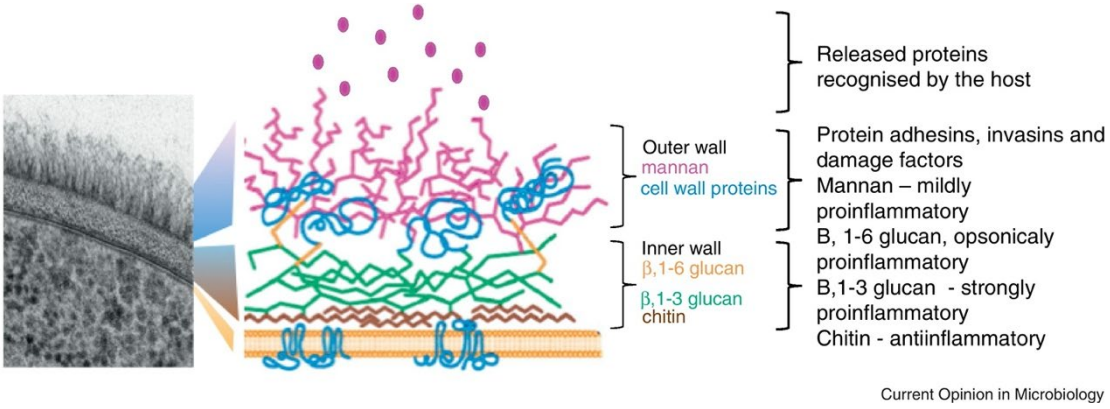

Figure 1-3: Transmission electron micrograph showed sections of the *C. albicans* cell wall and a schematic representation of the major components and the role of those components in immune activation and suppression.[17]

1.3.1 *Glucan*

Glucan is the most important structural polysaccharide of the *C. albicans* cell wall and represents 50-60% of the dry weight of this matrix. The *C. albicans* inner cell wall constructs a network composed primarily branches of β-1,3- and β-1,6-glucans, to which other components of this structure are covalently linked. The β-1,3 – glucans is synthesized by a number of enzymes located in the plasma membrane named glucan synthases.

The β-1,6-glucans are side chains of variable lengths and distributions, they can form complex structures stabilized by interchain hydrogen bonds. They act as a linking components binding different cell wall proteins to the β-1,3-glucan chitin core through glycosylphospatidyl inositol (GPI) proteins.[18]

1.3.1.1. Bgl2

In fungal cell wall, major polysaccharide components are glucans (including β-1,3 glucan and β-1,6-glucan). As can be seen on Figure 1-3, glucan is synthesized as

linear polymer, therefore, many enzymes are required to form the branching of polysaccharide from the linear polymer. Bgl2 is a manno protein from the surface of *C. albicans* and was identified as major β-1,3-glucosyltransferase.[8] Bgl2 is categorized as non-covalent and secreted protein in *C. albicans* cell wall proteins matrix. Bgl2 has substrates within the cell wall and remain primarily cell associated.[8]

Since Bgl2 is a β-1,3-glucosyltransferase and a highly conserved cell wall protein, disruption of the *C. albicans BGL2* gene interferes cell wall integrity, and the mutant also became attenuated in virulence for mice when compared to its parent strain.[19] Chen *et al.* investigated the morphological role of Bgl2p during the biofilm formation and found that after 24h of cultivation, both the wild-type SC5314 and the *bgl2Δ/bgl2Δ* mutant strains exhibited similar amount of biofilm. However, while wild-type strain composed of long filamentous cells and few yeast cells, the *bgl2Δ/bgl2Δ* mutant showed excess numbers of yeast cells in addition to some filamentous cells. This studied demonstrated a delay in the transition to filamentous form in the *bgl2Δ/bgl2Δ* mutant during initial step of biofilm formation. Further screening the expression level of some well-known transcription factors associated with hypha-associated genes showed that the deletion of *BGL2* gene stimulates a repression of the *CPH2/TEC1* pathway and leads to a delay in the transition of filamentous cells during biofilm formation.[7,20]

1.3.2 *Chitin*

The chitin content of the cell wall varies according to the morphological phase of the yeast. It represents 1-2% of the dry weight of yeast cell wall while in filamentous form, it can reach up to 10-20%. The chitin content in the *C. albicans* hyphae wall is three times higher than that of yeast.[21] Chitin is synthesized from n-acetylglucosamine by chitin synthase, which deposits chitin polymers in the extracellular space. Chitin chains can form tight antiparallel hydrogen-bonded structures associated with high insolubility, cell shape, viability and integrity.[8]

Chitin plays an important role in the interaction of *C. albicans* with the host. Chitin-deficient mutant strains showed attenuated virulence in the immunocompetent and immunosuppressed hosts[22,23].

Chitin is important for caspofungin resistance in some *Candida* species, especially *C. albicans.* For example, strains with increased levels of chitin in the *C. albicans* cell wall had higher resistance to echinocandin and showed a systematic *in vivo* infection model of candidiasis.[16,24]

1.3.3 *Glycoproteins*

As shown in Figure 1-3, the outer layer of *C. albicans* cell wall is packed with mannoproteins. One major class of this layer is glycosylphosphatidyl inositol (GPI)-anchored proteins.[16] N-linked mannans are composed of α-1,6-mannose backbone with α-1,2-oligomannose sidechains. O-linked mannans are found associated with cell wall glycoproteins. Some protein mannosyltransferases are responsible for the first steps in the O-linked mannans biosynthesis, adding a mannose residue to a serine or threonine residue. Some other additional mannoses are synthesized by α-1,2-mannosyltransferases with produces a short α-1,2-mannose chain. The last step results in the addition of an α-1,3-mannoses by α-1,3-mannosyltransferases.[22]

Mannan layers are less rigid compared to β-glucans and chitin, therefore, they do not influence the cell shape. However, they have low permeability and porosity which refer to the resistance of yeast cell to antifungal drugs and host defense mechanisms.[17] In addition, since the outer mannan layer covers the inner layers of the cell wall, it is considered to play an important role in the invasion process. Mannans has been described as pathogen associated molecular pattern (PAMP) ligands and many host receptors are proven to participate in their recognition.[25–27]

1.3.3.2. ECM33

Ecm33 is a glycosylphosphatidyl inositol-linked cell wall protein which is required for cell wall integrity and the yeast-to-hyphal transition. It is also required for normal virulence in the mouse model which is hematogenously disseminated candidiasis.[28] A mutation of *ECM33* in *Saccharomyces cerevisiae* increases the sensitivity to Calcofluor White[28]. In *C. albicans,* the filamentation process of *ecm33Δ/ecm33Δ* was delayed in YEPD with serum, and fewer hyphal from were produced.[29] In addition, the *ecm33Δ/ecm33Δ* mutant had delayed hypha formation in liquid and solid media. Consistent with these *in vitro* defects, this mutant had severely attenuated virulence in the mouse model of hematogenously disseminated candidiasis[28].

Examination of *ecm33Δ/ecm33Δ* mutants by transmission electron microscopy revealed that the cell wall of these strains had an abnormally electron-dense outer mannoprotein layer, which may represent a compensatory response to reduced cell wall integrity.[30] Therefore, these results suggested that Ecm33 is required for normal cell wall architecture as well as normal function and expression of cell surface proteins in *C. albicans*[30].

Wachtler *et al.* constructed 26 mutants in order to define the genes of *C. albicans* that mediate adhesion, invasion and damage during interaction with oral epithelial cells. One of the most interesting results was the *ecm33Δ* mutant showed significant reduction in adhesion (45%), and also had a higher decreased ability (more than 60%) to invade into epithelial cells, this finding suggested that there is a distinct adhesion and invasion function of *ECM33* gene.[31]

1.4 Human saliva

Within the human body, oral cavity is one of the most heavily colonized by microorganisms. Although there are several antimicrobial systems in human saliva, apparently the saliva also contains substrates for growth of different bacteria and yeast

cells. In the oral cavity, saliva plays an important role in supporting the maintenance or even excessive of microbial colonization[32]. Studies have showed that salivary constituents can be used by *C. albicans* for growth and survival and adherence to teeth surfaces. In addition, it was found that *C. albicans* can grow in human saliva without addition of glucose in more than 400 hours[32].

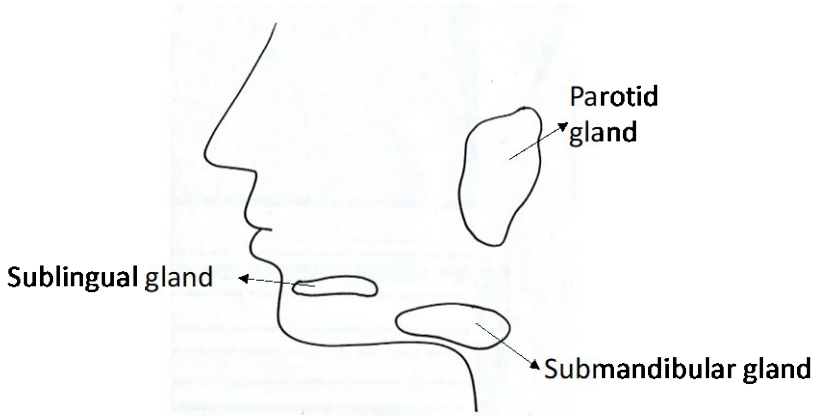

Figure 1-4: Overview of salivary gland anatomy.

Saliva is secreted from different location of saliva glands, which are the parotid, submandibular and sublingual glands.[33] Under stimulated conditions, the major glands produce about 90% of saliva production, whereas minor glands in the mouth and lips produce the rest of needed saliva.[34] The main composition of saliva is a number of electrolytes (sodium, potassium, calcium, magnesium, bicarbonate, and phosphate), proteins (immunoglobulins, enzymes, mucins, bRPs) and nitrogenous producs (urea, and ammonia).[14,34,35] Because of these variety of components, saliva takes part in various interactions including hydration, buffering, tissue coating, mineralization, antimicrobial activities as well as facilitating taste, swallowing and speech.[35] The normal pH value of saliva is ranging from 6 to 7, which means that it is slightly acidic.[34]

1.4.1 *Salivary proteins:*

It is challenging to study the saliva proteome due to the relative composition of salivary proteins varies from person to person as well as physiological conditions. In

general, saliva is a relatively dilute complex mixture of protein including α-amylase, lysozyme, esterase, peroxidase, immunoglobulins, mucins, solid food and trapping of microorganisms.[33,34,36,37]

- Mucins: Mucins are large O-linked glycosylated proteins which have gel-forming properties.[38] There are two carbohydrate rich mucin populations in human saliva, which are MG1 encoded by *MUC5B* gene and MG2 encoded by *MUC7* gene.[38] These mucins are synthesized by different cells of the salivary glands, salivary gel-forming mucin MUC5B is a large polymeric disulphide bonded protein which can facilitate the dental plague formation, by contrast, non-gel forming mucin MUC7 is reported to possess inhibitory activity against a number of bacterial, fungal and viral strains.[38–40]
- Alpha-Amylase: this is the most abundant enzyme in saliva, which is mainly formed in the parotid glands and plays an important role in the adhesion of bacteria in the tooth surface by facilitating carbohydrate metabolism.[41]
- Proline-rich proteins (PRPs): the proline-rich proteins are described by high number of proline residue (25-42%), and they are categorized into three classes: basic (bPRPs), glycosylated (gPRPs), and acidic (aPRPs).[42] Acidic PRPs bind to hydroxyapatite, and calcium ions in the cavity and therefore they present in the acquired pellicle.[43] When absorbed onto hydroxyapatite, the acidic PRPs are capable of exposes receptors for bacterial attachement.[43] The basic PRPs, which are expressed only in parotid saliva, form insoluble complexes with tannin and tannic acid and therefore might play a role in protection against harmful dietary tannins.[44] Glycosylated PRPs also binds to microorganisms and might facilitate the adherence of bacteria to oral surfaces or clearance from the mouth based on their presence in the tooth pellicle or saliva.[44]
- Statherin: Statherin and its variants exhibit various functions such as binding to hydorxyappatite, and inhibiting crysal growth of calcium phosphate salts. These interactions suggest that it is a potential precursor of the acquired enamel pellicle.[43]

- Cystatins: Cystatins are widely distributed in human tissues and fluids including saliva, they are natural physiological inhibitors of cysteine proteinase and could protect the oral tissues against degradation of these enzymes.[45]
- Salivary IgA: Immunoglobulin A is the main class of antibodies present in the body secreted fluids including saliva, tears, and mucus of intestines. It is considered the first line defense against harmful environmental factors. The amount of secretory IgA in saliva is stimulated by various factors such as stress and physical activity. IgA is responsible for the agglutination of bacteria as well as inhibition of bacterial adhesion, and neutralization of viruses, toxins, and enzymes produced by microorganism.[45,46]
- Histatin: histatins are several low molecular-weight histidine rich peptides which are secreted from parotid and submandibular glands. Like PRPs, histatin 1 inhibits crystal growth of calcium phosphate salts but does not inhibit its spontaneous precipitations.[43]

1.5 Conclusion

Biofilm characteristics of *Candida* species depends on the ability to produce extracellular polymeric substances (EPS), display dimorphic growth, carbon source availability and some other factors.[47] More importantly, the expression of some adhesins which may proceed to biofilm formation, filamentation displays great complexity and diversity of this pathogenic yeast. Those factors not only have implication in the persistence of colonization and infections but also on antifungal resistance typically found in *C. albicans* cells. Despite many challenges, one of the most promising strategies is to reduce biofilm formation and virulence of *C. albicans* by preventing the adhesion to teeth, medical devices or cell surfaces.

While *C. albicans* is generally considered as a commensal, however, under certain circumstances, it can cause diseases. *C. albicans* is one of the principal causes of opportunistic mycoses worldwide. Adhesion is important for establishing the *C. albicans*-host interaction and may lead to biofilm and antibiotic resistance.

In previous work other reseachers discovered that two cell surface mannoproteins, Bgl2 (35 kDa) and another highly glycosylated mannoprotein (97.4 kDa) inhibited the attachment of *C. albicans* cells to bPRP-coated hydroxyapatite beads. The importance of cell wall proteins in biofilm formation is supported by studies showing that mutations of these proteins in *C. albicans* affect cell morphology, cell integrity, adherence to different surfaces, and compromise biofilm development.[7,48,49] In particular, Bgl2 on the surface of *C. albicans* is required for cell wall maintenance, and disruption of the *C. albicans BGL2* gene interferes cell wall integrity[50] and the dimorphic transition[51] and the null-mutant is attenuated in virulence for mice when compared to its parent strain[50]. The N-terminal sequencing of the 97.4 kDa putative *C. albicans* adhesin gave the amino acid sequence ANXXXLXXAXP which is consistent with the ANNSTLTTATP sequence in Ecm33, (residues 19-29) a glycosylphosphatidylinositol (GPI) – anchored cell wall protein. *C. albicans* Ecm33 is required for normal cell wall integrity and the yeast-to-hyphal transition. [28] Deletion of *ECM33* in *C. albicans* leads to delayed hypha formation and severely attenuated virulence in mouse model.[28] Therefore, both Bgl2 and Ecm33 are required for normal cell wall architecture as well as normal function and expression of cell surface proteins in *C. albicans.*[30] Thus, I hypothesized that the two mannoproteins Blg2 and Ecm33 mediate specific interactions of *C. albicans* with adhesion receptors in saliva-coated surfaces.

In this research, I investigated the roles of Bgl2 and Ecm33 in the adhesion of *C. albicans* to saliva-coated hydroxyapatite by generating null mutants of these genes and using a novel NR staining assay. I found that the two cell wall proteins Bgl2 and Ecm33 contribute to interaction between *C. albicans* and saliva-coated surfaces. Interestingly, I observed that the deletion of both genes triggered compensatory responses in *C. albicans* cells. In particular, the *ALS1* gene, encoding a major GPI-anchored adhesin, was overexpressed in the *bgl2Δ/ecm33Δ* double disrupted strain, and the deletion of all three

genes caused a significant decrease in the adhesion of *C. albicans* to saliva-coated hydroxyapatite (SHA) beads.

CHAPTER 2. DEVELOPING NILE RED STAINING ASSAY FOR DETECTION OF *C. ALBICANS* TO ADHERE TO HYDROXYAPATITE (TEETH MODEL).

2.1 Introduction

In order to detect and quantify the microbial adhesion to teeth surfaces, several assays have been developed including radiolabeling of the yeast[5], and/or microscopy using crystal violet (CV) staining[14]. In general, radiolabeling is considered high sensitivity and reproducibility compared to quantifying with microscopy or viable cell counts[14]. However, all of the above-mentioned assays based on radiolabeling require specialized handling and equipment, laborious work and time consuming. Therefore, a novel alternative method that is fast, cheap and sensitive to detect *C. albicans* adhesion to HA is much needed.

Recently, fluorescence dyes have been extensively used for many cell fluorescence imaging purposes. Although encoded fluorescence proteins have been used widely, many chemical fluorescence dyes are still useful when conjugated to proteins or ligands.[52] Several fluorescence dyes used for visualizing and quantifying different cells are available. In particular, CFDA (5-Carboxyfluorescein diacetate, acetoxymethyl ester), one of the esterase dependent green fluorogenic probes used for flow cytometric method was developed.[53] CFDA provides rapid quantitative information on the vitality and vigor of yeast cell cultures and it was validated by cell sorting and analysis of live, heat killed, and UV-treated yeast.[53] However, as CFDA fluorescence signals depend on the amount of esterase, I found that if I use CFDA for quantifying yeast cells, the esterase in human saliva presents in saliva-coated beads will greatly interfere with the adhesion assay results. In 2019, Anna Ligasova *et al.* published a new method using DAPI and Hoechst stains, which based on DNA staining, for the purpose of quantification of fixed adherent cells. In this research, DAPI fluorescence showed excellent linear correlations of cell

quantification based on the measurement of DNA content with the enhancement of SDS.[54] However, using DAPI staining method for fixed cells requires several washing steps, which will be a major drawback for quantification of yeast cells in the suspension.

In general, fluorescence dyes for *C. albicans* assay about adhesion to saliva-coated HA beads must meet some criteria, including: (i) chemical stability under experiment conditions, (ii) labelling of yeasts to high fluorescence intensity and with uniformity, (iii) relatively short labelling time, (iv) retention of the fluorescence dyes within the labelled yeasts, (v) minimum effects on yeast adherence to HA beads, (vi) controllable nonspecific fluorescence labelling of saliva in the adherence reaction.

Nile Red (NR, 9-diethylamino-5Hbenzo-α-phenoxazine5-5-one), a lipophilic, is one of the most used fluorescencent dyes which can bind to intracellular neutral[55]. NR is almost nonfluorescent in water and other polar solvents but shows increased fluorescence signals in nonpolar environments[56]. Due to this characteristic, NR has proven for detection and quantification of intracellular lipid droplets in various organisms including yeasts, algae, filamentous fungi, bacteria and mammalian cells such as macrophages[55].

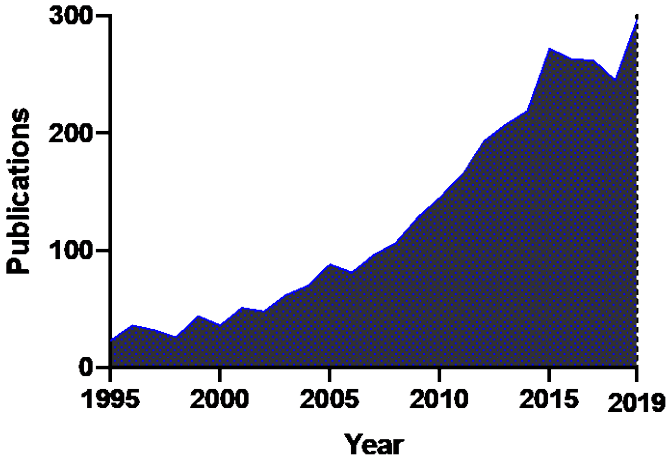

Figure 2-1: Number of publications from 1995 to 2019 concerning the use of NR. Source: Scopus, Keyword: NR, Last updated 9th March 2020.

In general, NR is dissolved in an organic solvent that serves as a stain carrier to enhance the permeability of NR into cells. Once NR has diffused into yeast cells, it binds

to the intracellular neutral lipid droplets, resulting in a yellow-gold fluorescence. The magnitude of the fluorescence signals depends on various parameters including stain carriers, cells numbers, cell growth, incubation time and NR concentrations[57]. Therefore, it is crucial to develop a fluorescent staining method using NR for *C. albicans* strains. This method needs to ensure that interactions between NR and intracellular lipids droplets give fluorescence values that are relevant to the calibration from fluorescence to neutral lipid contents and thus, cells numbers. This chapter focuses on the use of NR as a fluorescence dye to quantify *C. albicans* which can be used for further investigation of yeast cells that adhered to saliva-coated HA beads.

2.2 Materials and methods:

2.2.1 *Autofluorescence of NR in different solvents*

NR in acetone stock and DMSO stock were diluted into 3 different solvents to the final concentration of 1 µg/ml, in particular, sample (A) 1 µg/ml NR in KCl buffer containing 10% Acetone, (B) 1 µg/ml NR stock in Acetone in KCl buffer containing 10% DMSO, (C) 1 µg/ml NR in KCl buffer containing 10% DMSO. Two hundred µl of above mentioned solutions were put into different wells in 98 wells plate. Emission spectrum of stained cells in different solvents was measured at fixed excitation 530nm at different time frame.

2.2.2 *Excitation and Emission spectrum:*

SC5314 cells was inoculated in GSB medium to OD_{600nm} = 5, yeast cells were harvested by centrifuging at 5000 rpm in 5 minutes and diluted in KCl buffer to OD_{600nm} = 1. Cells were then stained with NR in 1 µg/ml in KCl buffer containing 10% DMSO. After 1 hour staining, 200 µl of stained cells and 200 µl of NR 1 µg/ml in KCl containing 10% DMSO were put in to wells in 98 wells plate. Excitation spectrum of stained cells in different solvents was measured at fixed emission 570nm. Emission spectrum of stained cells in different solvents was measured at fixed excitation 530nm.

2.2.3 *Emission spectrum of NR in the presence of C. albicans SC5314 at different cell numbers*

SC5314 cells was inoculated in GSB medium to OD_{600nm} = 4- 5, yeast cells were harvested by centrifuging at 5000 rpm in 5 minutes and diluted in KCl buffer to OD_{600nm} ranging from 0.1 to 1. Fluorescence spectra of stained cells were measured at excitation 530 nm.

2.2.4 *The correlation between NR fluorescence and cell numbers*

SC5314 cells was inoculated in GSB medium to OD_{600nm} = 5, yeast cells were harvested by centrifuging at 5000 rpm in 5 minutes and diluted in KCl buffer to different OD_{600nm} numbers using ASV11D spectrophotometer. Fluorescence intensity of NR-stained cells at 530 nm excitation and 570 nm emission wavelengths.

2.2.5 *The correlation between different concentration of NR fluorescence*

SC5314 cells was inoculated in GSB medium to OD_{600nm} = 5, yeast cells were harvested by centrifuging at 5000 rpm in 5 minutes and diluted in KCl buffer to OD_{600nm} = 1. Cells were then stained with different concentration of NR ranging from 0.25 µg/ml to 4 µg/ml in KCl buffer containing 10% DMSO. After 1 hour staining, 200 µl of stained cells and 200 µl of NR 1 µg/ml in KCl containing 10% DMSO were put in to wells in 98 wells plate. Fluorescence intensity of NR-stained cells at 530 nm excitation and 570 nm emission wavelengths.

2.2.6 *Time-dependent assay*

SC5314 cells was inoculated in GSB medium to OD_{600nm} = 4- 5, yeast cells were harvested by centrifuging at 5000 rpm in 5 minutes and diluted in KCl buffer to OD_{600nm} = 1. Cells were then stained with different concentration of NR 1 µg/ml in KCl buffer containing 10% DMSO. Fluorescence intensity were measured of NR-stained cells at 530 nm excitation and 570 nm emission wavelengths at different time.

2.2.7 *Fluorescence of cells in different solvents and time measurement*

SC5314 cells was inoculated in GSB medium to OD_{600nm} = 4- 5, yeast cells were harvested by centrifuging at 5000 rpm in 5 minutes and diluted in different solvents to OD_{600nm} = 1. Fluorescence intensity of stained cells in different solvents was measured at excitation 530 nm after 5 minute, 30 minutes, 60 minutes, and 80 minutes staining.

2.2.8 *Fluorescence intensity of* **C. albicans** *cells in different growth phases*

SC5314 cells was inoculated in GSB medium to mid-log phase (OD_{600nm}=1.5) and stationary phase (OD_{600nm}=4.5). Yeast cells were then harvested by centrifuging at 5000 rpm in 5 minutes and diluted in KCl buffer to OD_{600nm}=1 Fluorescence signals were measured at excitation wavelength 530 nm and emission wavelength at 570 nm after 60 minutes of staining of cells with 1 µg/ml NR in KCl buffer containing 10% DMSO.

2.3 Results and discussion:

2.3.1 *Effect of different solvent to autofluorescence 1µg/ml NR*

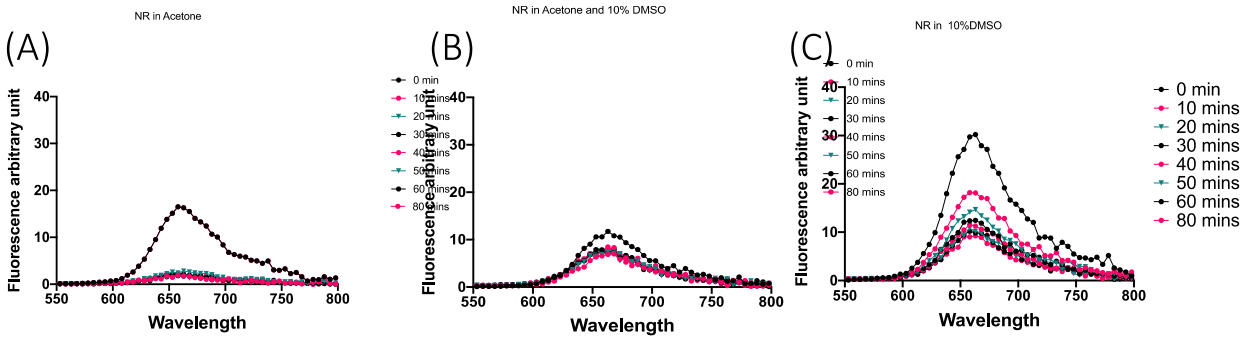

Figure 2-2: Effect of different solvent in NR staining. (A) Emission spectra of 1 µg/ml NR in KCl buffer containing 10% acetone, (B) Emission spectra of 1 µg/ml NR stock in acetone in KCl buffer containing 10% DMSO, (C) Emission spectra of 1 µg/ml NR in KCl buffer containing 10% DMSO.

In general NR is dissolved in organic solvents and serves as a stain carrier to enhance the diffusion of NR into cells. Previous studies suggested that the addition of solvents such as acetone or DMSO at different concentration can be used for enhancing fluorescence signal.[19] Therefore, I investigate the possibility of using DMSO, acetone at different concentrations, and combination of these two solvents at different time of fluorescence acquisition. Fluorescence spectra was measured at excitation 530nm. This result showed that NR gives the best fluorescence intensity when in KCl buffer containing 10% DMSO.

2.3.2 *Excitation and Emission spectrum*

As NR assay is sensitive, different excitation/emission wavelengths have been used for measurements of various biological samples[56], therefore, in this experiment, I re-examined the excitation and emission spectrum using VariosSkan LUX fluorescence spectrophotometer using 96 well plate.

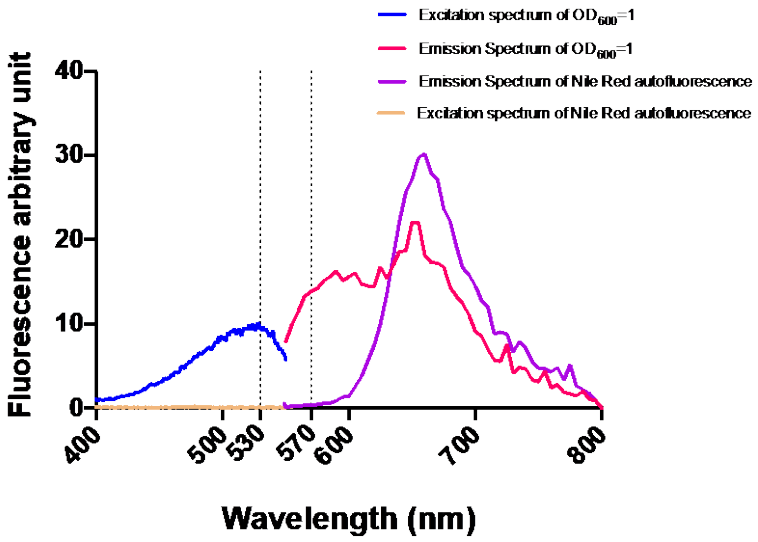

Figure 2-3: Excitation spectrum of NR stained *C. albicans* SC5314

As shown in Figure 2-3, although 650 nm was the best emission wavelengths for measuring NR fluorescence, but the autofluoresence of this solution is also very high. In contrast, the autofluorescence of emission wavelength at 570 nm was relatively low. Therefore, it would be better to choose 570 nm as emission wavelengths rather than 650nm.

2.3.1 *Emission spectrum of NR in the presence of* C. albicans *SC5314 at different cell numbers*

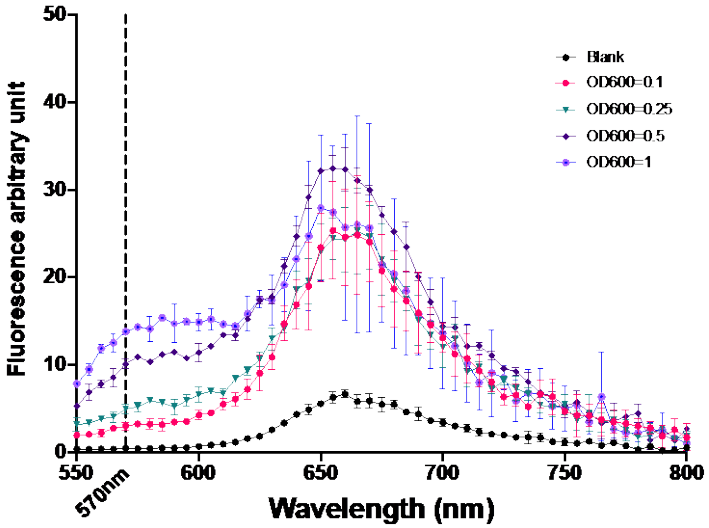

Figure 2-4: Emission spectrum of NR in the presence of *C. albicans* SC5314 at different cell numbers.

This figure showed that fluorescence intensities of NR at 570 nm were proportional to the cell number (dashed line) without autofluorescence. Autofluorescence signal (blank) increased above 600n m, and reached the highest (8 arbitrary unit) at 660 nm. Results are mean of three independent experiments.

2.3.2 *The correlation between NR fluorescence and cell numbers*

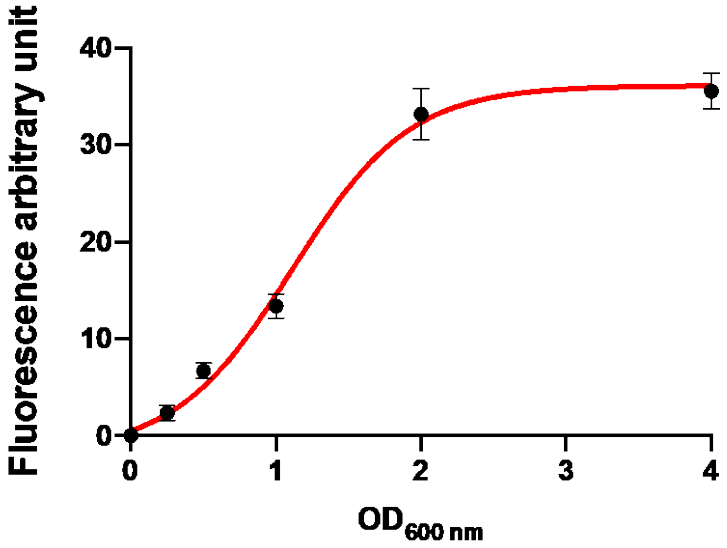

Figure 2-5: The correlation between NR fluorescence and optical density (600nm).

Figure 2-5 showed that the more NR stained cells number tested, the higher the fluorescence values. The fluorescence signals become stable or saturated when the number of cells reached OD_{600nm}=2. This phenomenon can be explained that due to the very high density of cells sink into the bottom of the well at OD_{600nm} above 2, the fluorescence signals that can be detected in this assay get saturated. This result indicates that the detection limit for this system would be best with the OD_{600nm} ranging from 0.1-2. Therefore, more tested was done with lower OD_{600nm} value in order to confirm the linear relationship of NR stained *C. albicans* and their fluorescence signals.

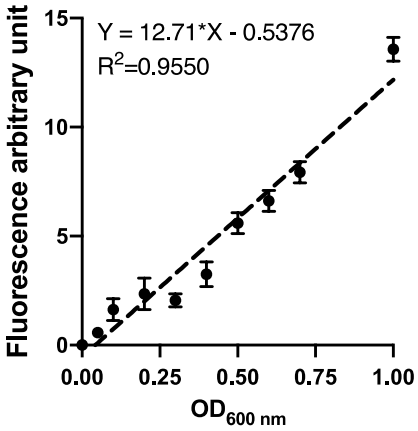

Figure 2-6: The correlation between NR fluorescence and optical density (600nm).

More detailed of NR stained at smaller OD_{600nm} was tested. *C. albicans* SC5314 cells were adjusted in different OD_{600nm} ranging from 0.125 to 1. Fluorescence signal was obtained after 60 minutes staining. Figure 2-6 clearly shows that there is a strong correlation and linear relationship between the NR fluorescence and the *C. albicans* cell number. This indicated that the NR fluorescence could be used to quantify the number of *C. albicans* cells adhered to hydroxyapatite beads.

2.3.3 *The correlation between different concentration of NR fluorescence*

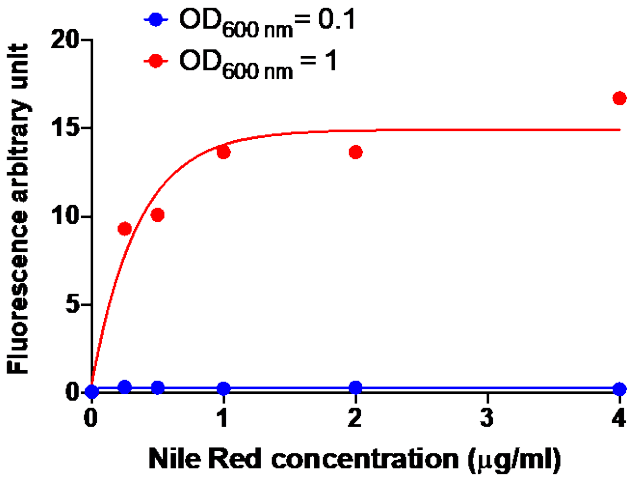

Figure 2-7: The correlation between different concentration of NR fluorescence at SC 5314 cells $OD_{600\ nm}$ =1 and $OD_{600\ nm}$ =0.1.

Figure 2-7 showed that the more NR added (>1 µg/ml) the fluorescence values becomes stable or saturated. This phenomenon can be explained that quenching effects might occurred because too little neutral lipid being available for staining, in addition, high NR/cells ratios may cause stacking of NR molecules which leads NR more exposed to quenchers in the samples.

2.3.4 *Time-dependent assay:*

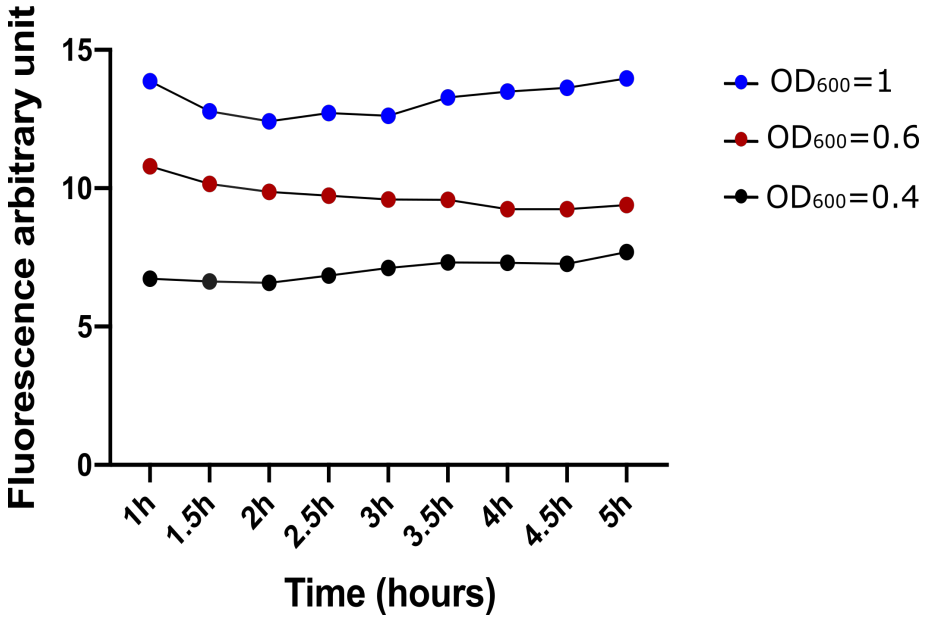

Figure 2-8: Time dependent of fluorescence signals at excitation 530nm and emission 570nm.

To test the stability of fluorescence signals of cells at different time of measurement, OD_{600} was fixed at 1 and stained with NR 1µg/ml. Emission fluorescence unit was measured at 570 nm, as showed in Figure 2-8, the fluorescence values becomes stable after 1-5h after staining.

2.3.5 *Fluorescence of cells at different NR concentrations*

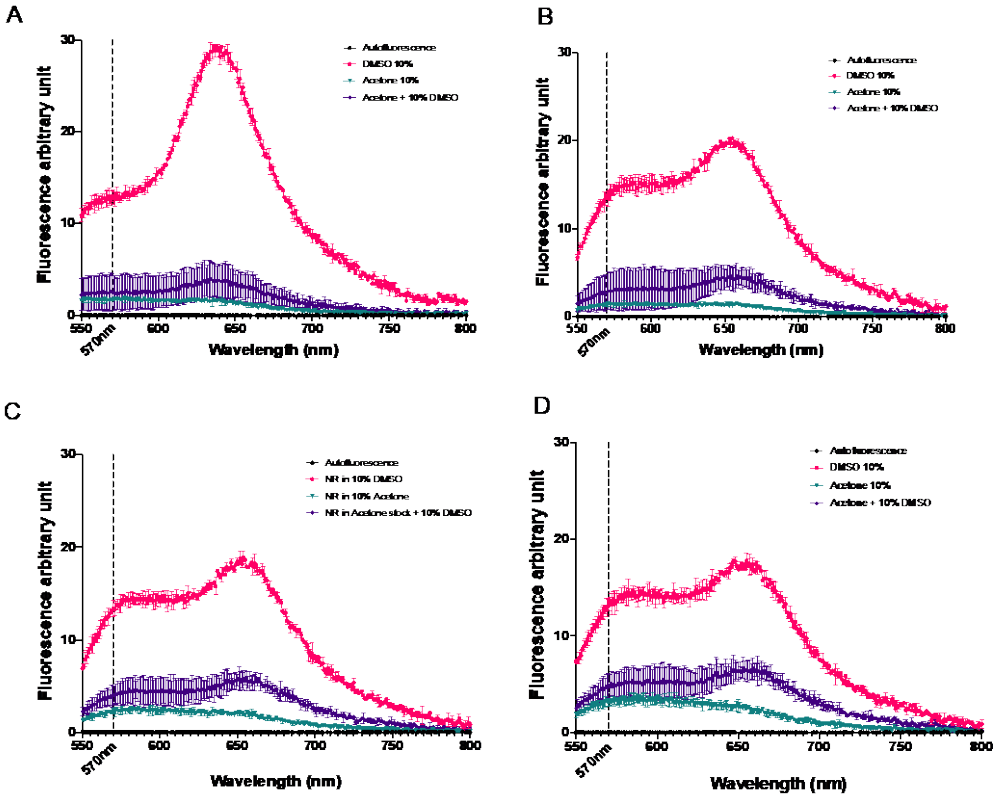

Figure 2-9: Fluorescence of cells in different solvents and time measurement. SC5314 cells was inoculated in GSB medium to OD_{600nm} = 4-5, harvested and diluted cells to OD_{600nm} = 1. Fluorescence intensity of stained cells in different solvents was measured at excitation 530nm. (A) After 5 minute staining, (B) After 30 minutes staining, (C) After 60 minute staining, (D) After 60 minute staining

In general organic solvent served as a stain carrier to enhance the diffusion of NR into yeast cells. Some studies suggested that addition of solvents such as acetone or DMSO can be used for this purpose.[2] Therefore, in addition to autofluorescence investigation that was mentioned above, I also investigated the effect of 10% DMSO, 10% acetone and combination of those two solvents at different exposure time on fluorescence intensity. The results also clearly showed that DMSO10% is the best solvent that enhances the fluorescence signals. In addition, this data also strengthen the stability of fluorescence signals after even 1-1.5h of staining.

2.3.6 *Fluorescence intensity of* C. albicans *cells in different growth phases*

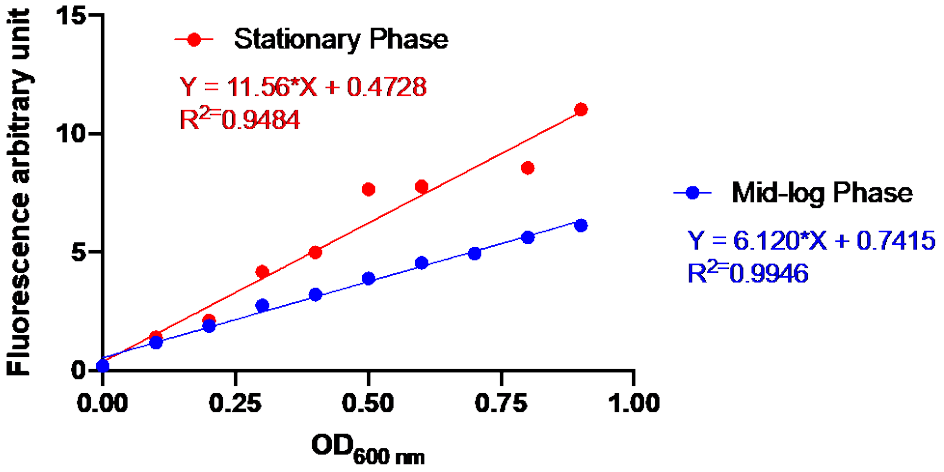

Figure 2-10: Fluorescence intensity of NR-stained mid-log phase (OD_{600nm}=1.5) and stationary phase (OD_{600nm}=4.5) *C. albicans* SC5314 cells grown in GSB medium. Cells were harvested and diluted at different OD_{600nm} and then stained with 1µg/ml NR in KCl buffer containing 10% DMSO. Fluorescence signals were measured after 60 minutes of exposure of cells to NR.

The main importance for using fluorescence staining using different fluorescence dyes is that the fluorescence signals depends on the metabolic state of cell populations. As metabolic state can be related to the cell density of the cell population, this may lead to non-linear dependence of the signals on the cell numbers.[54] In case of using NR staining yeast cells, as can be seen on Figure 2-10, the lipid amount in the stationary cells is different from that in the mid-log cells, however, most of stationary cells have similar lipid amount each other. Therefore, the fluorescence signals always showed excellent linear correlations with cell numbers even in different growth states. Cells at mid-log phase contain less lipid constituents than cells grow at stationary phase, therefore, the fluorescence intensity of NR stained cells at mid-log phase was less than in stationary phase. However, the fluorescence signals were always in a linear dependence with cell populations. This data was in agreement with all other above mentioned results where cells at stationary phase (OD_{600nm} =4-5) were used.

2.4 Conclusion

Cell quantification is a common procedure for many assays. In particular, assays used to detect and quantify the microbial adhesion to teeth surfaces have been developed. Some of the most common assays used radiolabeling of the yeast[5], and/or microscopy using crystal violet (CV) staining[14]. Although radiolabeling is considered high sensitivity and reproducibility compared to quantifying using microscopy or viable cell counts[14], all of the above-mentioned assays based on radiolabeling require specialized handling and equipment, laborious work and time consuming.

NR (NR) is a lipophilic fluorescent dye that has been used to stain mammalian cells[58], bacteria[59], microalgae,[56,57,60] and yeast cells[61]. I developed a novel adhesion assay based on NR staining of *C. albicans* cells to overcome the limitations of radiolabelling and microscopy techniques. First I optimized and validated the assay. I determined the optimum excitation (530 nm) and emission wavelengths (570 nm) to use to measure NR fluorescence, and ensured there was no autofluorescence of yeast cells at these wavelengths. The best solvent for the NR also investigated, in particular, NR gave the highest fluorescence signal in KCl buffer containing 10% DMSO as staining carrier and the signal remained stable 1 hour after staining. Then, I found that there was a linear relationship between the NR fluorescence and the *C. albicans* cell number (OD_{600} ranging from 0.125 to 1). This indicated that the NR fluorescence could be used to quantify the number of *C. albicans* cells adhered to hydroxyapatite beads.

In this chapter, I have optimized different parameters to used NR as a fluorescence dye to develop a novel method for quantifying *C. albicans* cells adhesions to hydroxyapatite beads. There are many advantages of using NR compared with conventional methods including: (i) robust and reliable staining, (ii) fluorescence signals are stable up to 5 hours after staining, (iii) no washing steps are needed, this is especially important for *C. albicans* adhesion assays as I need to wash cells that do not attached to

HA beads twice, the cell numbers can be small and if staining with fluorescence dyes need washing, human errors might easily occurred, (iv) NR offers a much cheaper and easier solution, and (v) NR has been widely used and it is safe for human.

The method of using NR as a novel fluorescence dye is suitable for cells quantifications which provides high linearity.

CHAPTER 3. THE ROLE OF BGL2P AND ECM33P IN THE ABILITY OF *C. ALBICANS* TO ADHERE TO SALIVA-COATED ORAL SURFACE.

3.1 Introduction

C. albicans is one of the most frequently isolated fungal pathogens of human, it affects immunocompromised patients ranging from premature infants to AIDS patients. Systemic infections due to *C. albicans* attributed mortality rate of 30-50%.[62] *C. albicans* is always a diploid organism and has 8 sets of chromosome pairs, its genome size is about 16 Mb (haploid). The complete sequence of *C. albicans* are available at http://www.candidagenome.org/.[63,64]

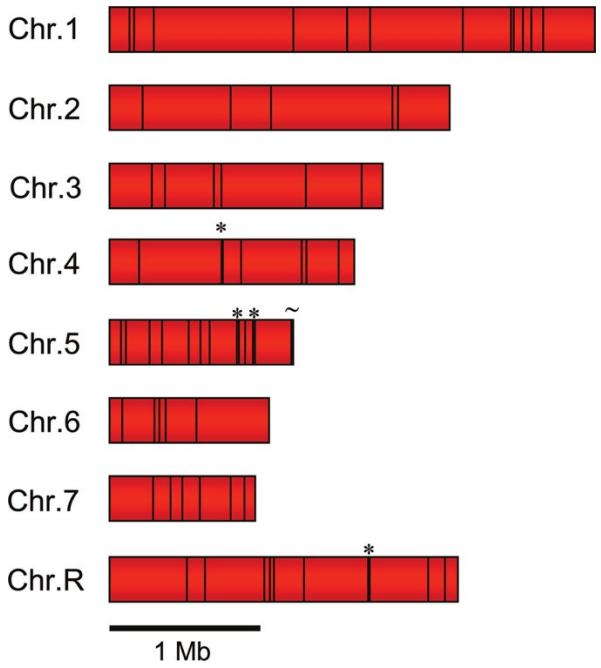

Figure 3-1: Chromosomal map of C. albicans-specific genes. Chromosomal coordinates for all 65 expressed C. albicans-specific genes were downloaded from the CGD and plotted as a function of chromosome location. Asterisks denote clusters of genes at the same loci; indicates the presence of a gene at the telomere.[64]

In recent years, the free *Candida* Genome Database is established to facilitate the genome sequences research about *C. albicans.*[63] Several gene manipulation techniques are available to allow useful genetic approaches to study gene functions in *C. albicans*

including gene disruption, controlled gene expression, protein tagging, gene re-intergration, and overexpression.[63] All of the mentioned techniques have the same principles, which are based on homologous recombination between exogenous complementary sequences and genomic sequences in *C. albicans.*[63] In general, genes that are not essential for survival in *C. albicans* can be disrupted or replaced by transformation with DNA cassettes containing selectable markers such as auxotrophic or drug-resistant marker.[63,65]

The *SAT1*-flipper cassette consists of a nourseothricin resistance marker *C. albicans SAT1* gene and the FLP-mediated gene which encodes a site-specific recombinase FLP, and two FLP recombination sites. Target genes disruption plasmids are usually created by subcloning upstream and downstream regions of the target gene onto both sides of the *SAT1*-flipper cassette.[63] After that, the linearized of target gene deletion cassette is introduced into *C. albicans* and successful transformant will be selected in YPD medium containing nourseothricin. The transformants which contain *SAT1*-flipper cassette will be inserted into the *C. albicans* genome and the target gene in one allele is deleted accordingly. The *SAT1*-flipper region is the liberated by transferring the transformants to YPD medium, under the regulation of maltose promoter of FLP gene, Flp recombinase expression is activated. To delete the second allele of the target gene, the process is repeated one more time. This method requires many cloning steps and is therefore somewhat laborious, however, it has the advantage of deleting certain genes in most of *C. albicans* strains, including clinical isolates that lack auxotrophic markers.[66] Based on the same techniques, the re-insertion of target gene cassette can be constructed and used to generate revertant of null mutants.

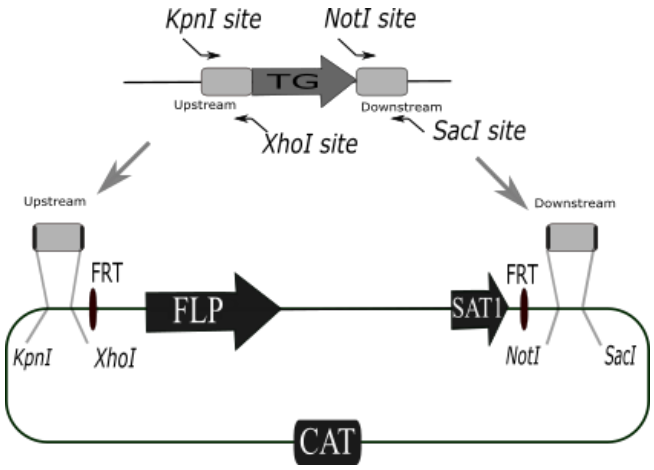

Figure 3-2: Plasmid construction. TG: Target gene (BGL2. ECM33. ALS1)

Comparing with classical cloning *SAT1*-flipper cassette method, the deletion cassette construction procedures are considered laborious and time consuming due to several cloning steps. In our experiment, in addition to constructing the deletion cassette using classical cloning strategy, I used an alternative method which relies on PCR-based strategies, using long primers (i.e. 45-100 nt) to flank both sides of the re-insertion cassette which contain both upstream region, target gene sequence, *SAT1*-flipper cassette, and downstream region. Although this technique relies heavily on PCR-based cloning strategy, but it is much faster and can eliminate the hurdle of choosing restriction enzymes that are appropriate for cloning.

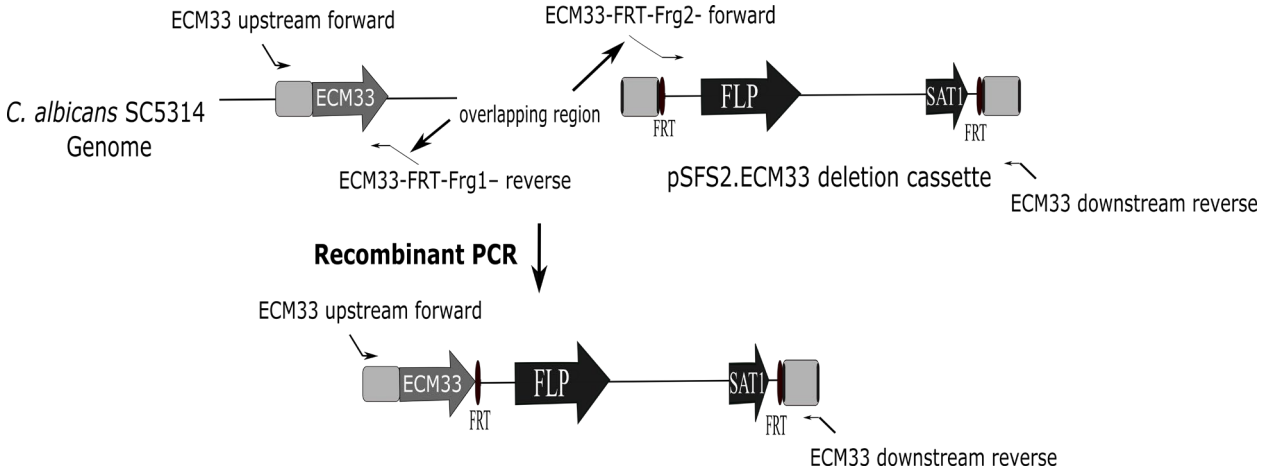

Figure 3-3: Construction of ECM33 reintegration cassette using PCR-based cloning strategy.

In previous work the other reseachers discovered that two cell surface mannoproteins, Bgl2 (35 kDa) and another highly glycosylated mannoprotein (97.4 kDa) inhibited the attachment of *C. albicans* cells to bPRP-coated hydroxyapatite beads. The importance of cell wall proteins in biofilm formation is supported by studies showing that mutations of these proteins in *C. albicans* affect cell morphology, cell integrity, adherence to different surfaces, and compromise biofilm development.[7,48,49] In particular, Bgl2 on the surface of *C. albicans* is required for cell wall maintenance, and disruption of the *C. albicans BGL2* gene interferes cell wall integrity[50] and the dimorphic transition[51] and the null-mutant is attenuated in virulence for mice when compared to its parent strain[50]. The N-terminal amino acid sequencing of the 97.4 kDa putative *C. albicans* adhesin gave the sequence ANXXXLXXAXP which is consistent with the ANNSTLTTATP sequence in Ecm33, (residues 19-29) a glycosylphosphatidylinositol (GPI) – anchored cell wall protein. *C. albicans* Ecm33 is required for normal cell wall integrity and the yeast-to-hyphal transition. [28] Deletion of *ECM33* in *C. albicans* leads to delayed hypha formation and severely attenuated virulence in mouse model.[28] Therefore, both Bgl2 and Ecm33 are required for normal cell wall architecture as well as normal function and expression of cell surface proteins in *C. albicans.*[30] In this research, I hypothesized that two mannoproteins Blg2 and Ecm33 mediate specific interactions of *C. albicans* with adhesion receptors in saliva-coated surfaces. This interaction is an initial event in the colonization of human oral cavity, which leads to oral diseases such as candidiasis and denture stomatitis. Therefore, I propose that the deletion of these two genes triggered compensatory responses in the yeast cells.

3.2 Materials and methods:

3.2.1 *Strains and growth conditions:*

C. albicans strains used in this study were listed in Table 3-1. Yeast cells were maintained in YPD medium (1% [w/v] yeast extract, 2% [w/v] peptone, 2% [w/v] glucose) containing 50 % [v/v] glycerol at -80°C. They were routinely grown in glucose/salts/biotin medium (GSB) (10 [g/l] glucose, 1.0 [g/l] $(NH_4)_2SO_4$, 2.0 [g/l] KH_2PO_4, 0.05 [g/l] $MgSO_4.7H_2O$, 0.05 [g/l] $CaCl_2.2H_2O$; and 0.05 [mg/l] biotin)[67].

3.2.2 *Gene disruption and re-insertion*

C. albicans gene sequences were obtained from the Candida Genome Database web site (http://www.candidagenome.org/cgi-bin/seqTools). The *bgl2* null mutant (BGL2M4) and the *BGL2*-restored strain (BGL2MK2 from BGL2M4) had been constructed previously by our group using the *SAT1* flipper method.[7] In the present study, the same method was used to construct the *ecm33* null mutant (ECM33M4). In brief, the *SAT1*-flipper method was used to construct the mutants by using *SAT1*, a nourseothricin resistance marker. An *ECM33* deletion cassette was constructed using the plasmid pSFS2. One *Kpn*I-*Xho*I fragment of the upstream region (564 bp) of the *ECM33* gene was amplified from SC5314 genomic DNA with *ECM33* upstream forward and reverse primers. Another *Not*I-*Sac*I fragment of the downstream region (555 bp) of the *ECM33* gene was amplified with primers *ECM33* downstream forward and reverse primers. These two fragments were inserted into pSFS2 on both sides of the *SAT1*-flipper. The resulting plasmid was digested with *Kpn*I and *Sac*I to excise *ECM33* deletion cassette.

A recombinant PCR method was used to construct the revertant of the *ecm33*Δ strain (revertant ECM33MK2 containing a functional *ECM33* gene), and revertant of *bgl2*Δ/*ecm33*Δ strain (revertant BEMK2). Firstly, using SC5314 genomic DNA as a template, a fragment containing *ECM33* and its upstream region was PCR amplified using the ECM33 upstream forward primer and the ECM33-FRT-Frg1-reverse primer (Table 3,

Fig. S7(b)), which contained an overlapping region (15 nucleotides) with the ECM33-FRT-Frg2-forward primer. An ECM33 deletion cassette, which contained the nourseothricin resistance marker *SAT1,* the *FLP* recombinase, and the *ECM33* downstream region, was PCR amplified using ECM33 deletion cassette and the ECM33-FRT-Frg2-forward primer and ECM33 downstream reverse primer. These two DNA fragments were gel extracted and purified and used as templates for generating an *ECM33* reinsertion cassette using the ECM33 upstream forward primer and ECM33 downstream reverse primer (Supplementary Figure S7). The resultant *ECM33* re-insertion cassette contained the *ECM33* upstream region, *ECM33* coding region and *SAT1*-flipper followed by the *ECM33* downstream region.

3.2.3 *Transformation of* C. albicans

C. albicans SC5314 cells were transformed with DNA fragments using the lithium acetate method[68] with slight modifications. The overnight-cultured of *C. albicans* SC5314 cells were diluted to 10^7 cells/ml in 50 mL fresh YPD medium and grown at 30°C for two generations (~ 4 h). The cells were collected by centrifuging at 5000 rpm for 5 minutes and washed with distilled water. Washed cells were resuspended in 600 µl distilled water, and 100 µl of cell suspension was mixed with transformation mixture (72 µL 1 M lithium acetate, 100 µL of 2.0 mg/mL single-stranded carrier DNA, 68 µL *ECM33* deletion cassette suspension). The transformation mixture was incubated at 30^0C with rotatory mixing at 20 rpm for 2 h before heat shocking in a 42°C water bath for 45 min. Cells were harvested by centrifuging at 5000 rpm for 5 minutes and resuspended in 1 mL fresh YPD medium before being incubated for 4 h at 30°C with shaking at 200rpm for recovery. Finally, the cells were collected by centrifuging and resuspended in 200 µl YPD and, then the cell suspension were spread on YPD plates containing 200 µg/mL nourseothricin and incubated at 30°C for 1-2 days.

The same procedure was repeated using *ECM33* re-insertion cassette or *BGL2* re-insertion cassette into BEM4 (*bgl2*Δ*, ecm33*Δ) double mutants.

3.2.4 *Preparation of saliva-coated hydroxyapatite (SHA) beads*

Saliva-coated hydroxyapatite beads were prepared as previously described[5] with modifications. Unstimulated whole saliva collected from 8 donors was stored on ice, and an equal volume of saliva from each donor was pooled. The pooled saliva was clarified by centrifugation at 3000g for 1 h and the supernatant was diluted with an equal volume of KCl buffer (2 mM KH_2PO_4, 2 mM $K_2HPO_4.3H_2O$, 5 mM KCl, 1 mM $CaCl_2$, pH 6.5). Proteinase inhibitor (Protease Inhibitor Cocktail, EDTA free, Nacalai tesque, Japan) was added to the diluted saliva at a final concentration of 1 x protease inhibitor solution to prevent proteolysis of saliva proteins. Prepared saliva solution was aliquoted in glass tubes and stored at -80°C.

Hydroxyapatite beads (Micro-Prep Ceramic Hydroxyapatite Type I 80 µm diameter, Bio-rad) were suspended in KCl buffer, washed with dH_2O 10 times and dried in an oven at 60°C. For each adhesion assay 12 mg beads were placed in a 1.5ml eppendorf and hydrated by incubation in 1 mL KCl buffer at 4°C for 16 h prior to use in an adhesion assay. The KCl buffer was aspirated from the hydrated beads and 1 ml saliva solution (50% [v/v] in KCl buffer) was added. The tube was mixed with end-over-end rotation at 15 rpm at room temperature for 1 h to allow adsorption of saliva proteins to the hydroxyapatite beads. The supernatant was aspirated, and the beads were washed twice with 1 mL KCl buffer. As a negative control, hydroxyapatite beads were incubated in KCl buffer (non saliva-coated beads [NSHA]).

3.2.5 *Assay of* **C. albicans** *adhesion to SHA beads*

One colony of yeast cells of SC5314 (wild type), BGL2M4 (*bgl2*Δ), ECM33M4 (*ecm33*Δ), and BEM4 (*bgl2*Δ, *ecm33*Δ) were pre-cultured in 5mL GSB medium overnight at 30°C. Cells were harvested by centrifuging 5000 rpm for 5 minutes ad then diluted in KCl buffer to OD_{600} of 1.

SHA beads were incubated with stationary phase cells to determine the adhesion ability of yeast cells to the saliva-coated beads. Yeast cells (1 ml) of OD_{600nm} ranging from 0.05 to 1 using ASV11D spectrophotometer in KCl buffer were incubated with SHA or NSHA beads at room temperature with end-over-end mixing for 1.5 h. After incubation, loosely adhered *C. albicans* cells were removed by washing the beads twice with 1 ml KCl buffer. Cells on the beads were stained with NR (final concentration 1 µg/ml in 10% DMSO) for 1 h, and fluorescence intensity associated with the cells was measured using a Varioskan Lux LL154104 (Thermo Fisher) at 530 nm and 570 nm, excitation and emission wavelength, respectively. The fluorescence intensity was determined using a fluorescence-intensity standard curve generated by mixing NR with various number of yeast cells. (Supplement Figure S6).

*Adhesion ability was calculated by following formula. Adhesion ability of wild type strain SC5314 was considered as 100%.

$$Adhesion\ ability\ (\%) = \frac{(Fluorescence\ intensity\ of\ input\ cells - Fluorescence\ intensity\ of\ unattached\ cells)}{Fluorescence\ Intensity\ of\ input\ cells} x100\%$$

As other reference experiment, 1mg of Skim milk (Nacalai Tesque), 1mg BSA (Nacalai Tesque), and FBS (Gibco) were diluted in KCl buffer and protein concentration of each sample was determined by Lowry method using (Bio-Rad DC Protein Assay; cat no. 500-0116). As the protein concentration of 50% whole saliva in KCl buffer contained 1.3 mg/ml protein, the SM, BSA and FBS and were diluted accordingly to the same amount of protein presented in 50% saliva in KCl buffer, those solution were used to coat HA beads in the same way with saliva-coated hydroxyapatite beads mention above.

3.2.6 *The susceptibility of* C. albicans *strains to Calcofluor white (CFW)*

SC5314 (wild type), BGL2M4 (*bgl2*Δ), ECM33M4 (*ecm33*Δ), and BEM4 (*bgl2*Δ, *ecm33*Δ) cells were cultured in 5 mL YPD medium overnight at 30°C and diluted in YPD to an OD_{600} of 1. The cells were then serially diluted (10-fold) in YPD medium. Three

microliters of each dilution were spotted onto YPD plates containing 12.5 μg/mL CFW, and incubated at 30°C for 24h. Susceptibility of cells to CFW was indicated by their inability to grow on this medium.

Table 3-1: Strains list

Strain	Genotype	Source/Reference
SC5314	Wild type strain	[69]
BGL2M4	SC5314, *bgl2Δ*::*FRT*/*bgl2Δ*::*FRT*	[70]
BGL2MK2	SC5314*, bgl2Δ::FRT/BGL2Δ::FRT*	[70]
ECM33M4	SC5314*, ecm33Δ*::*FRT*/*ecm33Δ*::*FRT*	[70]
ECM33MK2	SC5314, *ecm33Δ*::*FRT*/*ECM33Δ*::*FRT*	this study
BEM4	SC5314*, bgl2Δ*::*FRT/bgl2Δ::FRT, ecm33Δ::FRT/ecm33Δ::FRT*	[70]
BEMK2	SC5314, *bgl2Δ::FRT/BGL2Δ::FRT, ecm33Δ::FRT/ECM33Δ::FRT*	this study

Table 3-2: Plasmid list

Name	Description	Source/Reference
pSFS2	Contains SAT1 flipper for constructing the revertant strains	[65]
pSFS2.BGL2	Deletes *BGL2* gene	this study
pSFS2.ECM33	Deletes *ECM33* gene	this study
pSFS2.ALS1	Deletes *ALS1* gene	this study

Table 3-3: Primers for BGL2, ECM33, ALS1 deletion and reintegration

Primer name	Sequence 5′–3′
BGL2 upstream forward	AAGGTACCGATGTTGCTATAAATAGTACTCACATGA
BGL2 upstream reverse	AACTCGAGATGGGAAGAAGTGGGTATAAGAATG
BGL2 downstream forward	AAGCGGCCGCTTTAACACTCAATTTCATTTTTTATCGTAACTTGG
BGL2 downstream reverse	AAGAGCTCCATGACAATATATTGTGCCAGTTC
ECM33 upstream forward	AAGGTACCTCTTCCCTCTGAACATTACATCTAG
ECM33 upstream reverse	AACTCGAGGAAGGGAAGAAACAAATGGAAAGG
ECM33 downstream forward	AAGCGGCCGCGGAACCAACACAAAGAAGTATCCAC
ECM33 downstream reverse	AAGAGCTCGGATTGAAAAACAAACGTGCAACTTCG
BGL2 check forward	CTGGTGGTAGTGTAATCATTACACA
BGL2 check reverse	TCCCATGTTGCCAAATTAACATTGC
ECM33 check forward	ACAGAGTTCAAACTTCTACACCATCTG
ECM33 check reverse	CATGGAATCACCGAATATGGAATTGC
SAT1 check forward	GTGAAGTGTGAAGGGGGAGAT
SAT1 check reverse	CTGACGAACAAGCACCTTAGG
on *BGL2* check reverse	AACACCAAGGTTGAAAGCCAAATCACC
on *ECM33* check forward	TCATCTTGAAGACTGAGGGTA
ECM33-Check1-R	TCACTGCACACAATCCATCCA
ECM33-Check2-R	GTTGGAAGCAGTAGCGGTGA
SAT1-Check-F	AGTCTCGAACGAAACAGCG
ECM33-FRT-Frg1– reverse	CTAGAAAGTATAGGAACTTCTTAGAATAAAGCAACACCAA
ECM33-FRT-Frg2- forward	TTGGTGTTGCTTTATTCTAAGAAGTTCCTATACTTTCTAG
BGL2-FRT-Frg1- Reverse	CTAGAAAGTATAGGAACTTCTTAGTTGAATTTACAGTCAA
BGL2-FRT-Frg2- forward	TTGACTGTAAATTCAACTAAGAAGTTCCTATACTTTCTAG
ALS1 upstream forward	AAGGTACCCTCGAATCTGCAATGAAAACG
ALS1 upstream reverse	AACTCGAGATTTCAATTGAGAGGAGGAAAGAGC
ALS1 downstream forward	AAGCGGCCGCGATCACCTTTTTGGCTTGATCT
ALS1 downstream reverse	AAGAGCTCGAAGCGAATGCTAGAAAATGAACTG
ALS1 check forward	CTGAAGGGAGATGGGAGAGAAT
ALS1 check reverse	GGTCAGGTGTGATCGAGGATA

on *ALS1* check reverse	ACCCAAAACAGCATTCCAAG	

3.3 Results and discussion

3.3.1 Construction of revertant strain of *bgl2Δ/ecm33Δ* double mutant

3.3.1.1. Positive control using SAT1 cassette

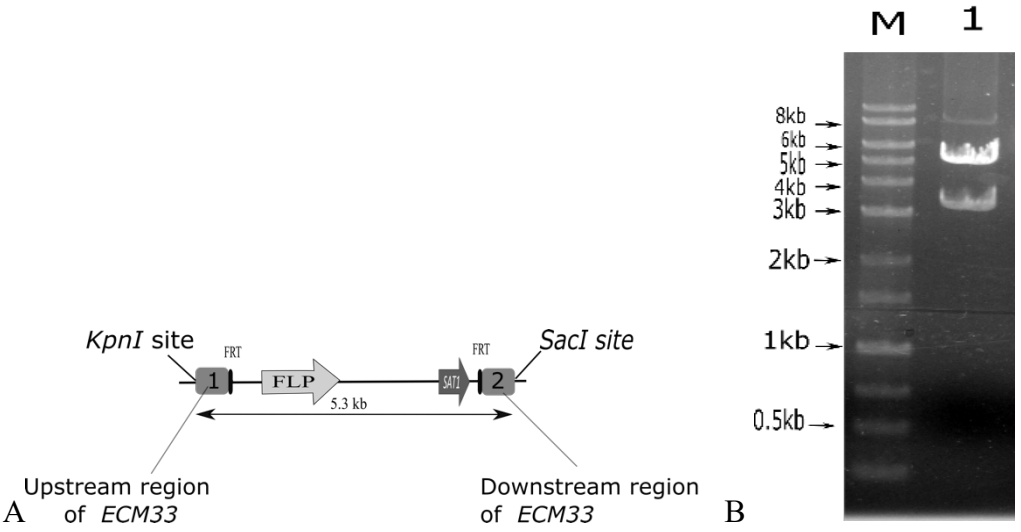

Figure 3-4: (A) Schematic of *ECM33* deletion cassette; (B) Lane 1: *Kpn*I and *Sac*I digested plasmid containing *ECM33* deletion cassette

The aim of this experiment is to use the *ECM33* deletion cassette which was constructed by previous student as a positive control for introduction of *ECM33* re-insertion cassette into *bgl2Δ/ecm33Δ* double mutant. Figure 3-4 showed that the *ECM33* deletion cassette which contains *ECM33* upstream region, *SAT1*-flipper, and the *ECM33* downstream region was generated by cutting the deletion cassette using *Kpn*I and *Sac*I.

3.3.1.2. Amplification of ECM33 fragment 1:

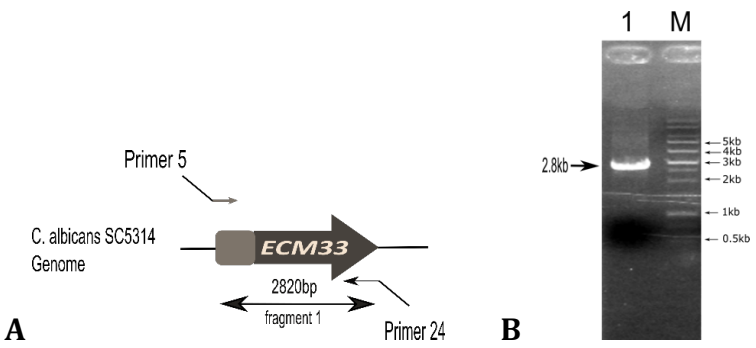

Figure 3-5: (A) Fragment 1 was amplified by primer 5 (*ECM33* upstream forward) and primer 24 (*ECM33* FRT-Frg1-reverse), (B) Lane 1: Fragment 1 was obtained and estimate the concentration of 100 ng/µl, Lane M: Marker 1kb

The aim of this experiment was to amplify fragment 1 which contains the *ECM33* coding sequence and the upstream region for re-insertion of *ECM33* into the double mutant *bgl2Δ/ecm33Δ* by PCR using genomic *C. albicans* as the template. Figure 3-5 showed that the *ECM33* fragment 1 (2820 bp) was successfully amplified with the concentration around 100 ng/µl.

3.3.1.3. Amplification of ECM33 fragment 2:

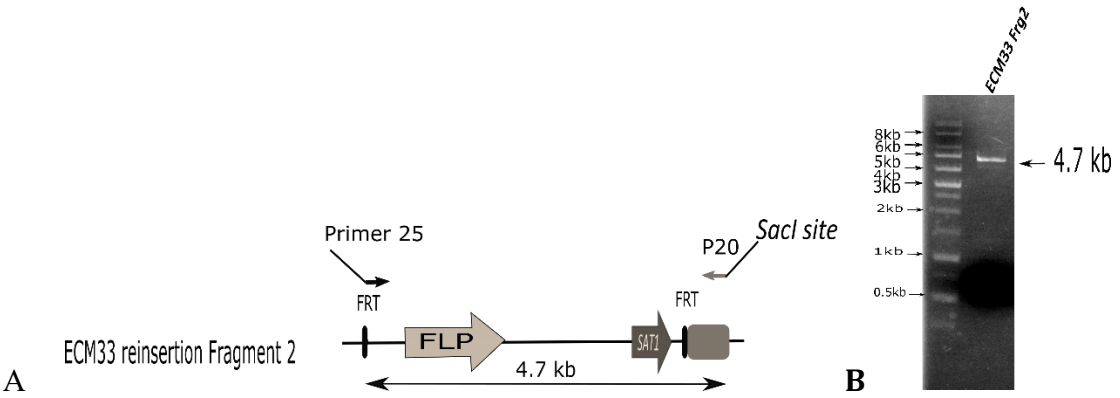

Figure 3-6: Fragment 2 was amplified by primer 25 (*ECM33* FRT-Frg2-forward), and primer 20 (*ECM33* downstream reverse), (B) PCR result Fragment 2 was purified and estimate the concentration of 100 ng/µl

The aim of this experiment was to amplify fragment 2 which contains the *SAT1*-flipper cassette and *ECM33* downstream region for re-insertion of *ECM33* into the double mutant *bgl2Δ/ecm33Δ.* The *ECM33* deletion cassette was used as the template for this step. Primer 24 (*ECM33* FRT-Frg1-reverse) and primer 25 (*ECM33* FRT-Frg2-forward) have an overlapping region so that *ECM33* fragment 2 has an 15 nucleotide overlapping region with and *ECM33* fragment 1. Figure 3-6 showed that the *ECM33* fragment 2 (4.7 kb) was successfully amplified with the concentration around 100 ng/µl.

3.3.1.4. ECM33 Fragment 1 and Fragment 2 for PCR recombination PCR

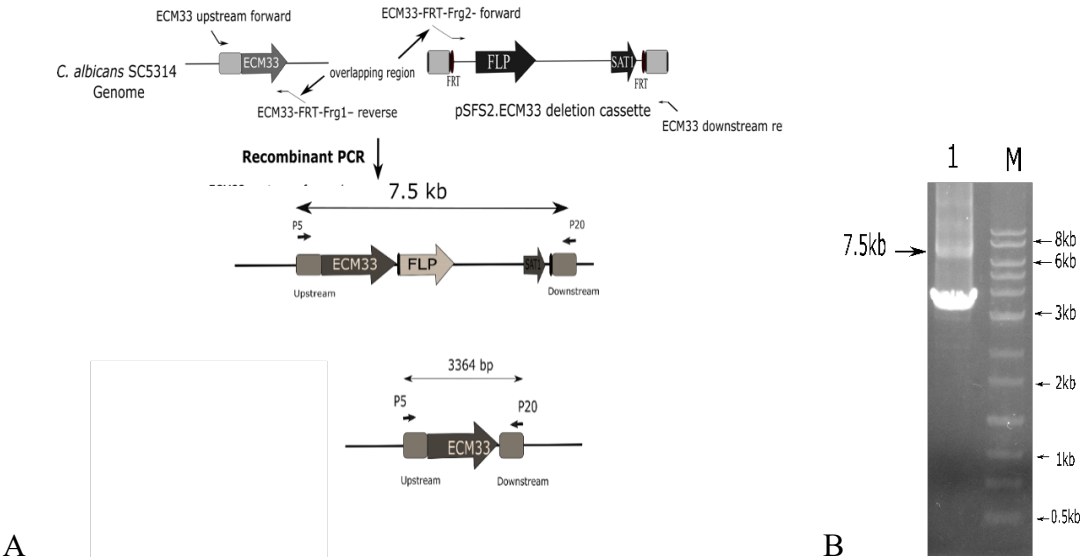

Figure 3-7: *ECM33* re-insertion cassette amplified by primer 5 and 20, templates are *ECM33* fragment 1 and ECM33 fragment 2 (described as the above). B: 1µl loaded of PCR products, M: Marker .

The aim of this experiment was to amplify *ECM33* re-insertion fragment which contains (i) *ECM33* upstream region, (ii) *ECM33* coding region, (iii) *SAT1*-flipper cassette, and (iv) *ECM33* downstream region. In this recombination PCR protocol, *ECM33* fragment 1 and *ECM33* fragment 2 were used as two templates for this reaction, and primer 5 and primer 20 was used to amplify the whole *ECM33* re-insertion cassette of 7.5 kb. Figure 3-7 showed that the *ECM33* reinsertion cassette (7.5 kb) was successfully amplified, however, due to the overlapping region of FRT sequence, there was another small band occurred at 3.3kb. The 7.5 kb fragment was then purified, and 0.18 µg of this cassette was used for the transformation step to re-insert *ECM33* coding region back into *bgl2Δ/ecm33Δ*.

3.3.1.5. Transformation result:

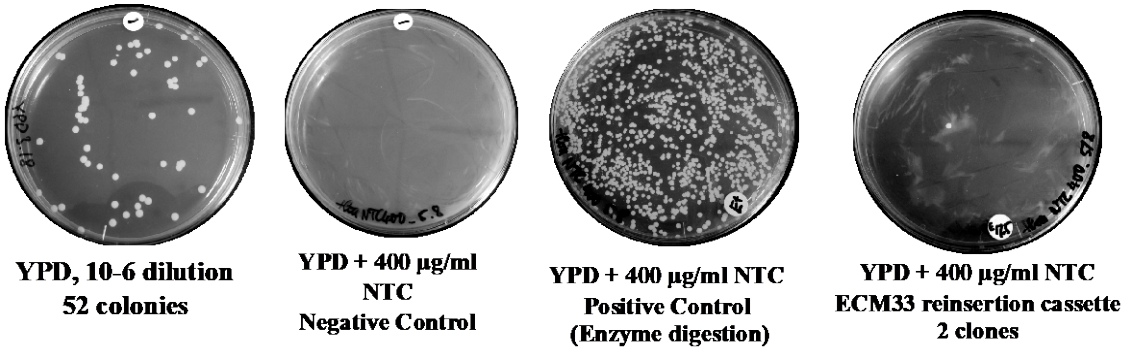

Figure 3-8: Transformation result of *ECM33* re-insertion cassette into bgl2Δ/ecm33Δ double mutant

Based on the dilution methods, about $9x10^8$ cells was plated on each plate of YPD containing 400µg NTC/ml. Using the positive control which was the *ECM33* deletion cassette generated by enzyme digestion method, as the amount of input DNA was around 3.2 µg, 1048 clones on positive control were obtained. Since recombination PCR method produce much little DNA amount, only 0.18 µg DNA of *ECM33* re-insertion cassette was used, 2 clones in ECM33 re-insertion transformation was obtained. There was no other colonies grew in negative control. As can be seen on Figure 3-8, the transformation efficiency of using high amount of DNA was much higher than the recombination PCR method, however, generation of *ECM33* re-insertion cassette by using conventional cloning methods is often considered time-consuming and laborious. Although the recombination PCR provided a much smaller amount of DNA, the *ECM33* re-insertion cassette could be constructed quickly using only PCR amplification protocols. The next steps were to test the 2 obtained clones in order to confirm the re-insertion of *ECM33* coding region into *bgl2Δ/ecm33Δ* mutant.

3.3.1.6. Confirmation of ECM33 reinsertion (1):

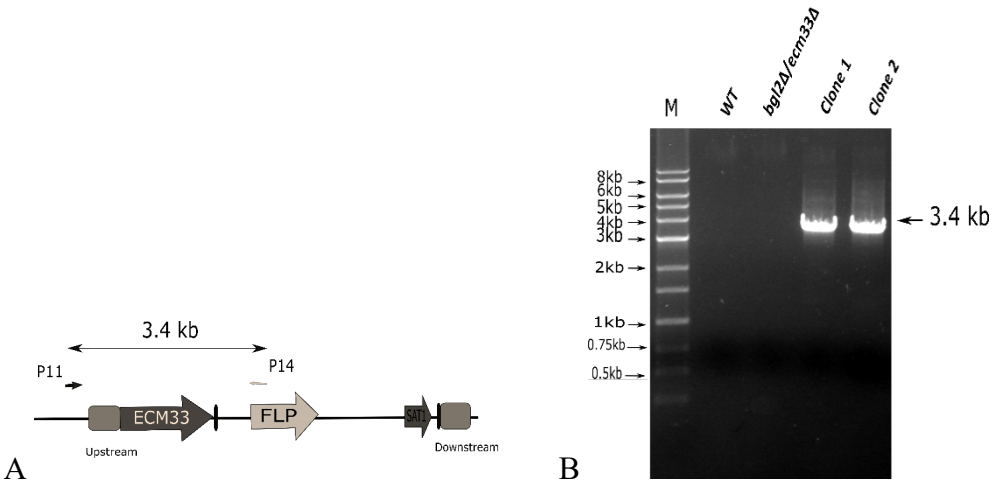

Figure 3-9: Testing the two clones after *ECM33* re-insertion cassette by using primer 11 and primer 14.

Genomic DNA of these two clones mentioned above were extracted, and the region which contained both *ECM33* upstream region and ECM33 coding region attached to *SAT1*-flipper cassette was amplified with primer 11 and 14. As can be seen on Figure 3-9, no band occurred WT and double mutant which showed that only the two obtained cloned contained *SAT1*-flipper cassette, and the expected band was at exactly 3.4kb. This result showed that *ECM33* reinsertion cassette has been successfully intergrated into *bgl2Δ/ecm33Δ* mutant.

3.3.1.7. Confirmation of ECM33 reinsertion (2):

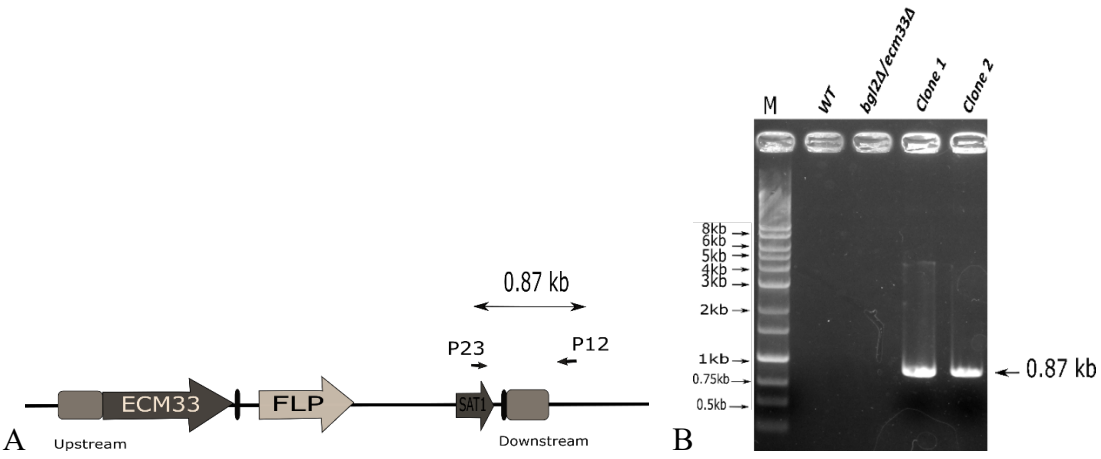

Figure 3-10: : Testing the two clones after ECM33 re-insertion cassette by using primer 23 and primer 12.

The aim of this experiment was to confirm that *SAT1,* the FRT, and *ECM33* downstream region were successfully inserted into *bgl2Δ/ecm33Δ* mutant. Using primer 23 and primer 12, and gDNA of two clones as templates, the results in Figure 3-10 showed that there were no band at WT and *bgl2Δ/ecm33Δ* double mutant which mean *SAT1*-flipper region, and there was an expected band at exactly 0.87 kb from clone 1 and clone 2. This result strongly confirmed that *ECM33* reinsertion cassette has been successfully transformed into *bgl2Δ/ecm33Δ* mutant. *SAT1* gene helped the two clones survive in strong NTC selection pressure.

3.3.1.8. Confirmation of ECM33 reinsertion (3):

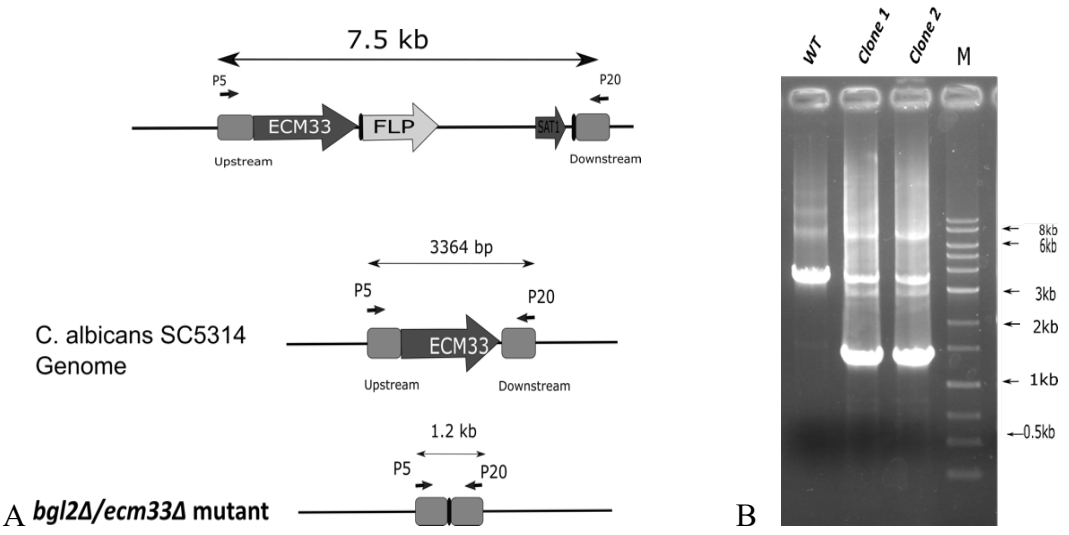

Figure 3-11: Testing the clones after *ECM33* re-insertion cassette

The aim of this experiment was to confirm that the whole *ECM33* re-insertion cassette was successfully transformed into *bgl2Δ/ecm33Δ* mutant. Using primer 5 and primer 20, and gDNA of two clones were used as primers and templates by PCR, the results in Figure 3-11showed that there was a band at 3.3 kb of WT and the two clones which mean *ECM33* upstream region, *ECM33* coding region, and *ECM33* downstream region presented. This result strongly confirmed that *ECM33* reinsertion cassette has been successfully transformed into *bgl2Δ/ecm33Δ* mutant, and *SAT1* gene helped the two clones survive in strong NTC selection pressure. The other bright band at 1.4kb showed that this two transformants still had one allele that lack *ECM33* coding region. The band at 3.3 kb of the two clones showed that some cells had flipped out *SAT1* during the liquid incubation overnight in order to obtain the gDNA. There was one band at 7.5 kb which showed that *ECM33* and *SAT1* cassette has been inserted into *bgl2Δ/ecm33Δ* double mutant. Once again, this result showed that ECM33 reinsertion cassette has been successfully intergrated into *bgl2Δ/ecm33Δ* mutant, and the *SAT1* region helped the 2 clones survive in strong NTC selection pressure. The next important step was to remove the *SAT1* region by cultivating these strains into YPD medium without NTC.

3.3.1.9. Removing SAT1 flipper in 2 clones:

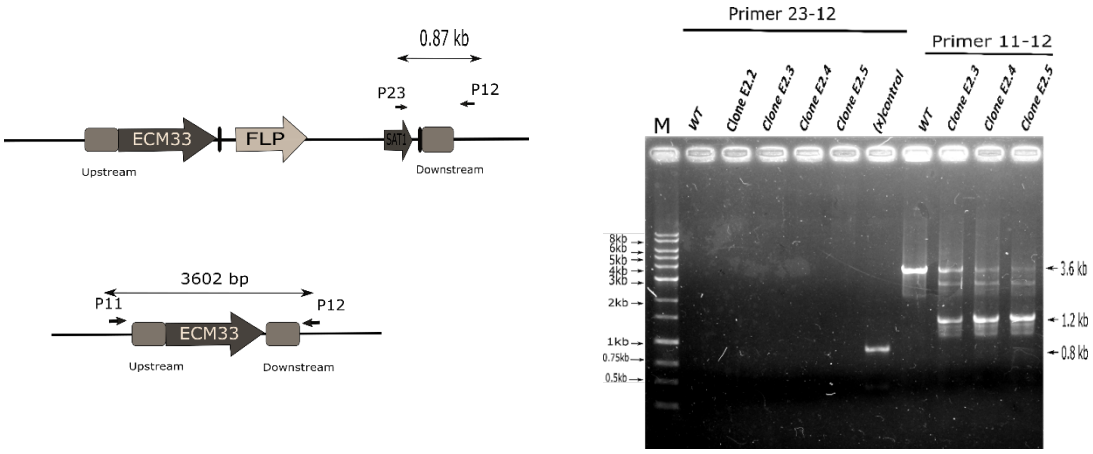

Figure 3-12: Removing SAT1 flipper in clone 1 and clone 2.

As stated before, the clone 1 and clone 2 were inoculated into YPD plates containing 25 µg/ml Nourseothricin for their smaller colony size compared to Nour^R strains, and then the new clones were inoculated in YPD liquid medium without nourseothricin to allow FLP-mediated excision of *SAT1* flippers**.** In summary, 5 colonies were selected and these gDNA were extracted and used as templates for different PCR amplification confirmation procedure. As a result, 4 of them showed no *SAT1* region (check by primer 23-12), and those 4 colonies contained *ECM33* coding region (check by primer 11-12). Finally, the sequence of *ECM33* in this revertant strain was confirmed by sequencing and comparing with *ECM33* sequence of WT SC5314.

In summary, *ECM33* was successfully re-inserted into *bgl2Δ/ecm33Δ*, the genome of this strain was *bgl2Δ::FRT/bgl2Δ::FRT/ecm33Δ::FRT/ECM33::FRT.* Therefore, the next step is to reinsert *BGL2* coding region into this revertant strain. The same procedure of using recombination PCR method was applied to re-insert *BGL2* coding region into *bgl2Δ::FRT/bgl2Δ::FRT/ecm33Δ::FRT/ECM33::FRT* strain.

3.3.2 *Protein concentration*

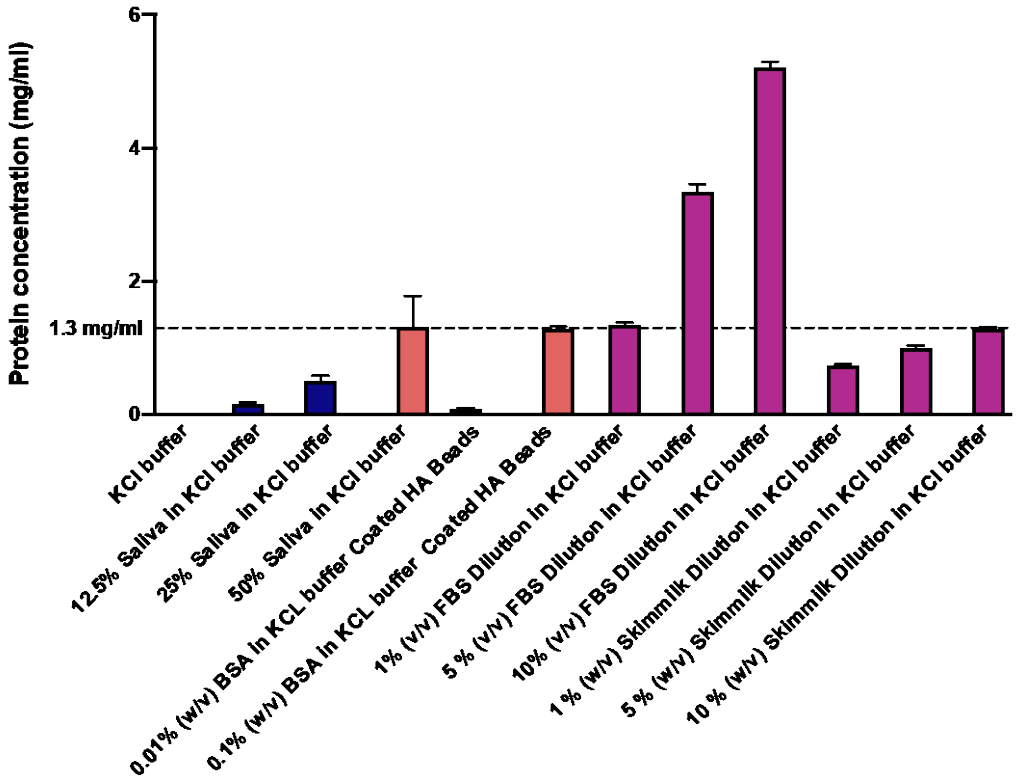

Figure 3-13: Protein concentration determination of different samples. Protein concentration of each sample was determined by Lowry method using (Bio-Rad DC Protein Assay; cat no. 500-0116). The measurements were carried out according to the manufacturer's instructions (Bio-Rad, Hercules, CA). The amount of 50% saliva diluted in KCl buffer is 1.3 mg/ml, therefore, 0.1% BSA coated HA beads, FBS 1/100 dilution, and skim milk dilution 1/10 in KCl buffer were used as controls for C. albicans adherence to hydroxyapatite beads. Results are mean of at three independent experiments.

Saliva plays an important role in oral surfaces and promotes the adhesions of *C. albicans* to the teeth. Therefore, it is important to identify the protein concentration in saliva in oral adhesion studies. In addition, to determine whether this promotion of adherence was specific to salivary proteins, other proteins including bovine serum albumin (BSA), fetal bovine serum (FBS), and skim milk (SM) were also recruited. In order to maintain the same concentration of proteins used in each experiment, the protein concentration of 50% saliva in KCl buffer were defined as 1.3 mg/ml, and therefore, the

same amount of protein in other solutions were determined and used for the study. In particular, 0.1% BSA in KCl buffer, 1% FBS dilution, and 10% skim milk with dilution in KCl buffer were used for next experiments.

3.3.3 *Adhesion assay with various protein coated HA beads*

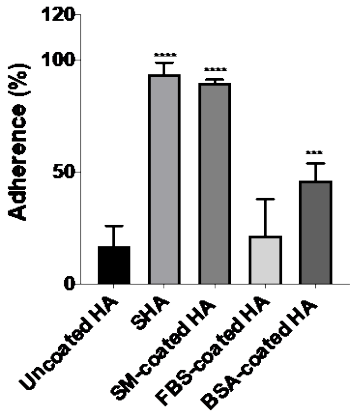

Figure 3-14: Adherence of C. albicans SC5314 cells to HA beads coated with various proteins. C. albicans cells (1 x 10^7) grown to stationary phase in GSB were incubated with uncoated, BSA-, FBS-, saliva- or skim milk-coated HA beads (12 mg) for 1 h at 28°C The percentage of the input C. albicans cells attached to the HA beads was calculated. Results are the means of at least three independent experiments.

All oral surfaces are coated with a salivary protein pellicle. Therefore, it is important to include saliva in oral adhesion studies. In this study, I used hydroxyapatite (HA) beads as a model for the tooth surface and so investigated the influence of human saliva on adherence of *C. albicans* cells to HA beads. HA beads were incubated in human saliva for 1 h (to make SHA beads), washed and then *C. albicans* cells (SC5314) were added. It was found that 93.8±4.9 % of the input yeast cells bound to the SHA beads, whereas only 17.0±9.0 % of SC5314 cells bound to uncoated HA beads (Figure 3-14). This result demonstrated that saliva promoted *C. albicans* adherence HA beads significantly (increased adherence 4.5-fold), and was consistent with our previous results [5,14]. To determine whether this promotion of adherence was specific to salivary proteins, HA beads were coated with other protein mixture: bovine serum albumin (BSA), fetal bovine serum (FBS), and skim milk (SM). It was found that FBS and BSA, unlike saliva,

did not promote adhesion greatly (by 0.28-fold and 1.74-fold, respectively). Interestingly, SM promoted the adhesion of *C. albicans* to HA beads to almost the same extent as saliva (4.3-fold). From these results, I inferred that saliva, and skim milk, act as receptors for *C. albicans* adherence to HA beads.

3.3.4 *Cells susceptibility:*

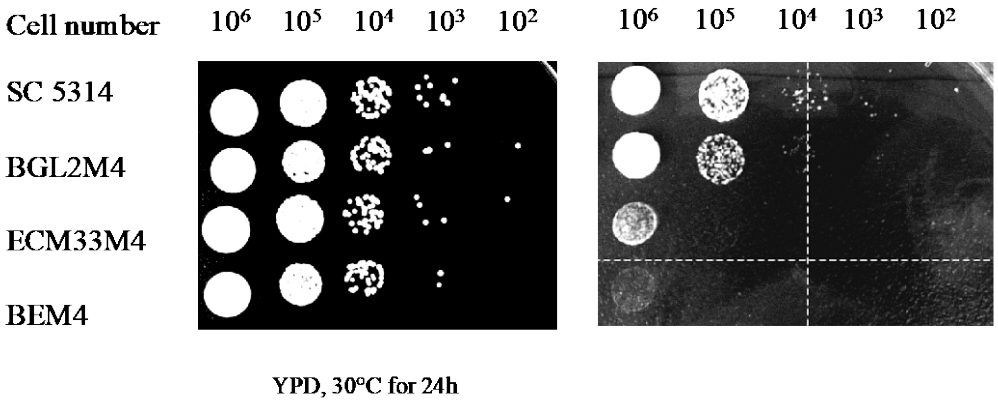

Figure 3-15: *C. albicans* strains deleted in BGL2 and ECM33 genes are hypersensitive to CFW. C. albicans SC5314, BGL2M4, ECM33M4, and BEM4 cells were serially diluted and spotted onto YPD agar plates and YPD agar plates containing 12.5 µg/mL CFW and incubated at 30 °C for 24 h.

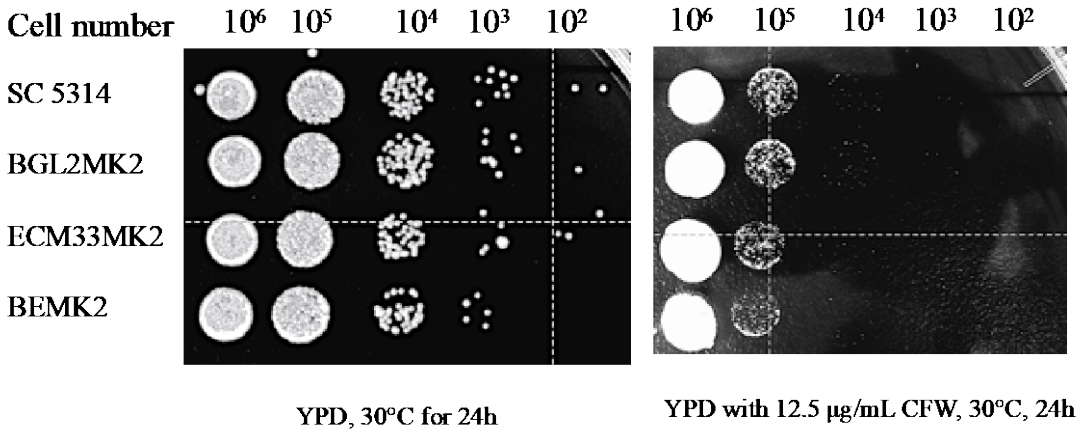

Figure 3-16: *C. albicans* strains SC5314 and revertant strain BGL2MK2, ECM33MK2 and BEMK2 restore sensitivity of CFW. Those strains were serially diluted and spotted onto YPD agar plates and YPD agar plates containing 12.5 µg/mL CFW and incubated at 30 °C for 24 h.

To investigate the impact of gene disruptions on *C. albicans* cell wall integrity, Itested the sensitivity of null mutants to calcofluor white (CFW), a chemical which interferers with cell wall construction.[71] *C. albicans* SC5314 (wild type), BGL2M4

(*bgl2*Δ), ECM33M4 (*ecm33*Δ), and BEM4 (*bgl2*Δ*, ecm33*Δ) cells were serially diluted and inoculated on YPD agar and on YPD agar containing CFW (12.5 µg/mL), and incubated for 24 h at 30°C. All of the null mutants were more susceptible to CFW than the wild type parental strain (Figure 3-15). BEM4 (*bgl2*Δ*, ecm33*Δ) showed the highest CFW sensitivity. This result indicated that when these cell surface mannoproteins are not expressed there is a decrease in the integrity of the *C. albicans* cell wall.

3.3.5 *Adhesion assay:*

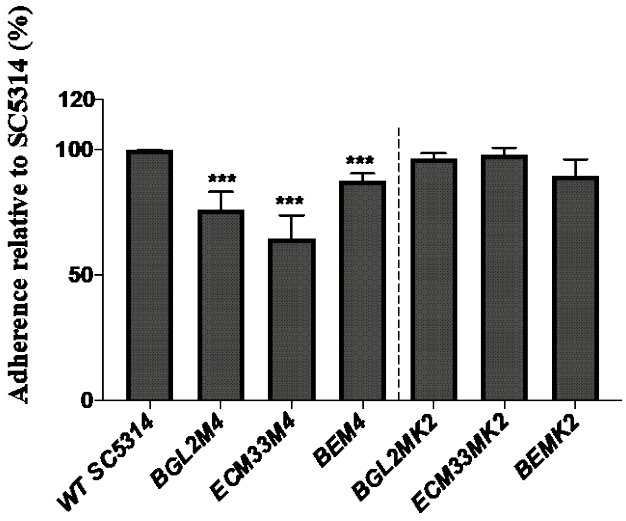

*Figure 3-17: Adherence of wild type cells and homozygous BGL2 and ECM33 single and double mutants to SHA beads. The C. albicans cells (1 x 107) and SHA beads (12 mg) were incubated for 1 h at 28°C.Percent attached cells is calculated relative to SC5314 which is set to 100%. Results are means of at least three independent experiments. Statistical significance is shown (**p > 0.01 ***p > 0.001).*

To determine whether Bgl2 or Ecm33 might be adhesins involved in adhesion to saliva-coated HA beads, the adherence of *C. albicans* null mutant strains to SHA beads was compared to that of the parental strain SC5314. The null mutants with the genes restored, BGL2MK2, ECM33MK2, and BEMK2 were used in adherence assays as controls. Deletion of both alleles on either the *BGL2* or the *ECM33* gene reduced *C. albicans* adherence by more than 20% compared to the adherence of the wild type strain. Returning the *BGL2* or the *ECM33* gene to the null mutants (BGL2MK2, ECM33MK2) restored their ability to bind to SHA beads (Figure 3-17). These results suggested that both Bgl2 and Ecm33 confer the ability to adhere to SHA beads. Surprisingly, I found

that disruption of the *ECM33* gene in the *BGL2* null mutant did not reduce adhesion further but in fact increased adhesion to a level that was not significantly different to that of the wild type (Figure 3-17; BEM4). Returning the *BGL2* and *ECM33* genes to the double disruptant BEM4 did not alter adhesion to SHA beads significantly (Figure 3-17; BEMK2). This increase in adherence when two potential adhesins had been deleted led me to hypothesize that there was a compensatory induction of expression of another adhesin in the *bgl2*Δ*, ecm33*Δ double mutant.

3.3.6 *Confocal images:*

In order to visualize the adhesion properties of *C. albicans* to saliva-coated beads, confocal images of different cell numbers of *C. albicans* SC5314 cells adhered to saliva-coated beads were obtained. As can be seen on Figure 3-18, the higher the OD_{600nm} number of cells added to the adhesion assay, the more cells adhered to the beads surfaces.

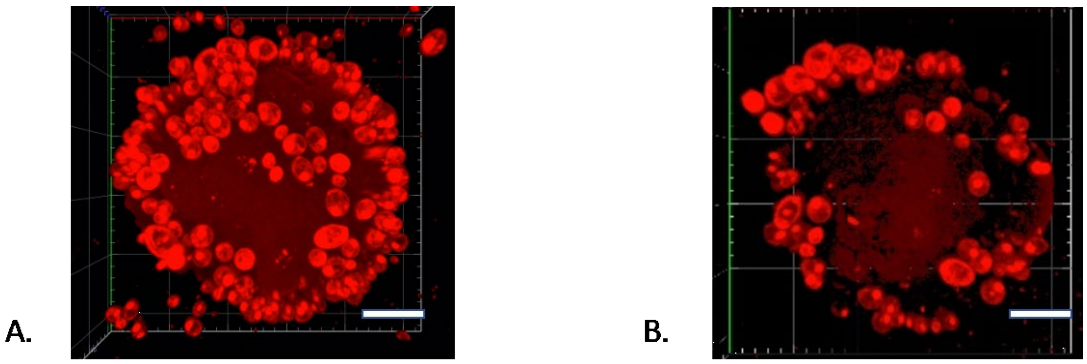

Figure 3-18: **Confocal microscopy Z-stack imaging to visualize *C. albicans* adhering to saliva-coated beads.** The *C. albicans* SC5314 cells that attached to hydroxyapatite beads were stained with NR. Confocal images were captured with a Zeiss LSCM780 laser scanning microscope. Scale-bar: 20 µm. (A) Bead that had been incubated with *C. albicans* SC5314 cells at $OD_{600nm} = 1$, (B) Bead that had been incubated with *C. albicans* SC5314 cells at $OD_{600nm} = 0.1$.

Theoretically, IMARIS (Bitplane, Inc.) software could be used for directly counting the numbers of cells that attached to each saliva-coated beads. This method

could be the one of the most accurate and direct way to quantitatively identify how many *C. albicans* cells interact with saliva-coated HA beads or other protein-coated HA beads. However, this methods is time consuming and requires a great deal of memory on disks. Therefore, quantitatively defining the number of cells that adhered to saliva coated beads surfaces by measuring the fluorescence signals of the unattached cells and total input cells gives more robust results. More detailed steps for constructing the z-stack 3D images was described in Figure 3-18.

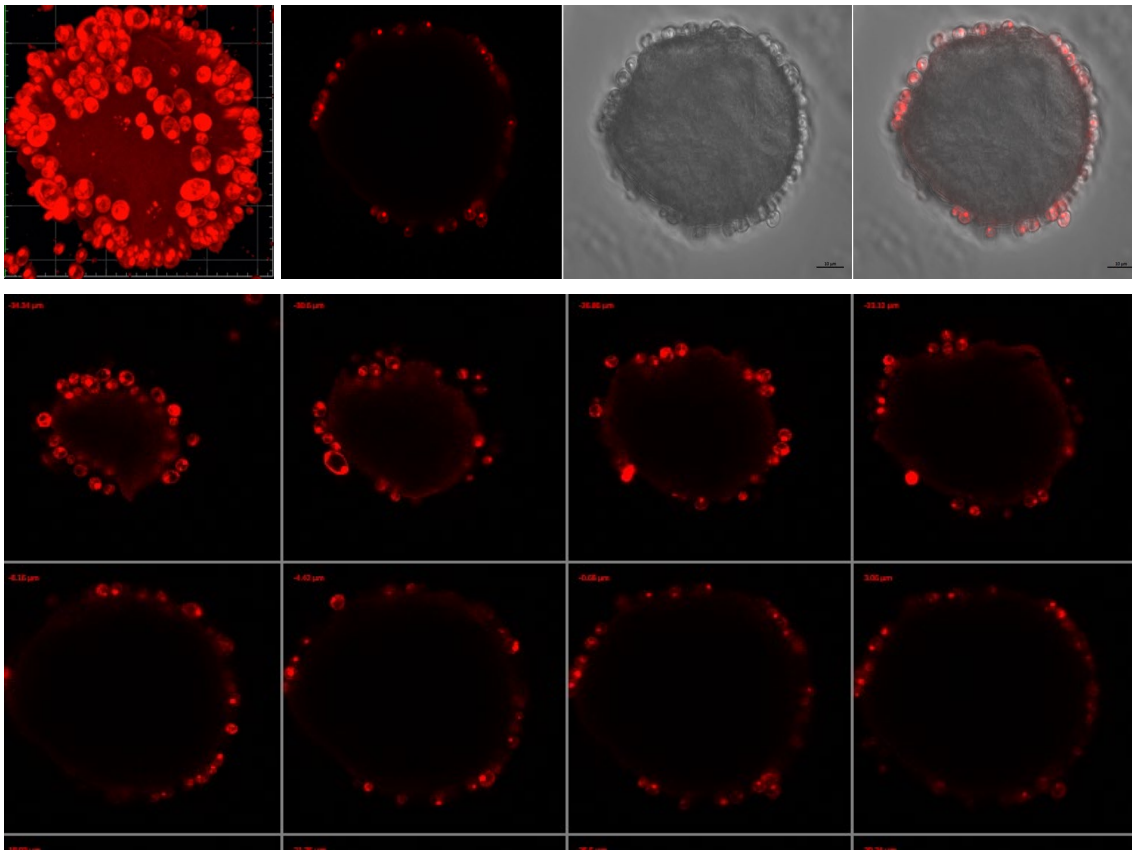

Figure 3-19: Confocal microcopy Z-stack imaging to visualize *C. albians* to saliva-coated beads. Z-stack images collected at 83.3 µm sections, total 246 slides were collected to construct the 3D image. Detection was made using excitation channel at 559nm, emission at 636 nm, the red fluorescence indicates yeast cells attached to saliva-coated beads. IMARIS (Bitplane, Inc.) software was used for confocal z-stack 4D and 3D reconstruction and counting the cells numbers.

3.4 Conclusion

C. albicans is an opportunistic pathogen responsible for 50-90% of oral candidiasis although it is considered commensal microorganism in healthy individuals[37]. In addition, it was found that *C. albicans* can grow in human saliva without addition of glucose in more than 400 hours[72]. The surface mannoproteins, which constitute the outer layer of cell wall in *C. albicans*, are believed to be the most likely candidates for putative adhesins. Previous studies have shown that two *C. albicans* cell wall proteins, Bgl2 and Ecm33, may mediate the interaction between the yeast and human teeth hydroxyapatite via saliva in human oral cavity[7]. This interaction is an initial event in the colonization of the oral cavity, which leads to oral diseases such as candidiasis and denture stomatitis yeast. The aim of this chapter was to investigate the roles of these two cell wall proteins Bgl2, Ecm33 in the adherence of *C. albicans* to saliva-coated hydroxyapatite as a model for the tooth surface. Since, *C albicans BGL2* and *ECM33* null mutants have been generated by previous students, these revertant strains of BEMK2 and ECM33MK2 were generated from *bgl2Δ/ecm33Δ* by using the *SAT1*-flipper gene disruption method. The novel method for measuring *C. albicans* adherence, based on labeling the yeast with the NR, was used to investigate the adherence of *C. albicans*. Labelling cells using NR is an effective way to count the cell and either by undirect method by quantifying the unattached cells or by direct method using 3D confocal images studies.

Hydroxyapatite beads coated with various proteins were incubated with *C. albicans* cells to determine the adhesion of the yeast to the beads, and then the cells attached to beads were stained with NR. The adhesion of the *bgl2*Δ and the *ecm33*Δ null mutants to saliva-coated hydroxyapatite beads was 76.4 % and 64.8% of the wild-type strain, respectively. Interestingly, the adhesion of the *bgl2*Δ*, ecm33*Δ mutant (87.7 %) was higher than that of both single mutants. This result suggested that the double mutant had developed a compensatory response to reduced cell wall integrity. In depth investigation

about the mechanism and possible compensatory phenomenon was continued in the next chapter.

CHAPTER 4. THE ROLE OF *ALS1* AS A KEY PROTEIN OF THE COMPLEMENTARY SYSTEM IN *BGL2* AND *ECM33* DOUBLE MUTANT OF *C. ALBICANS*

4.1 Introduction:

In the oral cavity, saliva performs a number of functions. It contains antimicrobial factors such as lysozyme, histatins, and amylase (to begin the breakdown of starch), but it also contains several nutrients that microorganisms can metabolise. Thus, saliva enables microbial colonization of the mouth and it has been reported that *C. albicans* could utilize salivary constituents for the growth and the colonization of tooth surfaces.[14,32] Salivary proteins adsorb to oral surfaces to form the acquired pellicle. The main component of human tooth enamel is hydroxyapatite and the adsorption of salivary proteins to hydroxyapatite can promote microbial adhesion. Salivary basic proline-rich proteins (bPRP) within the pellicle are thought to act as main receptors for the adherence of *C. albicans.*[73,74,19]

C. albicans possesses many surface proteins involved in adhesion that bind to host receptors. These include the ALS protein family, the Sap protein family, Hwp1, Ywp1, Pra1, and Csh1 which have been well studied.[8–13] In addition, surface mannoproteins, which constitute the outer layer of the cell wall, are thought to be major candidates for putative adhesins. Therefore, it is important to investigate the functions of cell wall mannoproteins in order to understand the adhesion mechanisms, biofilm formation and virulence of *C. albicans*.[3,7,14,15]

ALS family and adhesion

The *ALS* (agglutinin-like-sequence) gene family of *C. albicans* encodes a large cell surface glycoprotein containing three domains structure that are critical in the process of adhesion to host surfaces. The *ALS* family includes eight genes, from *ALS1* to *ALS7* and *ALS9,* whereas *ALS3* and *ALS8* are the same gene but reside at separate loci. In *C. albicans,*

ALS genes are found on three different chromosomes: chromosomes 3 (*ALS6* and *ALS7),* R (*ALS3),* and 6 (*ALS1, ALS2, ALS4, ALS5).*[14,27,75–77] Each Als protein contains a relatively conserved N-terminal domain, a central domain consisting of tandem copies of a highly conserved 108-bp unit of a repeated motif, and a serine-threonine-rich C-terminal domain that is relatively variable across the family (Figure 4-1).[8] The tandem repeats is a potential site for allelic variation, which occur within the same cells with differences in the two alleles borne by the diploid cell of between strains. For example, in the wild type *C. albicans* SC5314, the larger alleles of *ALS1, ALS2, ALS4, ALS5,* and *ALS9* are on one chromosome, while smaller alleles are on the other. The last two domains have multiple glycosylation sites that are considered to lead N-terminal binding domain from the attachment site.

Figure 4-1: Schematic of Als protein structure. Line drawing of a representative Als protein, using C. albicans Als3 as the example.[78] *The various domains are NT: N-terminal domain, T: T domain, TR: Tandem repeats, and CT: C-terminal domain.*

In the above chapter, I investigated the roles of Bgl2 and Ecm33 in the adhesion of *C. albicans* to saliva-coated hydroxyapatite by generating null mutants of these genes and using a novel NR staining assay. I found that the two cell wall proteins Bgl2 and Ecm33 contribute to interaction between *C. albicans* and saliva-coated surfaces. Interestingly, I observed that the deletion of both genes triggered compensatory responses in *C. albicans* cells. In this chapter, the *ALS* protein family, encoding a major GPI-anchored adhesin, was investigated whether there was a connection between the roles of those proteins in the adhesion ability of *C. albicans* to saliva-coated HA beads.

4.2 Material and Methods:

4.2.1 *Strains and growth conditions:*

C. albicans strains used in this study were listed in Table 3-1. Yeast cells were maintained in YPD medium (1% [w/v] yeast extract, 2% [w/v] peptone, 2% [w/v] glucose) containing 50 % [v/v] glycerol at -80°C. They were routinely grown in glucose/salts/biotin medium (GSB) (10 [g/l] glucose, 1.0 [g/l] $(NH_4)_2SO_4$, 2.0 [g/l] KH_2PO_4, 0.05 [g/l] $MgSO_4.7H_2O$, 0.05 [g/l] $CaCl_2.2H_2O$; and 0.05 [mg/l] biotin)[67].

Table 4: Strain list in this chapter

Strain	Genotype	Source/Reference
SC5314	Wild type strain	[69]
ALS1M4	SC5314, *als1Δ::FRT/als1Δ::FRT*	[79]
ABM4	SC5314, *bgl2Δ::FRT/bgl2Δ::FRT, als1Δ::FRT/als1Δ::FRT*	[79]
AEM4	SC5314, *als1Δ::FRT/als1Δ::FRT, ecm33Δ::FRT/ecm33Δ::FRT*	[79]
ABEM4	SC5314, *als1Δ::FRT/als1Δ::FRT, bgl2Δ::FRT/bgl2Δ::FRT, ecm33Δ::FRT/ecm33Δ::FRT*	[79]

4.2.2 *Gene disruption and re-insertion*

C. albicans gene sequences were obtained from the Candida Genome Database web site (http://www.candidagenome.org/cgi-bin/seqTools). The *als1* null mutant (ALS1M4); *als1, bgl2* null mutant (ABM4); *als1, ecm33* null mutant (AEM4); and *als1, bgl2, ecm33* triple mutant (ABEM4) had been constructed previously by our group using the *SAT1* flipper method.[7]

4.2.3 *qRT-PCR*

C. albicans cells were cultured in YPD medium at 30°C overnight. The culture was diluted to an OD_{600} of 0.2 in 50 ml fresh YPD medium, and incubated at 30°C until the OD_{600} reached 1–2. Total RNA was extracted using the glass beads lysis method[68]. The RNA samples were used as templates to synthesize first strand cDNA using the ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (Toyobo, Osaka, Japan). Real time PCR was performed using the THUNDERBIRD SYBR qPCR Mix (Toyobo) under the following conditions: 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C, 30 seconds at 56°C, 1 min at 72°C. *ACT1* mRNA was used for normalization, and gene expression levels were determined using Applied Biosystems software and the ΔΔCt method.

Figure 4-2: Primer for qRT PCR

Primer name	Sequence 5′–3′
ACT1 forward	TGTGTAAAGCCGGTTTTGCC
ACT1 reverse	TTGGATTGGGCTTCATCACC
ALS1 forward	AGCGGTTCTCATGAATCAGC
ALS1R reverse	CAGAAGAAACAGCAGGTGATGG
ALS2 forward	AGCGGTTCTCATGAATCAGC
ALS2 reverse	CAGAAGAAACAGCAGGTGATGG
ALS3 forward	TCGTTGACATGGACAAGAGC
ALS3 reverse	ACCAGCCCAAAACAGCATTC
ALS4 forward	TGCCGGTTATCGTCCATTTG
ALS4 reverse	ATGTGAAAGGTGCACGTTGC
ALS5 forward	ACCCTGTGTTGACAACAAGC
ALS5 reverse	ATAACCACTGTCGCAGTTGC
ALS6 forward	TCCAATGCTGGCAATTACGC
ALS6 reverse	AATGACCAACCCAACACAGC
ALS7 forward	GCTGTTTTAGGTTGGAGCTTGG
ALS7 reverse	TTGAAAACGCAGGGCATGAC
ALS9 forward	ATCGACCTTTTGTGGATGCG

4.2.4 *Assay of* C. albicans *adhesion to SHA beads*

One colony of yeast cells of SC5314 (wild type), ALS1M4(*als1*Δ), ABM4 (*als1*Δ/*bgl2*Δ), AEM4 (*als1*Δ/*ecm33*Δ), and ABEM4 (*als1*Δ/*bgl2*Δ/*ecm33*Δ) were pre-cultured in 5mL GSB medium overnight at 30°C. Cells were harvested by centrifuging 5000 rpm for 5 minutes and then diluted in KCl buffer to OD_{600} of 1. Adhesion assays were done in the same method described in chapter 3.

4.2.1 *Fluorescence signals of different strains*

One yeast colony of each 8 strains were inoculated in GSB medium overnight to OD_{600} =4-5. Cell pellet were harvested by centrifuging at 5000 rpm in 5 minutes and then were suspend in KCl buffer to $OD_{600\ nm}$ =1. Cells were then stained with NR in 10% DMSO with final concentration of 1 µg/ml. After 1 hour, two hundred µl of cell suspension were put in to 96 well plate and fluorescence signals were measured with excitation wavelength of 530 nm and 570 nm using Varioskan Lux system.

4.2.2 *Growth curve of different strains in GSB medium*

Overnight culture of 8 strains in YPD medium were harvested by centrifuging at 5000 rpm in 5 minutes and then were diluted to $OD_{600\ nm}$ =0.01 and inoculated in GSB medium with shaking 200 rpm. The OD_{600}nm values were measured after 2-4 hours and the growth curve were constructed.

4.2.3 *The susceptibility of* C. albicans *strains to Calcofluor white (CFW)*

One colony of yeast cells of SC5314 (wild type), ALS1M4(*als1*Δ), ABM4 (*als1*Δ/*bgl2*Δ), AEM4 (*als1*Δ/*ecm33*Δ), and ABEM4 (*als1*Δ/*bgl2*Δ/*ecm33*Δ) cells were cultured in 5 mL YPD medium overnight at 30°C and diluted in YPD to an OD_{600} of 1. The cells were then serially diluted (10-fold) in YPD medium. Three microliters of each dilution were spotted onto YPD plates containing 12.5 µg/mL CFW, and incubated at

30°C for 24h. Susceptibility of cells to CFW was indicated by their inability to grow on this medium.

4.3 Results and discussion

4.3.1 ALS1 *gene was over-expressed in the* bgl2Δ, ecm33Δ *strain*

Since the BEM4 (*bgl2*Δ, *ecm33*Δ) strain showed higher adhesion to SHA beads than BGL2M4 (*bgl2*Δ) or ECM33M4 (*ecm33*Δ), I examined the expression of a family of important *C.* albicans adhesin genes in these strains. The *ALS* (Agglutinin-Like Sequence) genes encode a family of GPI-anchored cell surface glycoproteins, eight of which (Als1-Als7 and Als9) have been reported to be involved in *C. albicans* adhesion.[27,75,80] Interestingly, qRT PCR indicated that *ALS1* mRNA was more than 6 fold higher in the *bgl2*Δ*, ecm33*Δ double mutant BEM4 (Figure 4-3) than that of *C. albicans* SC5314. In contrast, there was no significant increase in expression of any of the other *ALS* gene in BGL2M4, ECM33M4 and BEM4 (Figure 4-3). This indicates that the deletion of the *BGL2* and *ECM33* genes induces expression of *ALS1* which may be responsible for the increase in adherence of *C. albicans* to SHA beads.

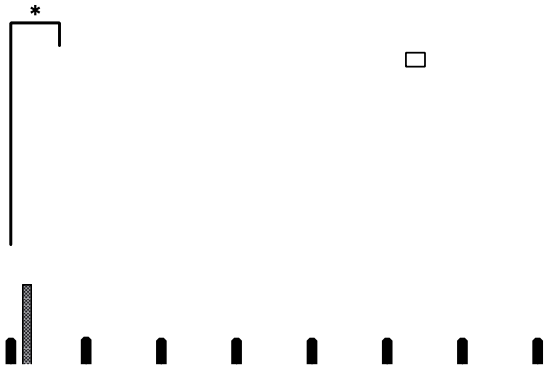

Figure 4-3: The transcriptional expression of ALS family genes of *C. albicans* wild type and mutant strains. Four strains of C. albicans were cultured in YPD medium and then total RNA was isolated for qRT-PCR assay. *C. albicans* ACT1 gene was used as internal control in this experiment. These results are mean of at least three independent experiments.

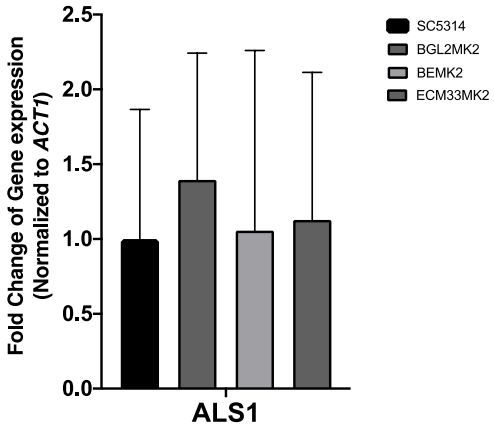

Figure 4-4: The transcriptional expression of ALS family genes of *C. albicans* wild type and mutant strains. Four strains of C. albicans were cultured in YPD medium and then total RNA was isolated for qRT-PCR assay. *C. albicans* ACT1 gene was used as internal control in this experiment. These results are mean of at least three independent experiments.

4.3.2 *The introduction of* ALS1 *gene disruptions in mutant strains and investigation of their effect on adherence to SHA beads*

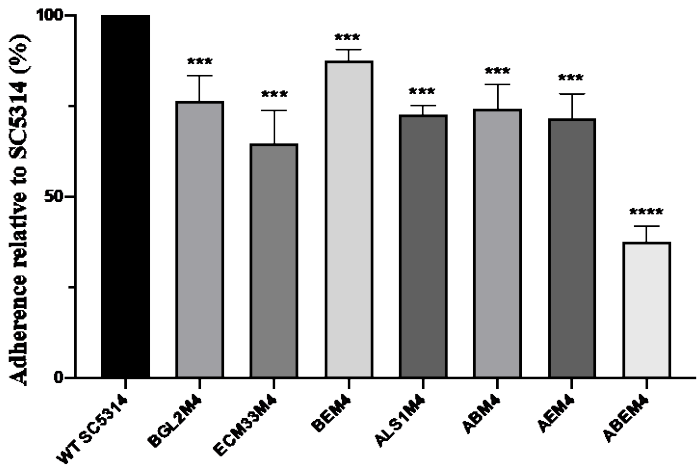

Figure 4-5: Adherence of ALS1, BGL2, and ECM33 single, double, and triple null mutants compared to the wild type strain *C. albicans* SC5314. The *C. albicans* cells (1 x 10^7) and SHA beads (12 mg) were mixed for 1 h at 28°C. After the incubation, the beads were washed twice with KCl buffer and all input cells and washed cells were stained with NR in KCl buffer containing 10% DMSO at the final concentration of 1 µg/ml. Results are means of at least three independent experiments. Statistical significance is shown (**p > 0.01 ***p > 0.001).

From these results, previously, *als1*Δ (ALS1M4), *als1*Δ and *bgl2*Δ (ABM4), *als1*Δ and *ecm33*Δ (AEM4), and *als1*Δ, *bgl2*Δ, and *ecm33*Δ (ABEM4) mutants were constructed from SC5314, BGL2M4, ECM33M4 and BEM4 strains.[79] After confirming the disruption of the *ALS1* gene in each strain, the adherence of these strains to SHA beads was measured. Deletion of *ALS1* in SC5314 (to form ALS1M4) reduced adherence ability and this percentage was as similar as (72.6±2.5 % of SC5314) as deletion of *BGL2* (76.4±13.0 %) or *ECM33* (64.8±9.1 %) (Figure 4-5). Deletion of *ALS1* in either a *bgl2*Δ or a *ecm33*Δ background did not reduce adhesion to SHA beads further (Figure 4-5). As predicted, however, deletion of *ALS1* in the *bgl2*Δ, *ecm33*Δ double disruptant (to form ABEM4) resulted in a significant reduction in adherence to SHA beads (37.6±4.3 % of

SC5414 adherence; Figure 4-5). These results indicated that reliance on Als1 for adherence to SHA beads is only triggered when both Bgl2 and Ecm33 are inactivated, and highlight the importance of all three proteins for the attachment of *C. albicans* cells to tooth surfaces via saliva in the oral cavity.

4.3.3 *Fluorescence signals of different strains:*

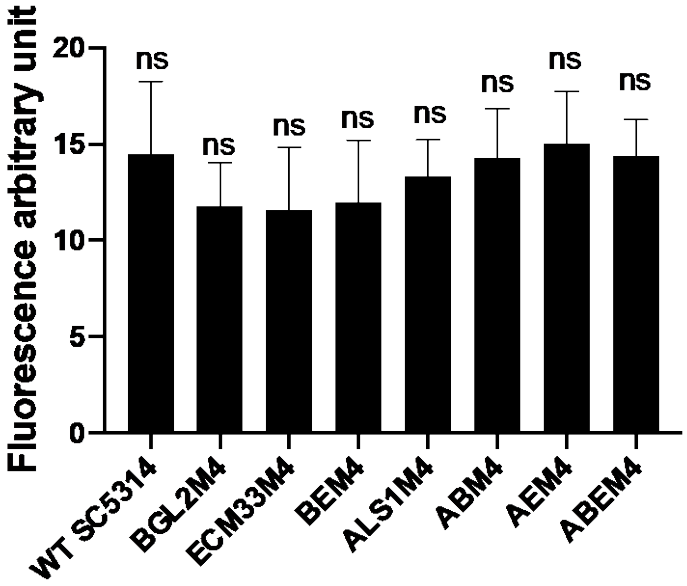

Figure 4-6: Fluorescence intensity of different strains at OD_{600nm} =1

Although the adhesion ability of each strain was measured by the ratio of the fluorescence intensity of attached cells vs. total input cell, therefore, the errors were already minimized. However, there might be a difference of lipid concentrations between *C. albicans* WT SC5314 and all other mutants. Therefore, fluorescence intensity of all strains constructed in this study were compared. Yeast cells of all strains were inoculated in GSB medium to OD_{600nm} = 4-5, these cells were harvested and diluted cells to OD_{600nm} = 1. Fluorescence intensity of stained cells was measured at excitation 530 nm and emission 570 nm. This result in Figure 4-6 showed that most cells have similar fluorescence signals with each other which might answer the questions of the level of lipid contents in the cells. By deleting either one, two, or three different genes that play

roles in cell membrane, the concentration of lipid did not change drastically, therefore, the fluorescence signals of NR stained are similar between those strains.

4.3.4 *Growth curve of different strains in GSB medium:*

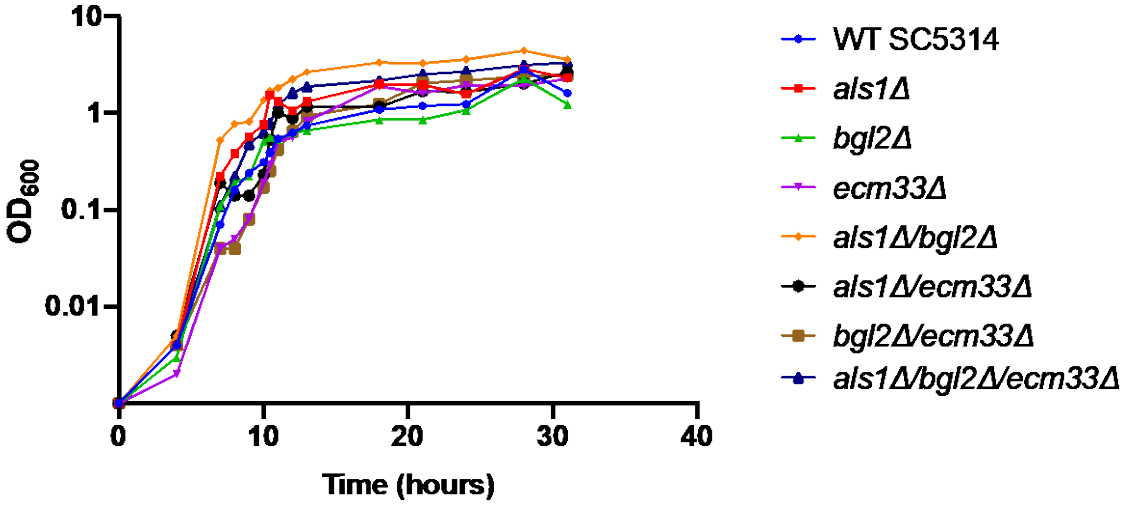

Figure 4-7: Growth curve of different strains in GSB medium

The aim of this experiment is to evaluate possible effect of deletion *BGL2, ECM33, ALS1,* double mutants and triple mutants of those genes to the cell growth. Overnight culture of these 8 strains were diluted to $OD_{600\ nm}$ = 0.01 and inoculated in GSB medium with shaking 200 rpm. The $OD_{600\ nm}$ values were measured after 2-4 hours and the growth curve. As shown in Figure 4-7, all strains had similar growth curve, which indicated that there were no strong effect on the cell growth by deleting those three genes in *C. albicans* SC5314 cells.

4.3.5 *Cells susceptibility*

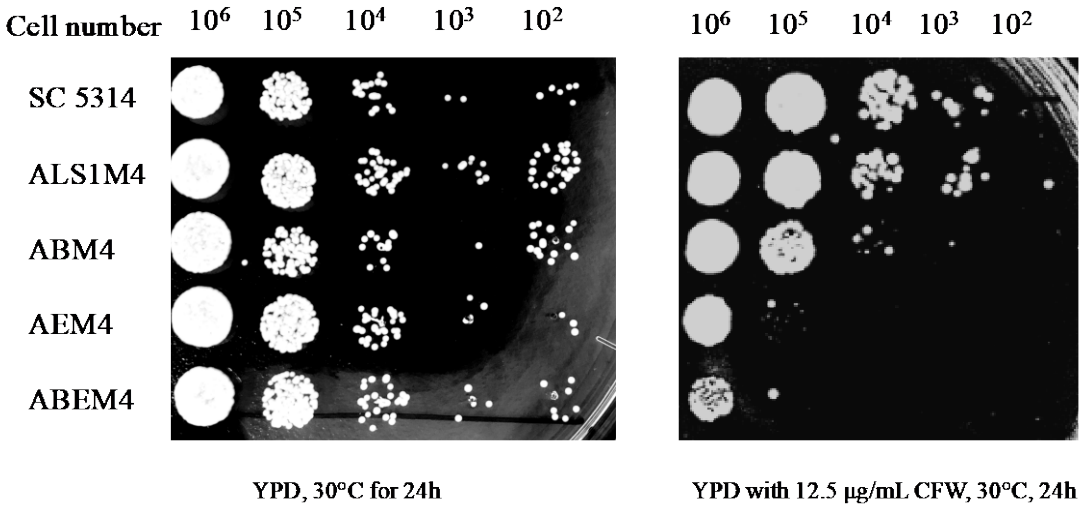

Figure 4-8: The susceptibility of C. albicans strains to Calcofluor white (CFW). SC5314 (wild type), ALS1M4(*als1*Δ), ABM4 (*als1*Δ/*bgl2*Δ), AEM4 (*als1*Δ/*ecm33*Δ), and ABEM4 (*als1*Δ/*bgl2*Δ/*ecm33*Δ) cells were cultured in 5 mL YPD medium overnight at 30^0C and diluted in YPD to an OD_{600} of 1. The cells were then serially diluted (10-fold) in YPD medium. Three microliters of each dilution were spotted onto YPD plates containing 12.5 µg/mL CFW, and incubated at 30°C for 24h. Susceptibility of cells to CFW was indicated by their inability to grow on this medium..

To investigate the impact of gene disruptions on *C. albicans* cell wall integrity, I tested the sensitivity of null mutants to calcofluor white (CFW), a chemical which interferers with cell wall construction.[71] SC5314 (wild type), ALS1M4(*als1*Δ), ABM4 (*als1*Δ/*bgl2*Δ), AEM4 (*als1*Δ/*ecm33*Δ), and ABEM4 (*als1*Δ/*bgl2*Δ/*ecm33*Δ) cells were serially diluted and inoculated on YPD agar and on YPD agar containing CFW (12.5 µg/mL), and incubated for 24 h at 30°C. All of the null mutants were more susceptible to CFW than the wild type parental strain (Figure 4-8). ABEM4 (*als1*Δ/*bgl2*Δ/*ecm33*Δ) showed the highest CFW sensitivity. This result indicated that lack of cell surface mannoproteins leads to a significant decrease in the integrity of the *C. albicans* cell wall.

4.4 Conclusion

As an opportunistic microorganism, *C. albicans* adheres to ligands that retain the yeast cells in the host through different surfaces.[14] These interactions prevent human fluids, such as saliva, from removing *C. albicans* from the host. Many methods were used to identify these interactions and the ability of the organism to bind to a variety of surfaces (cell, protein, or other substrate). Therefore, a number of *C. albicans* adhesins were detected including Als family of GPI-CWPs, Hwp1p, ECM33p, and extracellular hydrolytic enzymes.[81] Bgl2p (β-1,3-glucosyltransferase) and Ecm33p (a typical feature of glycophosphatitylinositol (GPI) – anchored protein) are known to be cell wall proteins and related to the cell wall integrity[6, 18]. In this study, it was confirmed that BGL2M4 (*bgl2Δ*) and ECM33M4 (*ecm33Δ*) showed higher sensitivity to CFW than SC5314 (wild type). Whereas the CFW susceptibility of BEM4 (*bgl2Δ*, *ecm33Δ*) was highest in all four strains. Then, it was suggested that the disruption of these double genes severely influenced the cell wall construction of *C. albicans*. Surprisingly, although the adhesion of BGL2M4 and ECM33M4 to SHA beads were 76.4% and 64.8% of SC5314, respectively, BEM4 strains showed 87.7 % of adhesion activity of the wild type and this activity was higher than BGL2M4 and ECM33M4. From these results, I assumed that a certain compensatory system to maintain the adherence activity was activated in BEM4 because lack of two cell wall proteins leads to drastic change in the cell wall integrity of the fungal cells. To investigate this assumption, *ALS* gene family, which encodes large cell surface glycoproteins and are critical in the process of adhesion to host surfaces, was focused. The *ALS* family includes eight genes, from *ALS1* to *ALS7* and *ALS9* and the transcriptional expression of these eight genes were analyzed. Interestingly, the expression level of *ALS1* in BEM4 is higher than those of wild-type and single gene disruptants. ALS1 is reported to take part in *C. albicans* adhesion to human epithelial, endothelial cells and even abiotic surfaces such as silicon[42]. This protein is considered

key component needed for biofilm, hyphal development of *C. albicans*[27,75] This result suggested that the double mutant had developed a compensatory response to overexpression of *ALS1* gene then recovered decrease in adhesion activity to SHA beads. In previous research, Chen *et al.* showed the *bgl2Δ* mutant (BGL2M4) took a delay in transition to filamentous cell morphology due to repression of transcription factor *CPH2/TEC1. TEC1* was identified as a transcriptional factor that is required for hypha formation and virulence.[82] In addition, *TEC1* is also reported to be an important transcription factor of *ECM33* and *ALS1*[83,84]. Therefore, I hypothesized that in case of reduction of *BGL2* and *ECM33*, the expression of *ALS1* may be induced by *TEC1*. To clarify this transcriptional mechanism, the further experiments on the protein network and functions of *C. albicans* outer cell wall constituents will be needed.

Collectively, in this chapter, I investigated the roles of Bgl2 and ECM33 in the ability of *C. albicans* to adhere to saliva-coated oral surface by generating several null mutants of these genes and a novel NR staining assay. I found that the two cell wall proteins Bgl2p, Ecm33p contribute to interaction between *C. albicans* and saliva-coated surfaces. However, I observed that the deletion of both two genes triggered compensatory responses in this yeast cells. In particular, the *ALS1* gene, encoding a major GPI-modified protein, was overexpressed in the *bgl2Δ/ecm33Δ* double disrupted strain. Then deletion of all of these three genes caused a significant decrease in the adhesion of *C. albicans* to saliva-coated hydroxyapatite (SHA) beads.

CHAPTER 5. CONCLUSION

Amongst *Candida* species, *C. albicans* is most commonly isolated from the oral cavity and is responsible for most superficial and systemic fungal infections[15].The oral cavity provides many saliva-coated surfaces and niches that are easily colonized by *C. albicans*. Therefore, it is important to investigate how this opportunistic yeast interacts with tooth surfaces when covered by saliva components. The yeast cell wall is the vital point of contact between *C. albicans* cell and the saliva-coated oral surfaces.[8] Because of this important role, the *C. albicans* cell wall has attracted a lot of attention over the last few decades.

A method used previously for measuring *C. albicans* adherence to saliva-coated surfaces involved labelling the yeast with [^{35}S]-methionine[5]. This method is reliable, reproducible and sensitive; however, it is time-consuming, expensive and poses a risk to human health. Recently, several alternative fluorescent staining strategies have been developed to provide fast detection and quantification of yeast cells. In my research, important criteria for choosing the optimum fluorescent dye to measure yeast adhesion were chemically stability under the experiment conditions, low-cost, safety of use, and no interference with other substances in saliva. I investigated using, carboxyfluorescein diacetate succinimidyl ester (CFDA) for staining of *C. albicans* cells as this compound is cleaved by intracellular esterase enzymes to form a fluorescent amine-reactive product. I found that CFDA stained *C. albicans* cells and gave a strong fluorescence signal but there were also many esterases in saliva resulting in a high background signal in our SHA bead adhesion assay. I then focused on NR, a commonly used fluorescent lipophilic dye that binds to intracellular neutral lipids. NR has low fluorescence in water and other polar solvents, but gives strong fluorescence signals in nonpolar environments.[55] I found, by using spectroscopy and confocal microscopy, that once NR was absorbed by *C. albicans* cells, the signal was stable, even after 2-5 h of staining. This characteristic is a major

advantage of using NR because once yeast cells were stained with this dye, no washing steps were needed. Therefore, I used NR to stain *C. albicans* cells for adhesion assays.

While uncoated HA beads showed low non-specific binding of 17% of SC5314 cells, I found that 89% yeast cells adhered to SM-coated HA beads, which is relatively equal to the amount of SHA beads. This is consistent with previous reports which have shown that bPRP and statherin promote attachment of *C. albicans* cells to hydroxyapatite beads[5,14]. Control proteins FBS and BSA did not promote much adherence of *C. albicans* cells to hydroxyapatite beads but, surprisingly, skim milk did. It would be interesting to determine which components of skim milk promote this adhesion.

The proteins Bgl2 (β-1,3-glucosyltransferase) and Ecm33 (a GPI–anchored protein) are cell wall proteins contributing to cell wall integrity[6, 18]. In this study, it was confirmed that BGL2M4 (*bgl2*Δ) and ECM33M4 (*ecm33*Δ) showed higher sensitivity to CFW than SC5314 (wild type) and the double disruptant (*bgl2*Δ, *ecm33*Δ; BEM4) showed even higher sensitivity. As well as having compromised cell wall integrity, BGL2M4 and ECM33M4 showed reduced adherence to SHA beads than SC5314. Surprisingly, the double disruptant BEM4 demonstrated increased adhesion to SHA beads. Deletion of ALS1 in either the *bgl2*Δ or the *ecm33*Δ background did not result in a strain with increased adherence to SHA beads (as was seen with the *bgl2*Δ, *ecm33*Δ strain). These results demonstrated that *ALS1* expression is only induced when both *BGL2* and *ECM33* are deleted, which was substantiated by the significant reduction in adherence of the *bgl2*Δ, *ecm33*Δ strain when *ALS1* was also disrupted. Als1p along with other Als proteins are well recognized adhesins, but their expression has been predominantly studied under hypha inducing conditions.[80,85] Much is known about *ALS1* expression during morphogenesis and biofilm formation where adhesin gene expression is under the control of transcriptional regulators such as Efg1p, Bcr1p, Tup1p and Nrg1p.[86,87] However, there have been very few studies on the expression of adhesins in yeast cells. Iare interested in

adhesin expression by yeast cells as they are responsible for the colonization of saliva-coated surfaces. *ALS1* is expressed by yeast cells when released from stationary phase, but expression decreases during yeast growth. In contrast, expression of *ALS1* is induced greatly during hyphal formation.[88] The present study shows that the expression of *ALS1* in yeast cells is increased in cells deleted in *BGL2* and *ECM33*. Chen *et al.* have previously reported that disruption of *BGL2* resulted in decreased expression of transcriptional regulator *CPH2* during yeast growth[51] and this may be responsible, in part, for changes in *ALS1* expression. Further experiments are needed to elucidate the transcriptional network controlling the expression of Bgl2p, Ecm33p and Als1p under yeast and mycelia growth conditions.

It must be acknowledged that I only looked for compensatory expression of *ALS* genes in the disrupted strains. While there is strong evidence that there is induction of *ALS1* when *BGL2* and *ECM33* are deleted, I cannot exclude the possibility that expression of other adhesins is also induced. Further research, including complete RNA sequencing of mutant strains, may provide a more complete mechanistic insight into the role of the cell wall protein network in the adhesion ability of *C. albicans*. This study does, however, provide strong evidence that Bgl2p, Ecm33p and Als1p contribute to *C. albicans* adherence to saliva-coated surfaces.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Approval to conduct the study was obtained from the Research Ethics Committee of Tokyo Institute of Technology in accordance with the Ethical Guidelines for Clinical Research and Ethical Guidelines for Epidemiological Research (issued by the Ministry of Health, Labour and Welfare and Ministry of Education, Culture, Sports, Science and Technology - Japan).

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APPENDIX

A1.Adhesion procedure