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By Jennafer M. Whybrew

Entitled Aerobic Uptake of Cholesterol by Ergosterol Auxotrophic Strains in Candida glabrata & Random and Site-Directed Mutagenesis of ERG25 in Saccharomyces serevisiae.

For the degree of Master of Science

Is approved by the final examining committee:

Martin Bard
Chair

N. Douglas Lees

Brenda Blacklock

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AEROBIC UPTAKE OF CHOLESTEROL BY ERGOSTEROL AUXOTROPHIC STRAINS IN *CANDIDA GLABRATA*

&

RANDOM AND SITE-DIRECTED MUTAGENESIS OF *ERG25* IN *SACCHAROMYCES CEREVISIAE*.

A Thesis
Submitted to the Faculty
of
Purdue University
by
Jennafer Marie Whybrew

In Partial Fulfillment of the
Requirements for the Degree
of
Master of Science

December 2010

Purdue University

Indianapolis, Indiana

For my family, thank you for all of your support.

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ABBREVIATIONS

bp	base pair
CSM	complete synthetic media
dNTP	deoxynucleotide 5' triphosphate
dH_2O	distilled water
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetate
ER	endoplasmic reticulum
EtOH	ethanol
EtBr	ethidium bromide
GC	gas chromatography
GTE	glucose/Tris-HCl/EDTA
HCl	hydrochloric acid
HIS	histidine
kb	kilobase
KOH	potassium hydroxide
LB	Luria-Bertani
LEU	leucine
LiAc	lithium acetate
MS	mass spectrometry
NaCl	sodium chloride
NaOH	sodium hydroxide
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol
SDS	sodium dodecyl sulfate
TAE	Tris-HCl/acetate/EDTA
TE	Tris-HCl/EDTA
URA	uracil
YPAD	Yeast, Peptone, Adenine, Dextrose complete media

UNITS

C	Celsius
g	relative centrifugal force of gravity
g	gram
mg	milligram
µg	microgram
ng	nanogram
l	liter
ml	milliliter
µl	microliter
sec	second
min	minute
hr	hour
M	molar
mM	millimolar
nM	nanomolar
nmol	nanomole
pmol	picomole
rpm	revolutions per minute
V	Volts
v	volume
w	weight

ABSTRACT

Whybrew, Jennafer Marie. M.S., Purdue University, December 2010. Aerobic Uptake of Cholesterol by Ergosterol Auxotrophic Strains in *Candida glabrata* & Random and Site-Directed Mutagenesis of ERG25 in *Saccharomyces cerevisiae*. Major Professor: Dr. Martin Bard.

Candida albicans and *Candida glabrata* are opportunistic human pathogens that are the leading cause of fungal infections, which are increasingly becoming the leading cause of sepsis in immunosuppressed individuals. *C. glabrata* in particular has become a significant concern due to the increase in clinical isolates that demonstrate resistance to triazole antifungal drugs, the most prevalent treatment for such infections. Triazole drugs target the *ERG11* gene product and prevent C-14 demethylation of the first sterol intermediate, lanosterol, preventing the production of the pathways end product ergosterol. Ergosterol is required by yeast for cell membrane fluidity and cell signaling. Furthermore, *C. glabrata,* and not *C. albicans,* has been reported to utilize cholesterol as a supplement for growth.

Although drug resistance is known to be caused by an increase in expression of drug efflux pumps, we hypothesize a second mechanism: that the overuse of triazole drugs has lead to the increase of resistance by *C. glabrata* through a 2-step process: 1) the accumulation of ergosterol auxotrophic mutations and 2) mutants able to take up exogenous cholesterol anaerobically in the body acquire a second mutation allowing uptake of cholesterol aerobically. Two groups of sterol auxotrophic *C. glabrata* clinical isolates have been reported to take up sterol aerobically but do not produce a sterol

precursor. Sterol auxotrophs have been created in *C. glabrata* by disrupting different essential genes (*ERG1, ERG7, ERG11, ERG25*, and *ERG27*) in the ergosterol pathway to assess which ergosterol mutants will take up sterols aerobically.

Random and site-directed mutagenesis was also completed in *ERG25* of *Saccharmoyces cerevisiae.* The *ERG25* gene encodes a sterol C-4 methyloxidase essential for sterol biosynthesis in plants, animals, and yeast. This gene functions in turn with *ERG26*, a sterol C-3 dehydrogenase, and *ERG27*, a sterol C-3 keto reductase, to remove two methyl groups at the C-4 position on the sterol A ring. In *S. cerevisiae*, *ERG25* has four putative histidine clusters, which bind non-heme iron and a C-terminal KKXX motif, which is a Golgi to ER retrieval motif. We have conducted site-directed and random mutagenesis in the *S. cerevisiae* wild-type strain SCY876. Site-Directed mutagenesis focused on the four histidine clusters, the KKXX C-terminal motif and other conserved amino acids among various plant, animal, and fungal species. Random mutagenesis was completed with a procedure known as gap repair and was used in an effort to find novel changes in enzyme function outside of the parameters utilized for site-directed mutagenesis. The four putative histidine clusters are expected to be essential for gene function by acting as non-heme iron binding ligands bringing in the oxygen required for the oxidation-reduction in the C-4 demethylation reaction.

CHAPTER 1

INTRODUCTION

1.1 Sterols-Structure and Function

Sterols are naturally occurring organic molecules produced by enzymes in the endoplasmic reticulum (ER) and are essential for animal, plant, and fungal cell function. Sterols are distributed in the plasma and cell membranes. Each kingdom utilizes a different primary sterol, each having slight variations in structure. Specifically, the primary animal sterol is cholesterol, fungal sterol is ergosterol, and plant sterols are stigmasterol and β-sitosterol. Figure 1.1 illustrates the sterol derivatives from the different kingdoms as well as the IUPAC numbering system for sterol molecules (37). The comparison in this figure clearly illustrates the similarity among the structures of the different end product sterols.

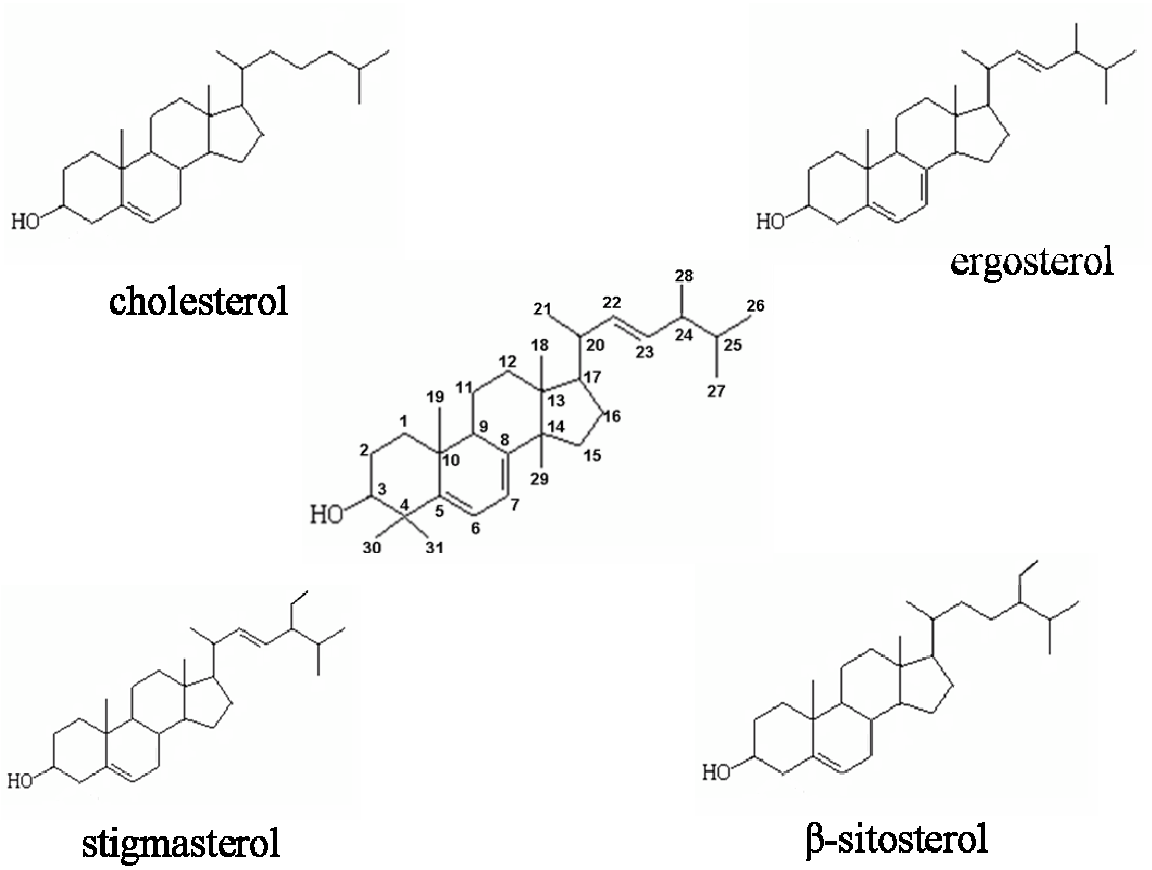

Figure 1.1 Sterol structures and IUPAC numbering system.

The general sterol structure is a substituted 4-ring steroid nucleus with a hydroxyl group at C_3, methyl groups at C_{10} and C_{13}, and a variable side chain at C_{17} about eight carbons long. The kingdom specific structural differences between sterols are generally in the number and position of double bonds and side chain substitutions. Of particular significance for this work is the slight variation between cholesterol and ergosterol, where cholesterol is a 27 carbon sterol structure with one double bond at C5-6 and a C_3 hydroxyl group (both standard for sterol structure) and ergosterol is a 28 carbon sterol structure with double bonds at C5-6, C7-8, and C22-23 and has an additional methyl group at C_{24} (5, 37).

In all kingdoms, sterols are associated with cell membrane structure. Interestingly, because sterol structures only have small variations from one another, it is possible for many different kingdom sterols to be substituted in the membrane in place of a sterol end product. For example, if a deleterious event occurs in the ergosterol biosynthetic pathway preventing the production of the ergosterol end product, cells can utilize cholesterol (the animal kingdom end product sterol) in place of ergosterol. Studies exploring this phenomenon of sterols have lead to a greater understanding of the biological functions sterols play in the cell. These molecules are known critical constituents of a cells plasma membrane and play significant roles in many biological functions including: membrane fluidity (1), membrane bound enzyme regulation (2), membrane permeability (3), endocytosis (4), and growth rates of fungal cells (38).

1.2 Sterol Biosynthesis

1.2.1 The Mevalonate Pathway

The first half of the biosynthetic pathway of sterols is referred to as the mevalonate pathway. This half of the pathway converts acetyl-CoA to farnesyl pyrophosphate (FPP) in nine steps (5). Acetyl-CoA is produced during glycolysis from the oxidative decarboxylation of pyruvate (41). In order for cells to produce sterols, acetyl-CoA must go through the mevalonate or isoprenoid pathway (41).

The nine steps of the isoprenoid pathway are illustrated in Figure 1.2 (42).

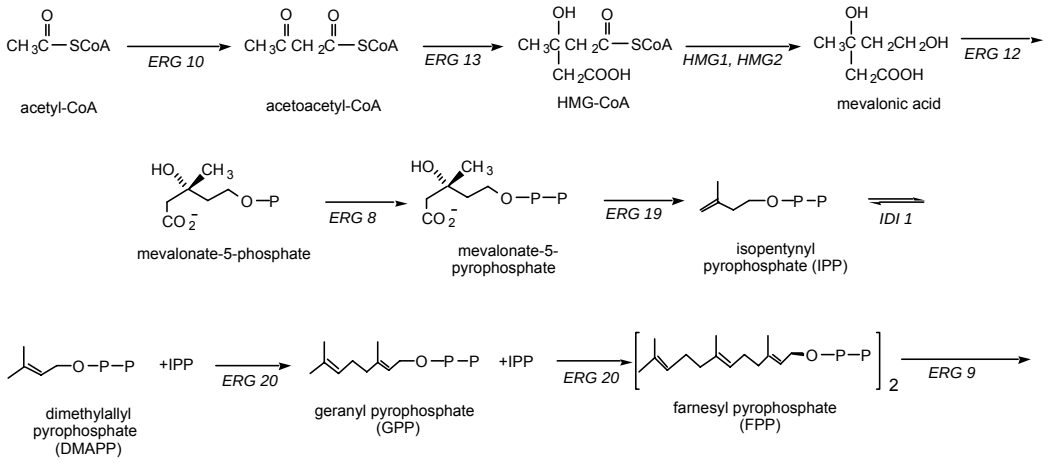

Figure 1.2 Isoprenoid Biosynthetic Pathway.

First, *ERG10,* an acetoacetyl-CoA thiolase, combines two acetyl-CoA molecules to produce acetoacetyl-CoA. Second, the *ERG13* gene product, an HMG-CoA synthase, produces HMG-CoA, which is then reduced to mevalonic acid by the HMG-CoA reductase product of *HMG1* or *HMG2.* Steps four and five phosphorylate mevalonic acid

in two steps: first the mevalonate kinase (*ERG12* gene product) produces mevalonate-5-phosphate and second the phosphomevalonate kinase (*ERG8* gene product) produces mevalonate-5-pyrophosphate. At step six, the *ERG19* gene product converts mevalonate-5-pyrophosphate to isopentenyl pyrophosphate (IPP), which is in turn converted to dimethylallyl pyrophosphate by the *IDI1* gene product, isopentenyl pyrophosphate isomerase. Finally, the *ERG20* gene product, farnesyl pyrophosphate synthase, converts IPP, in two steps, to farnesyl pyrophosphate (5). Farnesyl pyrophosphate is the starting product for the second half of the pathway, known as the ergosterol biosynthetic pathway.

1.2.2 Sterol Biosynthesis: Farnesyl Pyrophosphate to Ergosterol

This second part of the pathway is referred to as the ergosterol biosynthetic pathway and utilizes 13 gene-encoded enzymes to convert farnesyl pyrophosphate to the end product ergosterol in an 11-step process. Figure 1.3 illustrates this conversion process as a flow chart for easy reference.

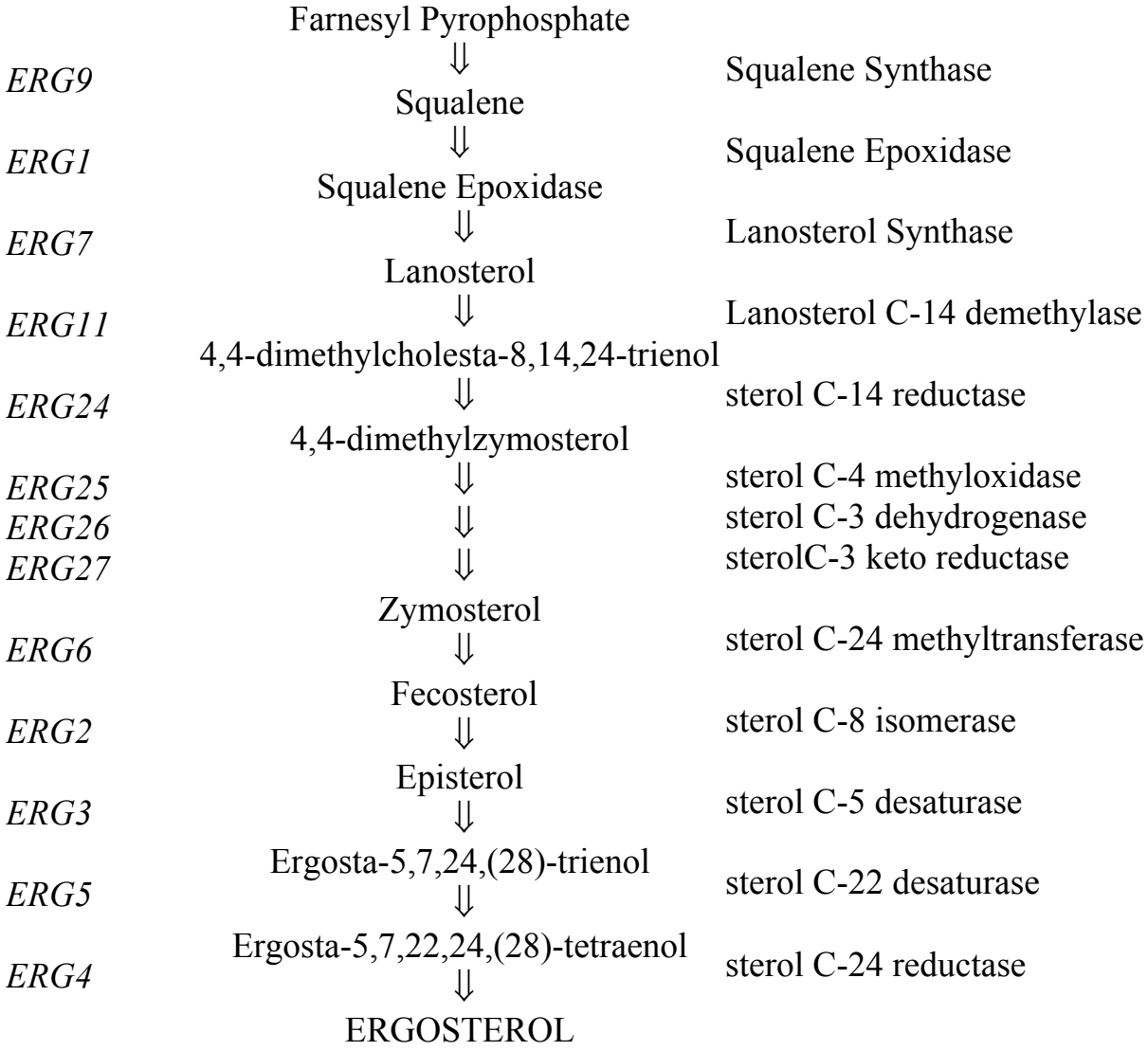

Figure 1.3 Flow chart diagramming the 11-step enzymatic process of the ergosterol biosynthetic pathway

First, *ERG9*, or squalene synthase, synthesizes squalene from farnesyl pyrophosphate. This is a molecular oxygen-requiring step of the pathway. Second, *ERG1*, or squalene epoxidase, converts squalene to squalene epoxide. Third, *ERG7*, or lanosterol synthase, transforms squalene epoxide into lanosterol, the first sterol precursor of the ergosterol pathway. Then, *ERG11* removes a methyl group from the C-14 position of lanosterol creating the 4,4-dimethylcholesta-8,14,24-tienol intermediate. *ERG24*, a sterol C-14 reductase, turns this intermediate into 4,4-dimethylzymosterol. The next three genes: *ERG25* (a sterol C-4 methyloxidase), *ERG26* (a sterol C-3 dehydrogenase), and *ERG27* (a sterol C-3 keto-reductase), function together with a scaffold protein *ERG28* to remove two methyl groups at the C-4 position on the sterol A ring creating zymosterol. These first nine genes and their encoded gene products (*ERG9, ERG1, ERG7, ERG11, ERG24, ERG25, ERG26, ERG27,* and *ERG28)* leading up to the production of zymosterol, a sterol precursor intermediate in the pathway, are essential for cell viability. An error or defect in this portion of the pathway is lethal to the cell.

Zymosterol is converted to fecosterol by the *ERG6* gene product sterol C-24 methyltransferase. *ERG2,* sterol C-8 isomerase, converts fecosterol to episterol. Next, two desaturases, *ERG3* (sterol C-5 desaturase) and *ERG5* (sterol C-22 desaturase), convert episterol to ergosta-5,7,24(28)-trienol and ergosta-5,7,24(28)-trienol to ergosta-5,7,22,24(28)-tetraenol, respectively. Finally, *ERG4* (sterol C-24 reductase) converts ergosta-5,7,22,24(28)-tetraenol to ergosterol. These genes involved after the production of zymosterol, *ERG6, ERG2, ERG3, ERG5,* and *ERG4,* are non-essential for growth because the resulting products can be used in place of ergosterol to support membrane

function (6). Figure 1.4 illustrates the chemical structure changes during the ergosterol biosynthetic pathway (42).

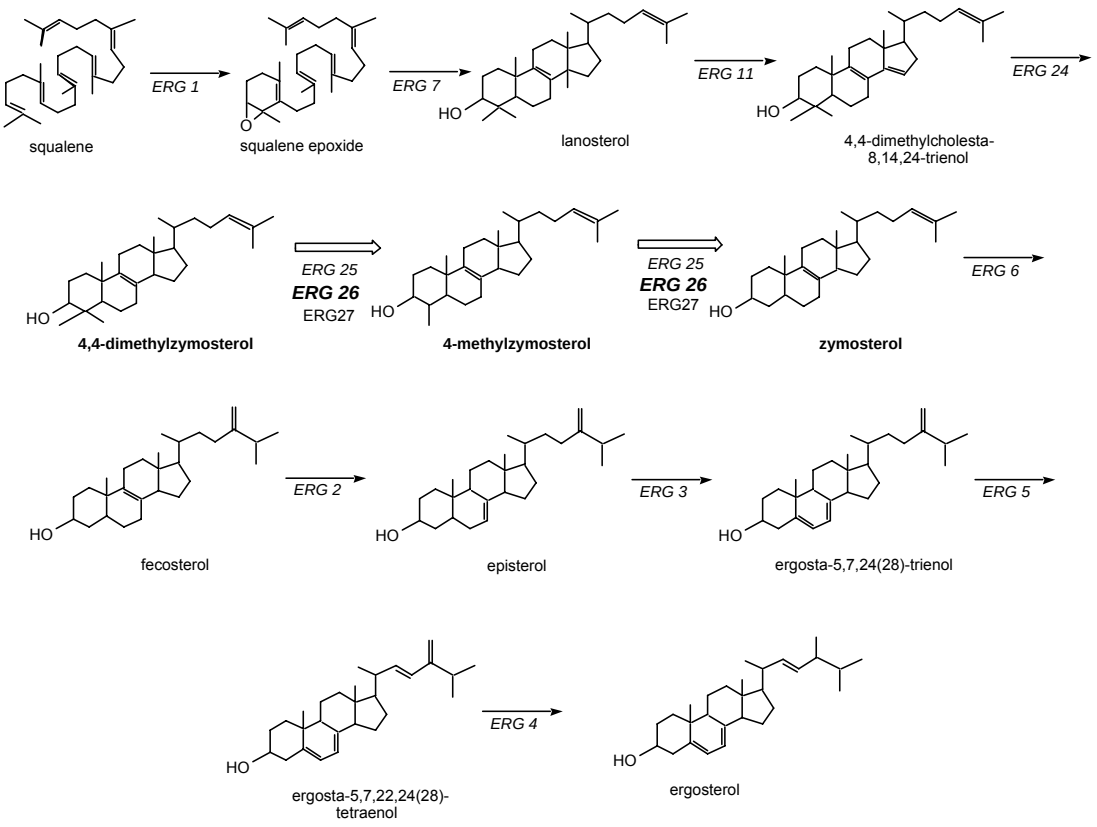

Figure 1.4 Ergosterol Biosynthetic Pathway

1.2.3 The Role of Heme in Ergosterol Biosynthesis

Heme consists of iron surrounded by a pyrrole ring system and is utilized to selectively bind molecules, in this pathway, molecular oxygen (41). Heme is bound to mitochondrial proteins known as cytochromes, which act as electron transporters in several different cellular functions (43). In yeast, the ERG11 and ERG5 encoded

enzymes are cytochrome P450's, while the ERG3 and ERG25 enzymes require cytochrome *b5* as a co-factor (44). These heme-requiring steps indicate sterols can only be endogenously produced aerobically due to the requirement for molecular oxygen.

Sterol auxotrophic strains can be studied in some species of yeast because of their ability to uptake exogenous sterol anaerobically. When endogenous sterol production is compromised, yeast cells are capable of utilizing alternative sterol sources (48). This phenomenon is known as aerobic sterol exclusion. This phenomenon suggests yeast cannot take up exogenous sterol aerobically, rather only anaerobically (5). Although the mechanism is not clear, studies have indicated that heme plays a significant role in this phenomenon (49, 64). Gallub and colleagues reported that a heme product participates in transforming lanosterol to ergosterol (49). Lewis and colleagues reported that a heme mutation is required for aerobic rescue of an erg mutation (64). Of significant concern for this study are the recent reports indicating some strains of *C. glabrata* (an opportunistic human pathogen) are capable of aerobic sterol uptake (51).

CHAPTER 2

MATERIALS AND METHODS

2.1 Strains, Media, and Growth Conditions

2.1.1 Bacterial Strains, Media and Growth Conditions

Escherichia coli strains used in these studies include DH5α™ competent cells [F^- ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-(Invitrogen, CA)] and XL10-Gold® Ultracompetent Cells [Tet^rΔ (*mcr*A)183 *Δ*(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* Hte [F' proAB *lac*I^q*ZΔ*M15 Tn*10* (Tet^r) Amy Cam^r] (Stratagene, La Jolla, CA)]. Bacterial strains were grown in Luria-Bertani (LB) media with the addition of 60 µg/ml of ampicillin for selection. LB media consisted of 10 g of pancreatic digest of casein, 5 g of yeast extract, and 10 g/L of sodium chloride (46) dissolved in milli-Q water and autoclaved for 25 minutes. Solid media required the addition of 2% (w/v) granulated Difco agar (Becton Dickinson, Sparks, MD) prior to autoclaving. Liquid cultures were grown at 37˚C in a walk in incubator with shaking at 225 rpm and solid media was grown in a 37˚C incubator for 16-20 hours.

2.1.2 Yeast Strains

Table 2.1 Yeast strains used and created in this study

Strain	Genotype	Source
S. cerevisiae		
SCY876	Matα, *upc2.1* hap Ty (hi), *ura3-1, his3-11,-15, leu2-3,-112, trp1-1*	S. Sturley (55)
D1	SCY876; *erg25*::*HIS3*	This Study
	From Random mutagenesis	
JWSC213	D1; with pRM213	This Study
JWSC2118	D1; with pRM2118	This Study
JWSC2148	D1; with pRM2148	This Study
JWSC21100	D1; with pRM21100	This Study
JWSC393	D1; with pRM393	This Study
JWSC41	D1; with pRM41	This Study
	From Site-directed mutagenesis	
JWSCF67A	D1; with pJWF67A	This Study
JWSCQ88A	D1; with pJWQ88A	This Study
JWSCQ98A	D1; with pJWQ98A	This Study
JWSCC101A	D1; with pJWC101A	This Study
JWSCL102A	D1; with pJWL102A	This Study
JWSCI115A	D1; with pJWI115A	This Study
JWSCE152A	D1; with pJWE152A	This Study
JWSCD153A	D1; with pJWD153A	This Study
JWSCY157F	D1; with pJWY157F	This Study
JWSCH160A	D1; with pJWH160A	This Study
JWSCY169F	D1; with pJWY169F	This Study
JWSCK170A	D1; with pJWK170A	This Study
JWSCH176A	D1; with pJWH176A	This Study
JWSCP182A	D1; with pJWP182A	This Study
JWSCE188A	D1; with pJWE188A	This Study
JWSCH191A	D1; with pJWH191A	This Study
JWSCR228A	D1; with pJWR228A	This Study
JWSCH236A	D1; with pJWH236A	This Study
JWSCY239F	D1; with pJWY239F	This Study
JWSCH258A	D1; with pJWH258A	This Study
C. glabrata		
CG2001HT	Δtrp1::Scura3Δhis3::ScURA3Δura3	Nakayama (63)
JWCGΔ*erg1*	CG2001HT; *erg1*::*HIS3*	This Study
JWCGΔ*erg7*	CG2001HT; *erg7*::*HIS3*	This Study
JWCGΔ*erg11*	CG2001HT; *erg11*::*HIS3*	This Study
JWCGΔ*erg25*	CG2001HT; *erg25*::*HIS3*	This Study

JWCGΔ*erg27*	CG2001HT; *erg27::HIS3*	This Study
97SQS	*Δura3 Δtrp1::Scura3::*P_{ScADH1}*-tetRGAL4-TRP1Δhis3::ScURA3 erg9::97tERG9-URA3*	Nakayama (63)
97SQS/ΔAUS1	*Δura3 Δtrp1::Scura3::PScADH1-tetRGAL4-TRP1Δhis3::ScURA3 erg9::97tERG9-URA3 Δaus1::HIS3*	Nakayama (63)

2.1.3 Yeast Media and Growth Conditions

All strains of *S. cerevisiae* and *C. glabrata* used in this study were grown non-selectively in either 1) YPAD nutrient rich media consisting of 1% w/v Yeast extract (Difco), 2% w/v Peptone (Difco), 120 mg/L Adenine hemisulfate (Sigma), and 2% w/v Dextrose (Sigma) or 2) Complete Synthetic Media (CSM) consisting of 0.79 g/L CSM (Q Biogene) [40 mg/l adenine, 20 mg/l arginine, 100 mg/l aspartic acid, 100 mg/l glutamic acid (monosodium sulfate), 20 mg/l histidine, 60 mg/l leucine, 30 mg/l lysine, 20 mg/l methionine, 50 mg/l phenylalanine, 375 mg/l serine, 200 mg/l threonine, 40 mg/l tryptophan, 30 mg/l tyrosine, 150 mg/l valine, 20 mg/l uracil (46)], 1.7 g/L yeast nitrogen base without amino acids (YNB) (Difco), 5 g/L ammonium sulfate (Fisher Scientific), and 20 g/L glucose (Sigma). Synthetic dropout media, where a specific nutrient is left out of the media based on nutritional requirements of the strain, were used for genetic marker selection. For *S. cerevisiae* the media lacked uracil and for *C. glabrata* the media lacked histidine. CSM media had the pH adjusted to 5.8 and 2%-granulated agar (Difco) for solid media. End product ergosterol or cholesterol was added to media from a 2 mg/ml stock solution in Tween80/EtOH (1:1 v/v) for both species to screen for sterol auxotrophy. For anaerobic growth, both liquid and solid cultures were used in the

anaerobic jars with the GasPak EZ system (Becton Dickinson, Sparks, MD). Yeast strains were grown at 30˚C.

2.2 DNA Manipulations

2.2.1 List of Plasmids Used in These Studies

Table 2.2 Plasmids used and created in this study

Plasmid	Description	Reference
pRS303	CEN6/ARS, *LacZ*, *HIS3*, amp^R (4.453 kb)	Hieter
pIU800	Bluescript(SR)+ vector with ERG25	Bard
p426ADH	2µ, *URA3*, prom-ADH, MCS: *SpeI,BamH*I,*SmaI* *EcoR*V,*EcoR*I, *Pst*I, *Hind*III,*Cla*I, *Sal*I, *Xho*I; CYC1-term	Mulbury
	From Random mutagenesis	
pRM213	p426ADH; *erg25*: no change, wild type	This Study
pRM2118	p426ADH; *erg25*, change: W276R	This Study
pRM2148	p426ADH; *erg25*: no change, wild type	This Study
pRM21100	p426ADH; *erg25*, change: R274G	This Study
pRM393	p426ADH; *erg25*, change: S45G	This Study
pRM41	p426ADH; *erg25*, change: H263L	This Study
	From Site-directed mutagenesis	
pJWH62A	p426ADH; *erg25*; H62 changed to A (*EcoRI/SalI*)	This Study
pJWF67A	p426ADH; *erg25*; F67 changed to A (*EcoRI/SalI*)	This Study
pJWQ88A	p426ADH; *erg25*; Q88 changed to A (*EcoRI/SalI*)	This Study
pJWQ98A	p426ADH; *erg25*; Q98 changed to A (*EcoRI/SalI*)	This Study
pJWC101A	p426ADH; *erg25*; C101 changed to A (*EcoRI/SalI*)	This Study
pJWL102A	p426ADH; *erg25*; L102 changed to A (*EcoRI/SalI*)	This Study
pJWI115A	p426ADH; *erg25*; I115 changed to A (*EcoRI/SalI*)	This Study
pJWE152A	p426ADH; *erg25*; E152 changed to A (*EcoRI/SalI*)	This Study
pJWD153A	p426ADH; *erg25*; D153 changed to A (*EcoRI/SalI*)	This Study
pJWY157F	p426ADH; *erg25*; Y157 changed to F (*EcoRI/SalI*)	This Study
pJWH160A	p426ADH; *erg25*; H160 changed to A (*EcoRI/SalI*)	This Study
pJWY169F	p426ADH; *erg25*; Y169 changed to F (*EcoRI/SalI*)	This Study
pJWK170A	p426ADH; *erg25*; K170 changed to A (*EcoRI/SalI*)	This Study
pJWH173A	p426ADH; *erg25*; H173 changed to A (*EcoRI/SalI*)	This Study
pJWH176A	p426ADH; *erg25*; H176 changed to A (*EcoRI/SalI*)	This Study
pJWP182A	p426ADH; *erg25*; P182 changed to A (*EcoRI/SalI*)	This Study

pJWE188A	p426ADH; *erg25*; E188 changed to A (*EcoRI/SalI*)	This Study
pJWH191A	p426ADH; *erg25*; H191 changed to A (*EcoRI/SalI*)	This Study
pJWR228A	p426ADH; *erg25*; R228 changed to A (*EcoRI/SalI*)	This Study
pJWH236A	p426ADH; *erg25*; H236 changed to A (*EcoRI/SalI*)	This Study
pJWY239F	p426ADH; *erg25*; Y239 changed to F (*EcoRI/SalI*)	This Study
pJWH258A	p426ADH; *erg25*; H258 changed to A (*EcoRI/SalI*)	This Study

2.2.2 Endonuclease Restriction Digest

Restriction digests were done with 1 µl DNA, 1-5 Units of restriction enzyme (Roche) and 2 µl of the corresponding 10X digestion buffer (Roche) to a final volume of 20 µl. The solution was incubated at 37˚C for 1-4 hours. Up to two enzymes would be used simultaneously if the digestion buffer were the same for both enzymes. If digestion buffers did not correspond, separate digests were done separated by EtOH precipitation. Final digested product was verified and quantitated by 1% agarose gel electrophoresis.

2.2.3 Agarose Gel Electrophoresis

Gel electrophoresis separates DNA fragments by size. These were utilizied to verify and quantify DNA. Preparing agarose gels consisted of dissolving 1% (w/v) agarose (Sigma) in 10X TAE buffer pH 8.0 (20 mM Tris-HCl, 20 mM Acetate, 0.5 mM EDTA). The gel was poured into a cast cleaned with 95% EtOH and a comb inserted to create wells for DNA samples. DNA samples were prepared with water and 10X blue juice loading dye to a total volume of 10 µl. A Hi-Lo ladder (Minnesota Molecular) and λ phage DNA molecular weight standards digested with *HindIII* were used as comparisons for DNA. Gels were run at 40-60 V for four to six hours and then stained in 10 ug/ml

EtBr solution for 10-20 minutes. DNA was visualized on the gels with a transilluminator and pictures were taken with a Kodak camera when required.

2.2.4 Ethanol Precipitation

Ethanol precipitation was used to precipitate and purify DNA. The same volume of 5 M sodium acetate was added to a volume of DNA. This solution was vortexed to mix well. Then two volumes of -20˚C 95% EtOH were added to the DNA and sodium acetate solution. This solution was then vortexed to mix well and incubated for five to ten minutes at room temperature. This was then pelleted at 13,000 rpm for twelve minutes at room temperature and the supernatant carefully removed. The pellet was washed with 100 µl of -20˚C 70% EtOH and pelleted at 13,000 rpm for twelve minutes at room temperature and again, the supernatant carefully removed. The pellet was dried in the hood for no more than ten minutes to remove residual EtOH. The pellet was then resuspended in 20 µl of TE pH8.0. The DNA was finally run out on a gel to quantify.

2.3 Transformations

2.3.1 Bacterial Transformations

Bacterial cells stored at -80˚C were gently thawed on ice. While cells are thawing, 1-10 µg plasmid DNA was aliquoted into microcentrifuge tubes. Once cells are thawed, they were swirled very gently in their original tube and 40 µl were pipetted onto the DNA. Cell and DNA solution were incubated on ice for 30 minutes and the remaining bacterial cells were replaced in the –80˚C freezer. After 30 minutes, the solution was heat shocked

at 42˚C for 45-60 seconds and then placed immediately back on ice for 2 minutes. 500-1000 µl of room temperature LB broth was added to reaction and mixture is incubated at 37˚C for 1 hour with 225 rpm of shaking. Finally, cells were plated onto LB + ampicillin plates and incubated for 16-20 hours at 37˚C.

2.3.2 Yeast Transformations

Yeast cells were freshly grown overnight (or occasionally for two nights when growing anaerobically) in appropriate liquid media for all transformations. If aerobic growth was appropriate, cell cultures were grown to an OD of 1.0. Cells were pelleted for 5 minutes at 3,000 X g. The supernatant was decanted and cells were washed twice with 25 mL sterile H_2O for 5 minutes at 3,000 X g. Yeast cells were resuspended in a 10% 10X TE [pH 7.5], 10% 1M Lithium Acetate [pH 7.5], and 80% dH_2O solution and incubated at 30˚C for 15 minutes. While the cells incubated, a transformation solution with 100 µg carrier DNA (denatured salmon sperm DNA) and 1-5 µg template DNA was combined in a microcentrifuge tube. This transformation solution containing DNA had 100 µl of the cell solution and 600 µl of PEG solution (10% 10X TE [pH 7.5], 10% 1M lithium acetate [pH 7.5], and 80% of a 50% PEG 3400 solution) was vortexed briefly, incubated for 30 minutes at 30˚C, and heat shocked at 42˚C for 15 minutes. This transformation solution was pelleted for 5 seconds at 3,000 X g and the supernatant decanted. An appropriate amount of liquid dropout media or sterile water, generally 500-1000 µl, was added to dissolve the pelleted cells. The cells were plated in 100 µl aliquots onto appropriate dropout media plates with sterol supplementation when required. Plates were incubated 3-5 days.

2.3.3 Spot Plate Assays

Spot plate assays were used to monitor different yeast strains' growth characteristics on different media. Cells were freshly grown in appropriate media for each strain. For example, strains with essential gene disruptions were grown anaerobically on YPD plates with sterol supplementation and strains not containing essential gene disruptions were grown aerobically on YPD. Either pelleting liquid cultures or scrapping cells from plates and dissolving cells in sterile water created cell solutions. The concentration of the cell suspension was measured on a spectrophotometer. Dilutions were made from this suspension to plate 5-10 µl spots with OD values ranging from 0.1 to 4.0. Depending on the parameters being tested, different sterols were added for supplementation to plates at different concentrations. Plates were poured to 25ml.

2.4 Preparation of DNA

2.4.1 Bacterial Plasmid DNA Preparation

Bacterial cells were grown in 2 ml cultures tubes of LB + ampicillin liquid overnight for 16-20 hours at 37˚C with 225 rpm shaking. Aliquots of 1.5 ml of cells were pelleted at 13,000 rpm for 3 minutes. The pellet was resuspended in 250 µl P1 buffer (Qiagen) or 100 µl GTE (50 mM glucose, 25 mM Tris-HCl, 10mM EDTA) by vortexing. Next, 250 µl P2 buffer (Qiagen) or 200 µl of lysis buffer (1% SDS and 200mM NaOH) was added to tubes and inverted gently 4-6 times. Cell debris was precipitated by adding 350 µl buffer N3 (Qiagen) or 150 µl KOAc (5.0 M KOAc, 29.5% v/v glacial acetic acid [pH 4.8]) and inverting gently 4-6 times. The solution was then pelleted at 13,000 rpm for

10 minutes. The supernatant was transferred to a spin column (Qiagen) or new microcentrifuge tube. For the Qiagen protocol, the supernatant was spun down for 1 minute, the column washed with 500 µl buffer PB and pelleted for 1 minute, the column washed with 750 µl buffer PE and pelleted for 1 minute, and finally the DNA eluted with 25 µl of elution buffer. For the second protocol, two volumes of 95% EtOH was added to the pellet to precipitate the DNA and pelleted for 5 minutes. Then the pellet was washed with 70% EtOH and finally eluted in 25 µl TE buffer.

2.4.2 Yeast DNA Preparation

Plasmid DNA was extracted from yeast cells using the Zymoprep yeast plasmid miniprep kit (Zymo Research). Yeast cells were grown overnight at 30˚C in either liquid media or on solid media; whichever was the most appropriate for the best growth of the strain. If liquid cultures were grown, 1.5 mL of sample was pelleted at 1,000 X g for 2 minutes to obtain a cell pellet that was then resuspended in 150 µl of Solution 1 and 2 µl of zymolyase. If solid media was used, cells were scraped from the plate and added to the 150 µl Solution 1 and 2 µl zymolyase solution and mixed. The cell solution was then incubated at 37˚C for 60 minutes. Once the incubation was completed, 150 µl of Solution 2 was added to the tube and mixed by inverting 4-6 times followed by the addition of 200 µl Solution 3. Samples were then pelleted at 13,000 rpm for 5 minutes. The supernatant was then decanted into a fresh microcentrifuge tube and 400 ul of isopropanol added for DNA precipitation. The tube was pelleted at 13,000 rpm again for 10 minutes. The supernatant was decanted and the pellet was resuspended in TE pH 8.0. The presence of DNA was verified by 1% agarose gel electrophoresis.

2.5 Gas Chromotography

2.5.1 Saponification

Yeast cells, grown on appropriate media, were grown to stationary phase overnight at 30˚C. Cells were pelleted in 50 mL conical tubes for 5 minutes at 5,000 X g and the supernatant decanted. Cells were washed twice with 25-50 mL 1% igepal solution and twice with 25-50 mL sterile H_2O with five-minute centrifugation times at 5,000 X g in between to wash off residual exogenous sterol. The washed cells were then resuspended in 4 ml alcoholic KOH (25% w/v) and transferred to glass tubes. These were then incubated at 87˚C for 2 hours. After incubating, cells were cooled to room temperature and 3 mL of n-heptane and 1 mL dH_2O were added to extract the non-saponifiable lipid fraction. Samples were run on GC the same day if possible or stored at –20˚C and run the following day.

2.5.2 Gas Chromatography

The gas chromatograph is an HP5890 series II that utilizes a fused silica DB5-MS capillary column and the Hewlett Packard CHEMSTATION software and uses nitrogen as the carrier gas for the sample through a 15-meter column. The semi-splitless mode was used with a starting temperature of 195˚C for one minute increasing to 240˚C in 20˚C/min increments and then 2˚C/min increments to a final temperature of 280˚C where the temperature held for five minutes. The injection volume per sample was 2 μl.

2.5.3 Gas Chromatography/Mass Spectrophotometery

The gas chromatograph/mass spectrophotometer is an HP6890 series that utilizes a 5% phenyl methyl siloxane (HP-5MS) column (30mL X 0.25mm, 0.25μm) and uses helium as the carrier gas for the sample through a 30-meter column. The splitless mode was used with a starting temperature of 100˚C for one minute increasing to 300˚C in 7˚C/min increments and then held for 15 minutes at the final temperature of 300˚C. The injection volume per sample was 3 μl.

2.6 DNA sequencing

DNA sequencing reactions were performed at the Biochemistry Biotechnology Facility (BBF) Indiana University School of Medicine. Primers (Invitrogen) used for sequencing are listed in Table 2.3. R-seqMCS is a reverse strand primer located at the 3' end past *ERG25*, F-seq426ADH is a forward primer located at the 5' end before *ERG25*, and primers with '*ERG25'* are located somewhere within the *ERG25* gene (F- designates it is on the forward strand and R- designates it is on the reverse strand). DNA sequence was obtained from both forward and reverse strands around the site-directed mutations and for the entire strand for random mutations. The sequence was analyzed using Gene Runner software and Chromas electropherogram viewer both on a PC. To be considered valid, the mutation must have been verified on both directions of the DNA strand.

Table 2.3 *ERG25* mutagenesis sequence primers

Primer Name	Sequence
R-seqMCS	5'-TCGGTTAGAGCGGATGTGGG-3'
F-seq426ADH	5'-GCACAATATTTCAAGCTATACCAAGC-3'
F-ERG25seq1	5'-GGTACAGTTACATGAACAATGATGTTTTGGCC-3'
R-ERG25seq1	5'-GGCCAAAACATCATTGTTCATGTAACTGTACC-3'

F-ERG25seq2	5'-CCGTTCTTCTATCTCATTTCTTGGTCGAGGCC-3'
R-ERG25seq2	5'-GGCCTCGACCAAGAAATGAGATAGAAGAACGG-3'
F-ERG25seq3	5'-GGGTTTTGGTACCGTTGGTATGCC-3'
R-ERG25seq3	5'-GGCATACCAACGGTACCAAAACCC-3'
F-ERG25seq4	5'-CCCATGGTCTTTGAACAAGATCATGCCATTCTGGGC-3'
R-ERG25seq4	5'-GCCCAGAATGGCATGATCTTGTTCAAAGACCATGGG-3'
F-ERG25seqA	5'-GATAGTAGCATAGAGGACTAAGG-3'
R-ERG25seqA	5'-CTTCTCTGGAGGCCTTAGC-3'
F-ERG25seqB	5'-GCCATGGTTCATCATCGACC-3'
R-ERG25seqB	5'-TTAGGGTGATCCATACACATAGAG-3'
F-ERG25seqC	5'-CACCGTCTATTCCACTACGG-3'
R-ERG25seqC	5'-ACGAAGAATAGACCAATTTCTAGAGC-3'
F-ERG25seqD	5'-AAGATCATGCCATTCTGGGCTG-3'
R-ERG25seqD	5'-TAGTTGGTTGTAACTTCCATCTTCT-3'

2.7 DNA Manipulations

2.7.1 Yeast Gene Disruptions

ERG1, ERG7, ERG11, ERG25, and *ERG27* gene disruptions were made in *C. glabrata* wild type strain 2001HT and the *ERG25* gene disruption was made in *S. cerevisiae* wild type strain SCY876 using histidine as the selectable marker. The pRS303 plasmid was used for PCR amplification of the *HIS3* gene with specifically designed primers (listed in Table 2.4) containing 60 base pairs of homology at the ends for the specific gene being disrupted. PCR reactions contained: 1 µg of pRS303 plasmid DNA, 100 pmol of forward and reverse primers (Invitrogen), 1.5 mM dNTPs (Stratagene), 5 µl 10X Taq polymerase buffer (Promega), 1µl of 1 Unit/µl Taq polymerase (Promega), and the volume brought to 50µl with autoclaved dH20. PCR program parameters are listed in Table 2.5. PCR products were verified and quantified via 1% agarose gel electrophoresis. Approximately 1 µg of PCR product was transformed into the wild type yeast strain

using the LiAc transformation protocol (see section 2.3). Growth was screened for complementation on synthetic drop out media (without histidine). Yeast sterol auxotrophy was screened on synthetic media with and without sterols anaerobically and aerobically. Yeast colonies that grew anaerobically with sterol but not aerobically without sterol were then qualified as the correct gene disruptions based on gas chromotagraphy analysis.

Table 2.4 PCR primers for yeast gene disruptions: *ERG* gene homology in capital letters, homology with pRS303 in lower case

Forward C.g. *ERG1* GCTATTTAGTCGCTTAACACCTTATCAAGTGCTCTCCTGAAAACAATCAAG GACCAAAAAggcgggtgtcggggctggc
Reverse C.g. *ERG1* ATAAGAGAAAATACAATGAGTCGTTTAAGTGCAAAACACGTCTATATCAA ATGTTAGTCCttgccgatttcggcctattg
Forward C.g. *ERG7* CATAAGTTTATAAATTTGTATATTGAAAAATTGGAAGTGCAACGGTGTTGT AAAGCAATAggcgggtgtcggggctggc
Reverse C.g. *ERG7* AGTTTAAAAAAATTTTCGTTCGTAGCGCGGTATATAATATTATGCAGTGTA TATAGGAAAttgccgatttcggcctattg
Forward C.g. *ERG11* ATCTCGTATAATCAGTAGTCAAGACTTGTGCTAAACATCTTTACAAAAAA ATGTATATAATGGGCGATCCCTTCATGTCCggcgggtgtcggggctggc
Reverse C.g. *ERG11* TCAGCGTATATCCCGTATACGAGCCAGACAGCAATATTGTTTGAAGTAGG TTTTGACCATTGATTATTGGAAGAAAATGttgccgatttcggcctattg
Forward C.g. *ERG25* ACTTGATAAGATAAGAATTTGGTAAACAGGATATCTATTCTTCTTTCTCA CATTTAGAGCCTTAGACAAAACAACAAGCCggcgggtgtcggggctggc
Reverse C.g. *ERG25* GGTTAATTCTGTTTGTTATTGAAAAAAACAAAATCAAATGAAAGCGAGT TAGTGAAAAAAAAGTATAGTGATATGTAGTCCGttgccgatttcggcctattg
Forward C.g. *ERG27* TCATGAAATCAACTGCTACAACTTCAATATCAGGTAATAAACAGGATAT TAACAATCATTggcgggtgtcggggctggc
Reverse C.g. *ERG27* GCTATTTTACCAGTTTCAACCACCGAAACAAAGGCCAACATTCCACAAA ATATGATACCTttgccgatttcggcctattg

Forward S.c. *ERG25*
TTAGTTGTAACTTTTTCTCTTTAGATAGTAGCATAGAGGACTAAGGAAA AGTAGTACAGCCATAAAAAAAAGAGGAAAAGggcgggtgtcggggctggc
Reverse S.c. *ERG25*
ATATAGTTTTTAGAATTAACCTGAATTAATTTAAAAATCATATATTAAAA TAAACAATTGTGAAGGTAAAAAGAAAGAGTttgccgatttcggcctattg

Table 2.5 PCR parameters yeast gene disruptions

Parameter	Temperature	Time (min)
1	94˚C	5:00
2	94˚C	0:30
3	53˚C	0:30
4	72˚C	2:00
5		Repeat 2-4 (x25)
6	72˚C	5:00
7	4˚C	∞

2.7.2 Site-Directed Mutagenesis

Strategene's QuickChange II XL Site-Directed Mutagenesis Kit was used to perform site-directed mutagenesis. Primers (Invitrogen) were created in a complementary fashion with a range of 10-21 base pairs bordering each nucleotide change taking codon bias into account. Table 2.6 lists all primer sets for site-directed mutagenesis in ERG25 in *S. cerevisiae*. A wild type DNA template, RM213, utilized for these reactions was created by inserting *ERG25* gene into a p426ADH vector, PCR reactions had a final volume of 50 µl and contained 5.0 µl 10X Quick Change Lightening reaction buffer, 1.5 µl QuickSolution, 5 µl dNTP mix, 1 µl of *PfuUltra* HF DNA polymerase, 100-200 ng dsDNA template (RM213), and 125 ng of forward and reverse primer (listed in Table 2.6). PCR parameters are listed in Table 2.7. Upon completion of the PCR reaction, 2 µl

of *Dpn*I restriction enzyme was added to the reaction tube and incubated at 37˚C for 1-2 hrs. This enzyme digests any remaining methylated parent vector and ideally leaves only vector with the mutagenized gene insert. Next, 3 µl of the digested DNA was transformed into XL10 Gold Ultracompetent Cells (Stratagene) supplied with the kit. Cells were plated onto LB + amp (60 µg/ml) and incubated at 37˚C for 16-20 hrs. Plasmids were extracted from bacterial cells and sequenced to confirm the presence of the intended mutation.

Table 2.6 *ERG25* site-directed mutagenesis primer sets: codon changes are underlined, italicized, and in bold, nucleotides changed from original are in lower case

Change	Primers
H62A	F-SDM-H62A 5’-GGTCTAATGTTCTTTTTATTG***gcT***GAATTTATGTATTTC-3’ R-SDM-H62A 5’-GAAATACATAAATTC***Agc***CAATAAAAAGAACATTAGACC-3’
F67A	F-SDM-F67A 5’-CATGAATTTATGTAT***gcC***TTTAGATGTTTGCC-3’ R-SDM-F67A 5’-GGCAAACATCTAAA***Ggc***ATACATAAATTCATG-3’
Q88A	F-SDM-Q88A 5’-CCATACTTTAGAAGATGGAAGTTA***gcA***CCAACTAAGATTCC-3’ R-SDM-Q88A 5’-GGAATCTTAGTTGG***Tgc***TAACTTCCATCTTCTAAAGTATGG-3’
Q98A	F-SDM-Q98A 5’-CCAAGTGCTAAGGAA***gcA***CTATACTGTTTGAAATCCG-3’ R-SDM-Q98A 5’-CGGATTTCAAACAGTATAG***Tgc***TTCCTTAGCACTTGG-3’
C101A	F-SDM-C101A 5’-GCTAAGGAACAACTATAC***gcT***TTGAAATCCGTTC-3’ R-SDM-C101A 5’-GAACGGATTTCAA***Agc***GTATAGTTGTTCCTTAGC-3’
L102A	F-SDM-L102A 5’-GGAACAACTATACTGT***gcG***AAATCCGTTCTTCTA-3’ R-SDM-L102A 5’-TAGAAGAACGGATTT***Cgc***ACAGTATAGTTGTTCC-3’
I1115A	F-SDM-I115A 5’-TTCTTGGTCGAGGCC***gcC***CCTATCTGGACCTTC-3’ R-SDM-I115A 5’-GAAGGTCCAGATAGG***Ggc***GGCCTCGACCAAGAA-3’

E152A	F-SDM-E152A 5’-GGTCTATTCTTCGTCTTG***GcA***GATACATGGCATTACTGGG-3’ R-SDM-E152A 5’-CCCAGTAATGCCATGTATC***TgC***CAAGACGAAGAATAGACC-3’
D153A	F-SDM-D153A 5’-GGTCTATTCTTCGTCTTGGAA***Gcc***ACATGGCATTACTGGG-3’ R-SDM-D153A 5’-CCCAGTAATGCCATGT***ggC***TTCCAAGACGAAGAATAGACC-3’
Y157F	F-SDM-Y157F 5’-GGAAGATACATGGCAT***TtC***TGGGCTCACCGTCTATTCC-3’ R-SDM-Y157F 5’-GGAATAGACGGTGAGCCCA***GaA***ATGCCATGTATCTTCC-3’
H160A	F-SDM-H160A: 5’-GGCATTACTGGGCT***gcC***CGTCTATTCCACTACGG-3’ R-SDM-H160A 5’-CCGTAGTGGAATAGACG***Ggc***AGCCCAGTAATGCC-3’
H164A	F-SDM-H164A 5’-CCGTCTATTC***gcC***TACGGTGTCTTC-3’ R-SDM-H164A 5’-GAAGACACCGTA***Ggc***GAATAGACGG-3’
Y169F	F-SDM-Y169F 5’-CGGTGTCTTC***TtC***AAGTACATTCACAAGCAACATCAC-3’ R-SDM-Y169F 5’-GTGATGTTGCTTGTGAATGTACTT***GaA***GAAGACACCG-3’
K170A	F-SDM-K170A 5’-CGGTGTCTTCTAC***gcG***TACATTCACAAGCAACATCAC-3’ R: SDM-K170 5’-GTGATGTTGCTTGTGAATGTA***Cgc***GTAGAAGACACCG-3’
H173A	F-SDM-H173A 5’-CAAGTACATT***gcC***AAGCAACATCAC-3’ R-SDM-H173A 5’-GTGATGTTGCTT***Ggc***AATGTACTTG-3’
H176A	F-SDM-H176A 5’-GTACATTCACAAGCAA***gcT***CACAGATACGCTGC-3’ R-SDM-H176A 5’-GCAGCGTATCTGTG***Agc***TTGCTTGTGAATGTAC-3’
H177A	F-SDM-H160A 5’-CACAAGCAACAT***gcC***AGATACGCTGCTCCATTCGG-3’ R-SDM-H160A 5’-CCGAATGGAGCAGCGTATCT***Ggc***ATGTTGCTTGTG-3’
P182A	F-SDM-P182A 5’-CACAGATACGCTGCT***gcA***TTCGGTCTTTCTGC-3’ R-SDM-P182A 5’-GCAGAAAGACCGAA***Tgc***AGCAGCGTATCTGTG-3’

E188A	F-SDM-E188A 5’-CGGTCTTTCTGCT***gcA***TATGCTCATCCTGCTG-3’ R-SDM-E188A 5’-CAGCAGGATGAGCATA***Tgc***AGCAGAAAGACCG-3’
H191A	F-SDM-H191A 5’-GCTGAATATGCT***gcT***CCTGCTGAAACTTTGTCTTTGGG-3’ R-SDM-H191A 5’-CCCAAAGACAAAGTTTCAGCAGG***Agc***AGCATATTCAGC-3’
L198A	F-SDM-L198A 5’-GCTGAAACTTTGTCT***gcG***GGTTTTGGTACCGTTGG-3’ R-SDM-L198A 5’-CCAACGGTACCAAAACC***Cgc***AGACAAAGTTTCAGC-3’
R228A	F-SDM-R228A 5’-GGATCACCCTA***gcA***TTATTCCAAGC-3’ R-SDM-R228A 5’-GCTTGGAATAA***Tgc***TAGGGTGATCC-3’
H236A	F-SDM-H236A 5’-CCAAGCTGTTGACTCT***gcT***TCTGGTTATGACTTCCCATGG-3’ R-SDM-H236A 5’-CCATGGGAAGTCATAACCAGA***Agc***AGAGTCAACAGCTTGG-3’
Y239F	F-SDM-Y239F 5’-GTTGACTCTCATTCTGGT***TtT***GACTTCCCATGGT-3’ R-SDM-Y239F 5’-ACCATGGGAAGTC***AaA***ACCAGAATGAGAGTCAAC-3’
H258A	F-SDM-H258A 5’-GGGCTGGCGCTGAACAC***gcC***GATTTGCATCATC-3’ R-SDM-H258A 5’-GATGATGCAAATC***Ggc***GTGTTCAGCGCCAGCCC-3’
H261A H262A	F-SDM-H261AH262A 5’-CCACGATTTG***gcTgcT***CACTACTTTATTGG-3’ R-SDM-H261AH262A 5’-CCAATAAAGTAGTG***AgcAgc***CAAATCGTGG-3’
T282A	F-SDM-T282A 5’-GGGATTACTGTCTAGAC***gcT***GAATCTGGTCC-3’ R-SDM-T282A 5’-GGACCAGATTC***Agc***GTCTAGACAGTAATCCC-3’
K306A, K307A	F-SDM-K306AK307A 5’-GAAAACAATGCTCAA***gcGgcG***ACTAACTAACCCACATCCG-3’ R-SDM-K306AK307A 5’-CGGATGTGGGTTAGTTAGT***CgcCgc***TTGAGCATTGTTTTC-3’

Table 2.7 PCR parameters for *ERG25* site-directed mutagenesis

Parameter	Temperature	Time (min)
1	95˚C	2:00
2	95˚C	0:20
3	61˚C	0:10
4	68˚C	10:00
5		Repeat 2-4 (x17)
6	68˚C	7:00
7	4˚C	∞

2.7.3 Random Mutagenesis

Random mutagenesis was undertaken in *S. cerevisiae ERG25* to generate point mutations at potentially novel sites in the gene. The method used is referred to as "gap repair" as described by Muhlrad et al. (47). First, an *ERG25* gene "insert" with a random mutation was created via PCR as diagrammed in Figure 2.1.

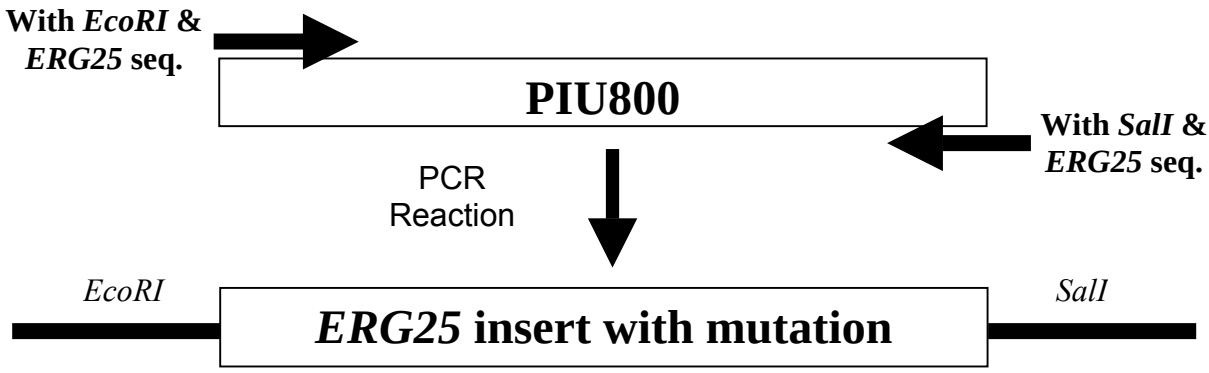

Figure 2.1 Diagrammatic scheme of random mutagenesis "insert" creation.

Using Taq polymerase, which introduces an error in replication in 1 of approximately 1000 base pairs, created the random mutation. The *ERG25* gene is 937 base pairs; therefore each PCR reaction should theoretically contain one mutation. Each

PCR reaction contained: 100 pmol of forward and reverse primers (listed in Table 2.8), 1 Unit Taq polymerase (Promega), 5 µl of 10X Taq buffer (Promega), 4 mM dNTPs (Stratagene), 100ng pIU800 plasmid DNA and brought to a volume of 50 µl with autoclaved dH_2O. The PCR program parameters are listed in Table 2.9. The PCR product, also referred to as the insert, contains *ERG25* with a potential mutation and *EcoRI* and *SalI* capped ends.

Table 2.8 Gap repair primers for insert. *ERG25* sequence lowercase, Restriction site underlined and in bold, plasmid sequence in uppercase

Forward 5'CCTCTTCTTGTTTTATCTAGAACTAGTGGATCCCCCGGGCTGCAG**<u>GAATTC</u>** atgtctgccgttttcaacaacgctaccc-3'
Reverse 5'GAATGTAAGCGTGACATAACTAATTACATGATGCGGCCCTCCTCGAG**<u>GTCGAC</u>** ttagttagtcttcttttgagcattg-3'

Table 2.9 PCR parameters Gap Repair

Parameter	Temperature	Time (min)
1	94˚C	5:00
2	94˚C	0:30
3	53˚C	0:30
4	72˚C	2:00
5		repeat 2-4 (X 34)
6	72˚C	5:00
7	4˚C	∞

Next, digesting p426ADH plasmid created a gapped vector, also referred to as the "backbone" as diagrammed in Figure 2.2.

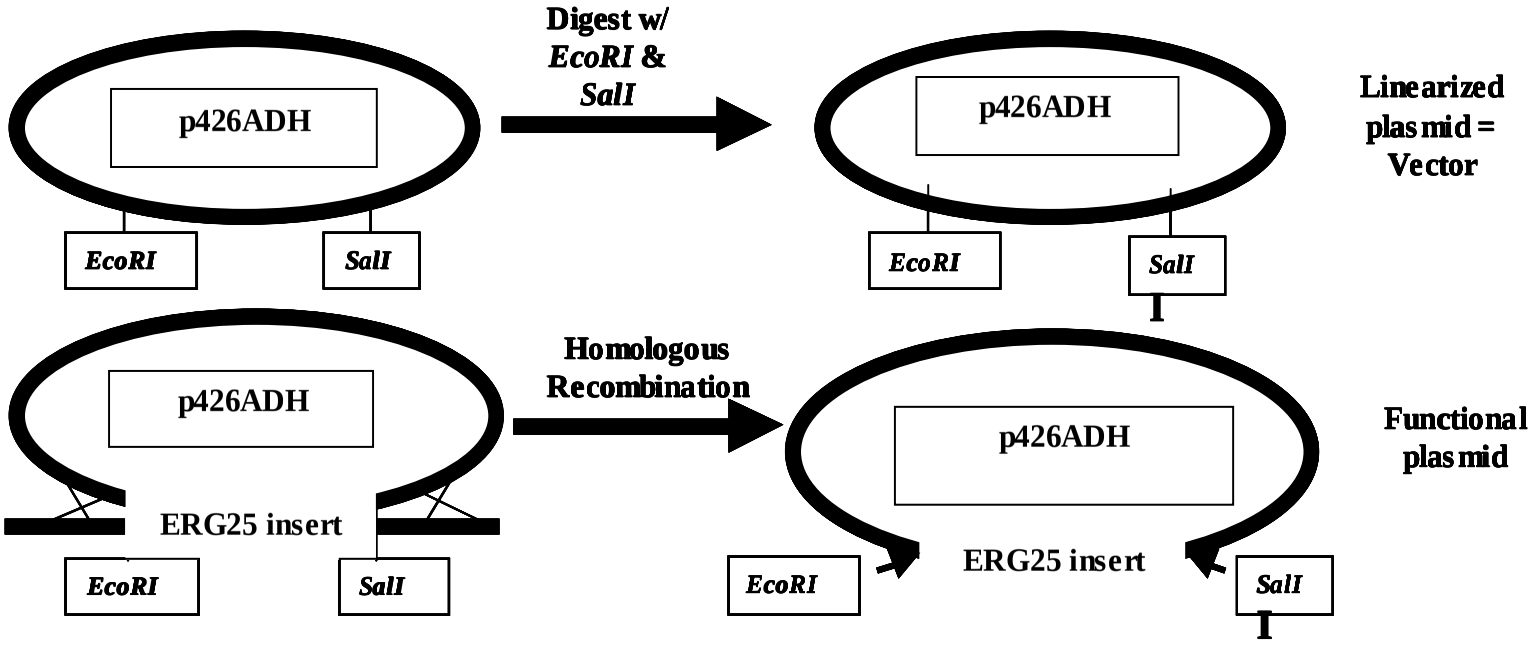

Figure 2.2 Diagrammatic scheme of creating the "vector" and functional plasmid for random mutagenesis

The p426ADH plasmid (7457 bp) was linearized via restriction digest with *EcoRI* and *SalI* endonucleases creating a 7430 bp plasmid vector with *EcoRI* and *SalI* capped ends. This vector and the insert were co-transformed into *S. cerevisiae* yeast strain D1 (Δ*erg25*) where homologous recombination between the vector and insert creates a functional plasmid while simultaneously transforming into yeast. Cells were plated onto CSM-ura + ergosterol plates and colonies were screened for complementation.

CHAPTER 3

AEROBIC UPTAKE OF EXOGENOUS CHOLESTEROL BY ERGOSTEROL AUXOTROPHS IN *CANDIDA GLABRATA*

3.1 Introduction

Many *Candida* species of yeast are opportunistic human pathogens (52). *Candida* species are the fourth leading cause of fungal bloodstream infections (28). When a *Candida* species infects an individual's bloodstream, the resulting disease is referred to as candidiasis, or more specifically candidemia. Individuals undergoing immunosuppressive therapies for cancer and organ transplantation, these with immunosuppressive diseases, such as HIV/AIDS, and/or surgical hospital patients are at a much higher risk for candidemia (7). From 1980 to 1990, 78.3% of nosocomial fungal infections were reportedly caused by *Candida* species (8). Candida infections are of particular concern due to the increasing resistance to current treatments (9) as well as the numerous studies showing an association between the disease and an increase in mortality rate and excessive length of hospital stays (8, 10, 11, 12, 13).

Several risk factors are recognized that may predispose higher risk individuals to acquiring sepsis from a *Candida* species infection. These include: prior cortiocosteroid use, the use of chemotherapy agents, neutropenia, burns, massive surgery, central catheters (29); hemodialysis, previous fungal infection and/or use of antimicrobial agents

(30); and organ malignancy (either solid or hematolgic) (31). Clinical symptoms of candidemia are generally non-specific and laboratory testing is complicated. Therefore it can be difficult to diagnose until late in the infection and is typically based on clinical status of the patient (14). Although early diagnosis and appropriate treatment of these infections is vital in decreasing the rate of mortality associated with such disease (17), a clearer understanding of *Candida's* increasing resistance to the current treatments as well as its increase in incidence also important for potentially creating new drugs for improved treatments and/or potential improvements in preventative methods of the spread of said disease.

Of the *Candida* species, *C. albicans* is the leading causative species of candidasis, although other *Candida* species are steadily increasing in their incidence of infection (15). *C. albicans* is found in approximately 48% of candidemia patients while approximately 24% of infected patients have *C. glabrata* growing in their bloodstream (14). Studies have shown that the increase in prophylactic use of antifungal drugs is associated with this increase of infection by non-albicans *Candida* species (16). Of these non-albicans *Candida* species, *C. glabrata* is of particular concern because of it's: significantly increasing occurrence, association with high mortality rate (19) and decrease in susceptibility to azole antifungal drugs (18) the most prevalent treatment for such infections.

The ergosterol pathway is an ideal target for antifungal drug treatments because the sterol itself and at least one step, such as *ERG24,* of its biosynthetic pathway are specific to fungi. Therefore, medications affecting sterol synthesis in the fungi causing the infection should not interfere with the sterol synthesis of mammalian cells. Several

types of antifungal drugs are available as treatment options targeting varying steps in the ergosterol pathway including: allylamines, thiocarbomates, pyridines, pyrimidines, morpholines, polyenes, and the most significant for this study, azoles.

Allylamines and thiocarbomates both target the *ERG1* gene product squalene epoxidase, as autoradiography studies have shown by an accumulation of radioactive squalene (23). Some ultrastructural studies also suggest these drugs interfere with cell wall biosynthesis (24). These drugs are most commonly used as topical medications and have been shown to work against *C. albicans* and *C. parapsilosis* (23).

Pyridines, such as pyrifenox, and pyrimidines, such as fenarimol, like azoles, target the *ERG11* gene product lanosterol C-14 demethylase. The mechanism used by these fungistatic antifungals is not clear (25), but these products are used most commonly in agriculture.

Morpholines target two steps in the ergosterol pathway increasing their fungistatic abilities. The target enzymes are the *ERG24* gene product, C-14 sterol reductase, and the *ERG2* gene product, C-8 sterol isomerase (26). Like the pyridines and pyrimidines, most morpholines are used in agricultural applications.

Polyenes are the major class of fungicidal antifungal drugs and do not target ergosterol biosynthesis. Rather, polyenes, such as Amphoteracin B, target ergosterol in the fungal cell membrane (27). These antifungal drugs open up channels in the cell membrane allowing leakage of ions in and out of the cell ultimately leading to cell death (53). These antifungals are known for their negative side effects including renal failure and resistance is common.

The antifungal drug group with the most significance to this study is also the most commonly used azole antifungal drugs. These azole drugs target the *ERG11* gene product by preventing the C-14 demethylation of the first sterol intermediate, lanosterol, thereby inhibiting the production of the pathways end product ergosterol (20). The azole ring of the drug binds nitrogen to the iron center of the heme interfering with the binding of oxygen to cytochrome P-450 (21). This in turn deactivates the enzyme preventing the C-14 demethylation of lanosterol. *ERG11* is an essential gene in the pathway. Lanosterol is the first sterol intermediate created in the pathway and is required for conversion to an active, functional sterol.

These azole drugs appear to be having a diminished effectiveness due to the recent increase in resistant fungal strains. Several mechanisms for resistance to these drugs include an increase in the expression of drug efflux pumps and alterations in genes encoding up regulation of target enzymes in the ergosterol pathway (20) as well as long-term use of antifungals leading to strains resistant to these drugs. Some studies have attributed *C. glabrata's* resistance to azole antifungal drugs to an increase in *CgCDR1* and *CgPDH1* transporters (32, 33) as well as *AUS1* sterol transporter (50). In this study, we suggest another mechanism: that the overuse of triazole drugs has led to the increase in resistance by *C. glabrata* through a 2-step process: 1) accumulation of ergosterol auxotrophic mutations due to prior azole treatment and 2) the ability of such mutants, able to take up exogenous cholesterol anaerobically in the body, to acquire a second mutation allowing uptake of cholesterol aerobically.

Several studies have reported and identified sterol auxotrophic strains in *C. glabrata*. Hazen and colleagues isolated six bile salt requiring *C. glabrata* strains from

ICU patients (60) urinary tracts. In collaboration with the Bard lab, these six isolates were found to be sterol-requiring and based on GC sterol profiles: two were *erg1*, three were *erg7*, and one had a single heme mutation (61). Rezusta and colleagues have also reported two *C. glabrata* sterol-requiring isolates (62), one of which was found to be *erg7* (unpublished). Interestingly, all patients harboring these clinical isolate specimens were previously treated with azole antifungals, supporting the first step of our hypothesized mechanism.

Sterol uptake by *C. glabrata* is more similar to sterol uptake by *S. cerevisiae* than *C. albicans* (22). Both *S. cerevisiae* and *C. glabrata* can take up exogenous sterol under anaerobic conditions or aerobically in heme deficient strains. This is referred to as aerobic sterol exclusion. The *S. cerevisiae UPC2* transcription factor regulates *AUS1* and *PDR11* genes, which are required for sterol uptake. It also has a heme-binding protein, *Rox1,* which will repress *UPC2* aerobically resulting in *AUS1* and *PDR11* being turned off aerobically along with sterol uptake (55). *C. glabrata* has two *UPC2* transcription factors; a short form and a long form, it also has an *AUS1* transporter; however, not a *PDR11*. On the other hand, *C. albicans* will not uptake exogenous sterols aerobically or anaerobically presumably due to a lack of *UPC2* gene(s).

In this work, sterol auxotrophs have been created in the *C. glabrata* strain 2001HT by disrupting different essential genes (*ERG1, ERG7, ERG11, ERG25,* and *ERG27*) in the ergosterol pathway to assess which strain will acquire mutations to allow the uptake of exogenous sterols aerobically. *ERG1, ERG7, ERG11, ERG25,* and *ERG27* are essential genes, indicating they are required to produce the end product ergosterol. *ERG1*, or squalene epoxidase, converts squalene to squalene epoxide. *ERG7*, or

lanosterol synthase, transforms squalene epoxide into lanosterol, the first sterol precursor of the ergosterol pathway. *ERG11* removes a methyl group from the C-14 position of lanosterol creating the 4,4-dimethylcholesta-8,14,24-tienol intermediate. *ERG25*, a sterol C-4 methyloxidase, oxidizes the alcohol to a carboxylic acid. *ERG27*, a sterol C-3 keto-reductase, reduces the ketone to an alcohol. Should one of these genes become disrupted, the pathway will stop and depending on the gene, will begin accumulating a sterol precursor (See Table 3.1).

Nakayama et al. have shown that a *C. glabrata* strain with its *ERG9* promoter replaced with a tet-promotor (*Cg*97SQS) will take up cholesterol from serum-enriched media aerobically for survival (63). The *ERG9* enzyme is squalene synthase and is essential for the ergosterol pathway. The squalene synthase converts farnesyl pyrophosphate to squalene. The 97SQS strain *ERG9* gene is under the control of a tet-promoter, which is regulated by doxycycline. Thus, *ERG9* expression is turned off in the presence of doxycycline preventing squalene synthesis. *AUS1* expression is required for the uptake of sterol. Experiments were initiated to compare the growth of two *C. glabrata* strains, one with the *AUS1* gene (97SQS) and one without the *AUS1* gene (97SQS/ΔAUS1) on media with different concentrations of cholesterol, human serum, or bovine serum and varying amounts of doxycycline. Based on Nakayama's work, we expected doxycycline to inhibit growth of both strains because both are under the control of the tet-promoter; however 97SQS would be better able to utilize exogenous cholesterol than 97SQS/ΔAUS1.

3.2 Results

Gene disruptions were created for each of *ERG1, ERG7, ERG11, ERG25,* and *ERG27* genes. First, a PCR product of the *HIS3* selectable marker and 60 base pairs of homology to each specific ERG gene was created using pRS303 and primers designed to target the *HIS3* marker in the plasmid with tails of ERG gene homology (See Tables 2.4 and 2.5 for primers and PCR parameters). Each PCR product was then transformed into *C. glabrata* 2001HT by homologous recombination in separate reactions and plated onto CSM-his supplemented with ergosterol and grown anaerobically for 3-5 days. Colonies were picked and plated into columns on CSM-his plus ergosterol and grown anaerobically again. These "master" plates were then replica plated onto CSM-his supplemented with and without ergosterol grown anaerobically. Colonies that grow only with ergosterol supplementation were considered as potential candidates for the gene disruption because sterol supplementation would be required for any essential gene disruption. Sterol profiles of the colonies were further analyzed via gas chromatography/mass spectrophotometry. Each gene knockout was verified when the appropriate ergosterol pathway intermediate accumulated on the GC/MS profile (refer to Table 3.1).

Table 3.1 Accumulating sterol precursors for specific ergosterol genes

Gene in pathway	Expected sterol precursor accumulated
ERG1	Squalene
ERG7	Squalene epoxide
ERG11	Lanosterol
ERG25	4,4-dimethylzymosterol
ERG27	Squalene epoxide

Each potential gene knockout was grown in 50mL CSM-his liquid media supplemented with cholesterol and grown anaerobically with shaking at approximately 225rpm for 2 days. Each sample was then saponified in a KOH/EtOH solution for 2 hours to separate the non-saponifiable sterols from rest of the yeast cells. The sterol samples were drawn out in heptane and run on the GC program as described in Chapter 2. The following five figures (Figures 3.1, 3.2, 3.3, 3.4, and 3.5) each display the GC profile for each of the *ERG1, ERG7, ERG11, ERG25,* and *ERG27* gene disruptions respectively. Where *ERG1* displays an accumulation of squalene*, ERG7* displays an accumulation of squalene epoxide*, ERG11* displays an accumulation of lanosterol*, ERG25* displays an accumulation of 4,4-dimethylzymosterol*,* and *ERG27*displays an accumulation of squalene epoxide, all as expected and verifying the correct gene disruptions were created.

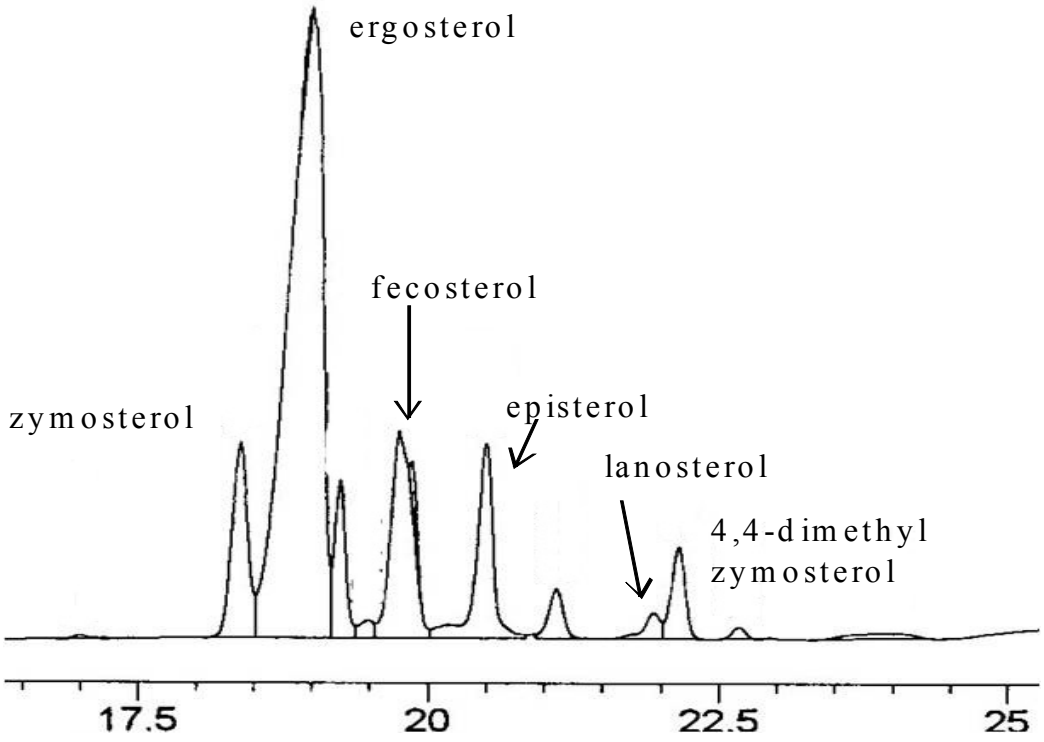

Figure 3.1 GC profile of wild type *Cg2001HT* strain grown with cholesterol aerobically

This GC profile illustrates the sterol profile of the wild type strain of *C. glabrata* used for these gene disruptions in this study. The largest sterol peak is ergosterol and every sterol precursor and intermediate is represented in the profile.

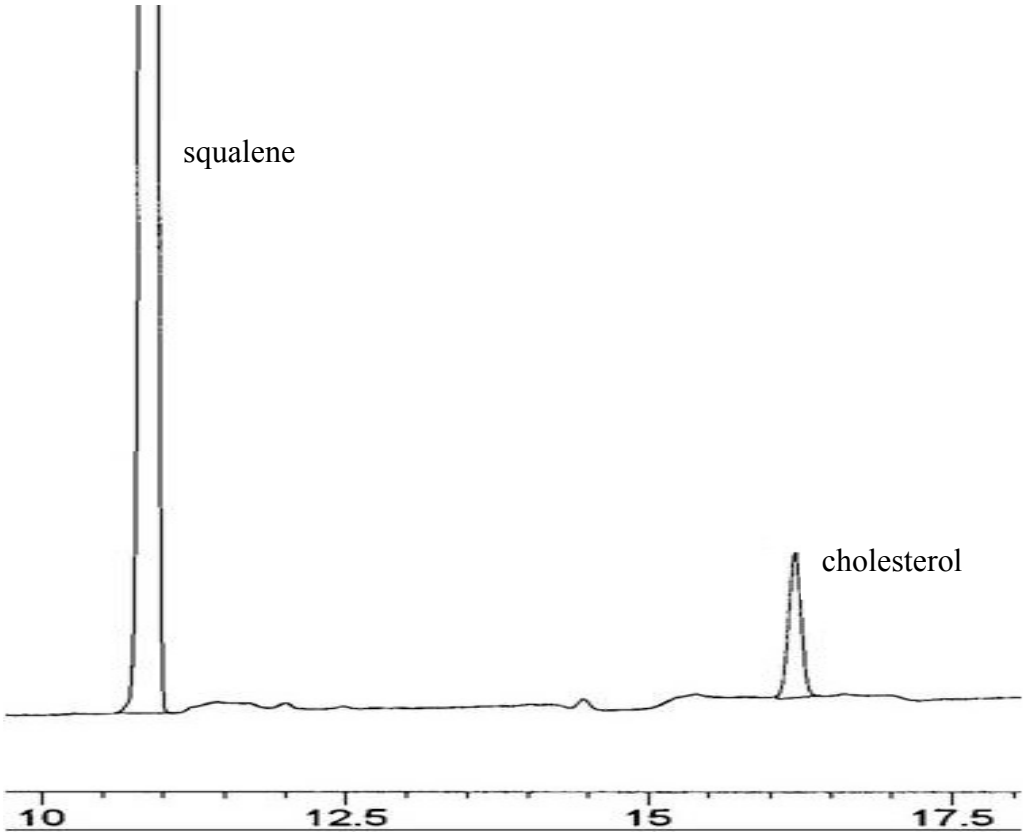

Figure 3.2 GC profile of *ERG1* in *C. glabrata*. Cells grown anaerobically with cholesterol for two days

This illustrates the GC profile of *ERG1* disruption in *C. glabrata*. As expected from what is known of the biosynthetic pathway, there is an accumulation of squalene. The second peak represents cholesterol, which is residual from the cells having been grown anaerobically in CSM-his + Cholesterol with shaking at approximately 225 rpm for 2 days.

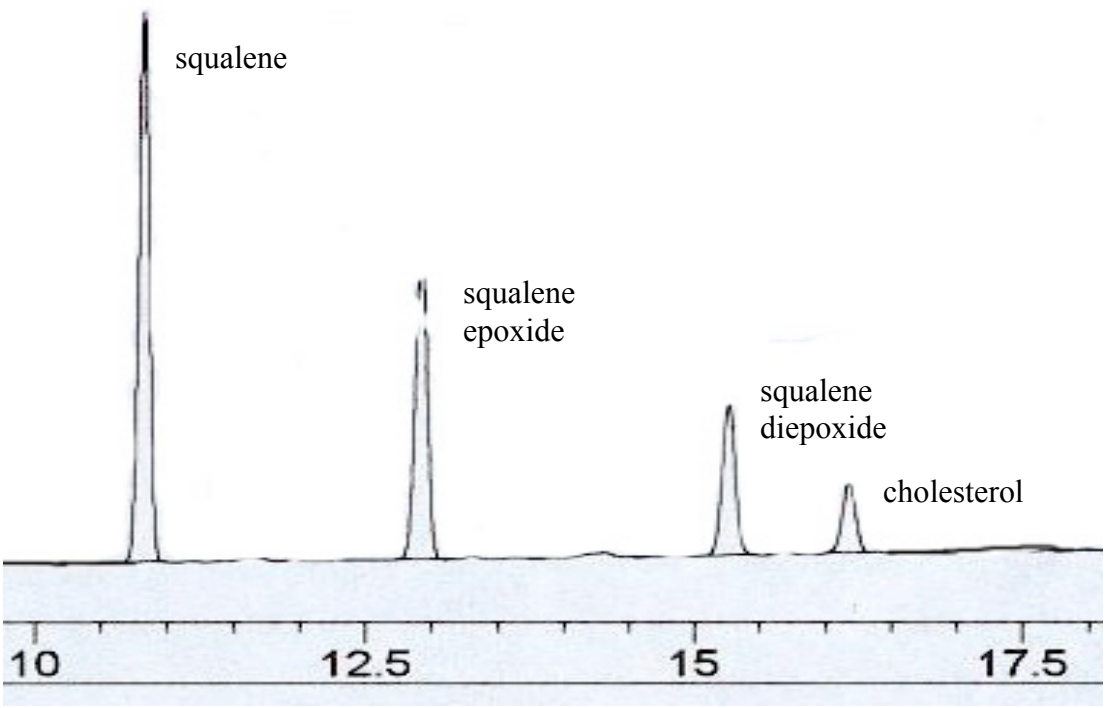

Figure 3.3 GC profile of *ERG7* in *C. glabrata*. Cells grown anaerobically with cholesterol for two days

This illustrates the GC profile of *ERG7* disruption in *C. glabrata*. As expected, there is an accumulation of squalene, squalene epoxide, and squalene diepoxide. Again, the fourth peak represents cholesterol, which is residual from the cells having been grown anaerobically in CSM-his+cholesterol with shaking at approximately 225 rpm for 2 days.

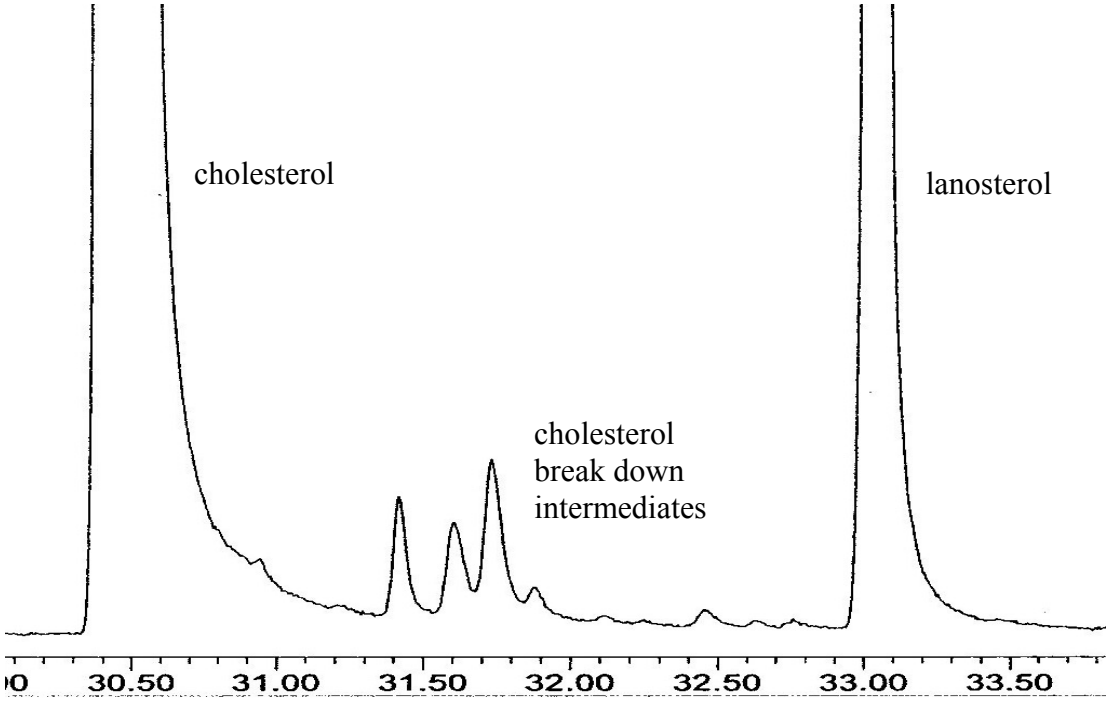

Figure 3.4 GC profile of *ERG11* in *C. glabrata*. Cells grown anaerobically with cholesterol for two days

This illustrates the GC/MS profile of *ERG11* disruption in *C. glabrata.* As expected, there is an accumulation of lanosterol. The other larger peak represents cholesterol, which is residual from the cells having been grown anaerobically in CSM-his+cholesterol anaerobically with shaking at approximately 225 rpm for 2 days. The remaining tiny peaks represent cholesterol break down intermediates.

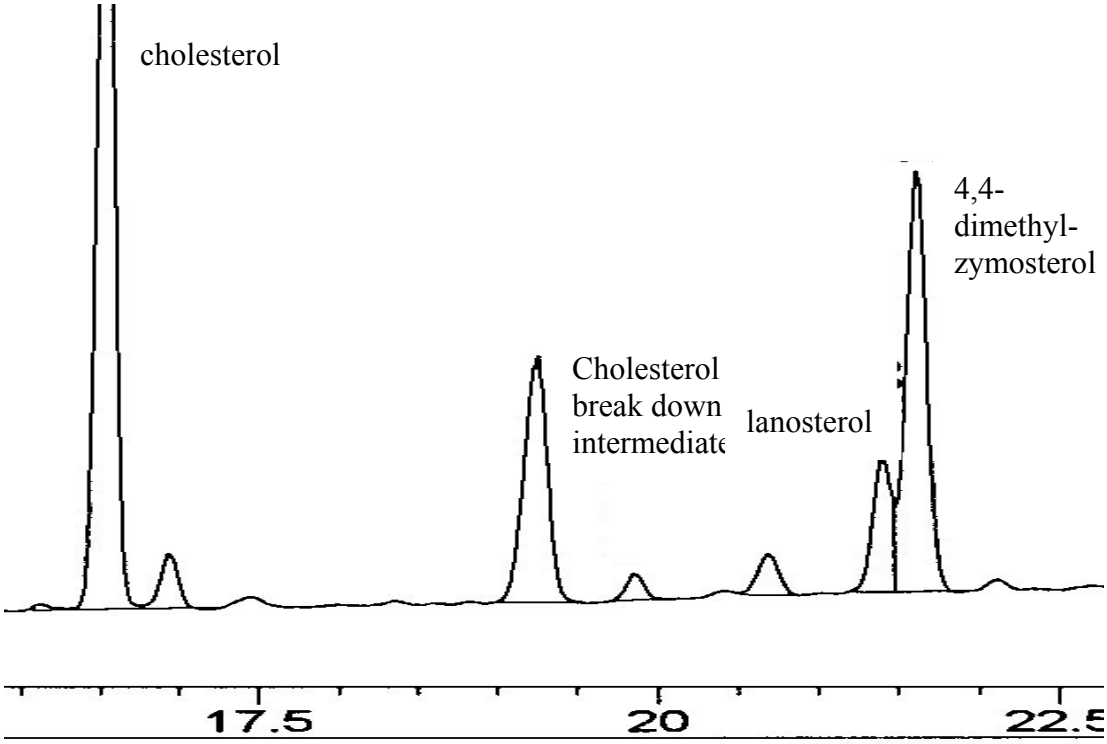

Figure 3.5 GC profile of *ERG25* in *C. glabrata*. Cells grown anaerobically with cholesterol for two days

This illustrates the GC profile of *ERG25* disruption in *C. glabrata.* As expected, there is an accumulation of 4,4-dimethylzymosterol. The other larger peak represents cholesterol, which is residual from the cells having been grown anaerobically in CSM-his+cholesterol with shaking at approximately 225 rpm for 2 days. The remaining tiny peaks, again, represent cholesterol break down intermediates.

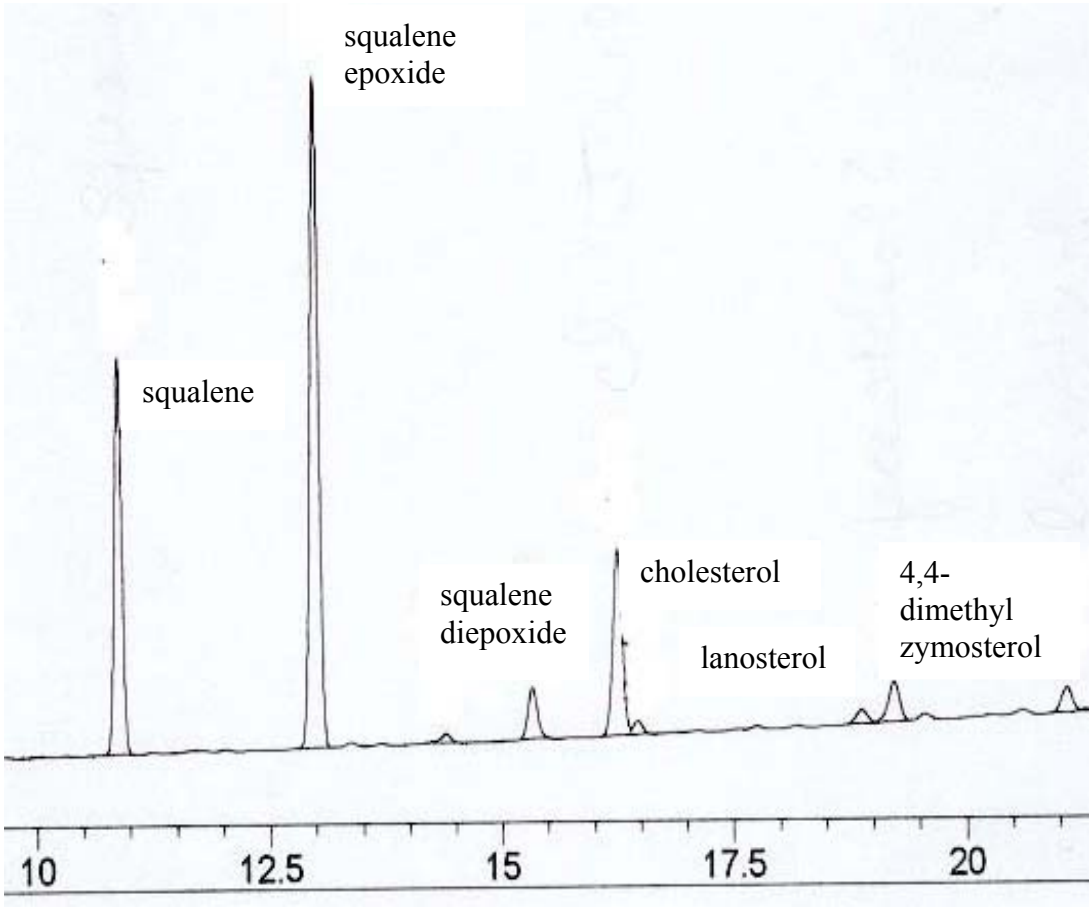

Figure 3.6 GC profile of *ERG27* in *C. glabrata*. Cells grown anaerobically with cholesterol for two days

This illustrates the GC profile of *ERG27* disruption in *C. glabrata.* As expected and similar to *ERG7*, there is an accumulation of squalene, squalene epoxide, and squalene diepoxide. Again, the third largest peak represents cholesterol, which is residual from the cells having been grown anaerobically in CSM-his+cholesterol with shaking at approximately 225 rpm for 2 days. There is a very small lanosterol peak.

The percentage of sterol accumulation was calculated based on GC profiles for each gene disruption and are listed in Table 3.2. Values are based on single peak values compared to total sterol precursor values of each GC profile.

Table 3.2 GC sterol profiles of Δ*erg1, Δerg7*, *Δerg11, Δerg25,* and Δ*erg27* strains. Values represent percent sterol. All samples were grown anaerobically in CSM-his + cholesterol with shaking at approximately 225 rpm for two days

	erg1	erg7	erg11	erg25	erg27
Sterol					
squalene	95	46	0	40	24
squalene-2,3-epoxide	0	29	0	0	42
squalene-2,3;22,23-diepoxide	0	16	0	0	4
cholesterol	5	8	80	30	16
ergosterol	0	0	0	0	0
lanosterol	0	0	20	0	3
4,4-dimethylzymosterol	0	0	0	25	0

After all five gene disruptions were verified by GC/MS, each strain was spot plated with OD values of 4.0, 2.0, 1.0, 0.5, and 0.1 onto YPD plates with no supplementation and with Tween 80 (an oleic acid detergent) and with cholesterol, cholesterol linoleate and cholesterol oleate supplementation and grown aerobically. The control plates, YPD and YPD+Tween80, should not show growth due to a lack of sterol supplementation that is required for ergosterol auxotrophic strains. Growth on any of the cholesterol and cholesterol ester supplemented plates indicates the ergosterol auxotrophs have acquired secondary mutations allowing for the aerobic uptake of cholesterol. Figures 3.6, 3.7, 3.8, 3.9, and 3.10 display the results for the spot plates of each of the *ERG1, ERG7, ERG11, ERG25,* and *ERG27* gene disruptions, respectively.

Figure 3.7 shows the spot plate results for *ERG1*. Control plates, YPD and YPD+Tween80, do not show any growth as expected due to the lack of sterol supplementation. Plates supplemented with cholesterol do acquire single colony growth aerobically on day 1 that increases daily. Plates supplemented with cholesterol esters, similarly to the control plates, also do not acquire any growth by day 3. All plates show full growth by day 1 when grown anaerobically.

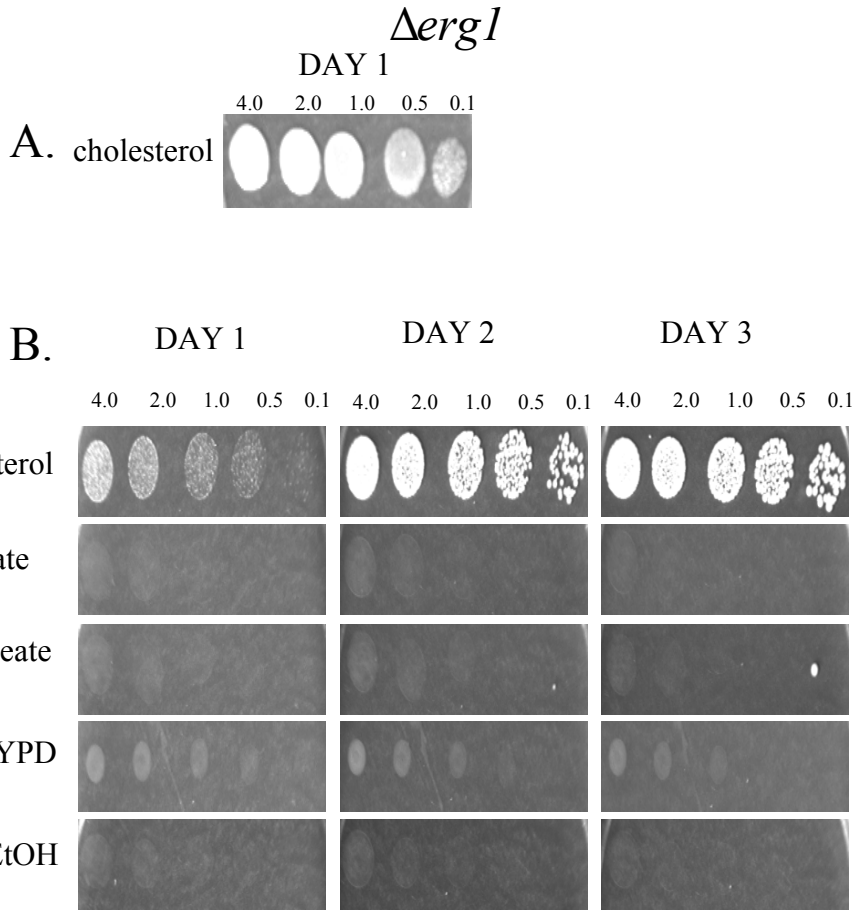

Figure 3.7 A) Δ*erg1* grown anaerobically with cholesterol. B) Spot plate analysis of Δ*erg1*. Strains grown on YPD media supplemented with 20 µg/ml sterol in Tween 80/EtOH (1:1 v/v)

Figure 3.8 shows the spot plate results for *ERG7*, which are similar to *ERG1*. Control plates, YPD and YPD+Tween80, do not show any growth as expected due to the lack of sterol supplementation. Plates supplemented with cholesterol do acquire single colony growth aerobically on day 1 that increases daily. Plates supplemented with cholesterol esters, similarly to the control plates, also do not acquire any growth by day 3. All plates show full growth by day 1 when grown anaerobically.

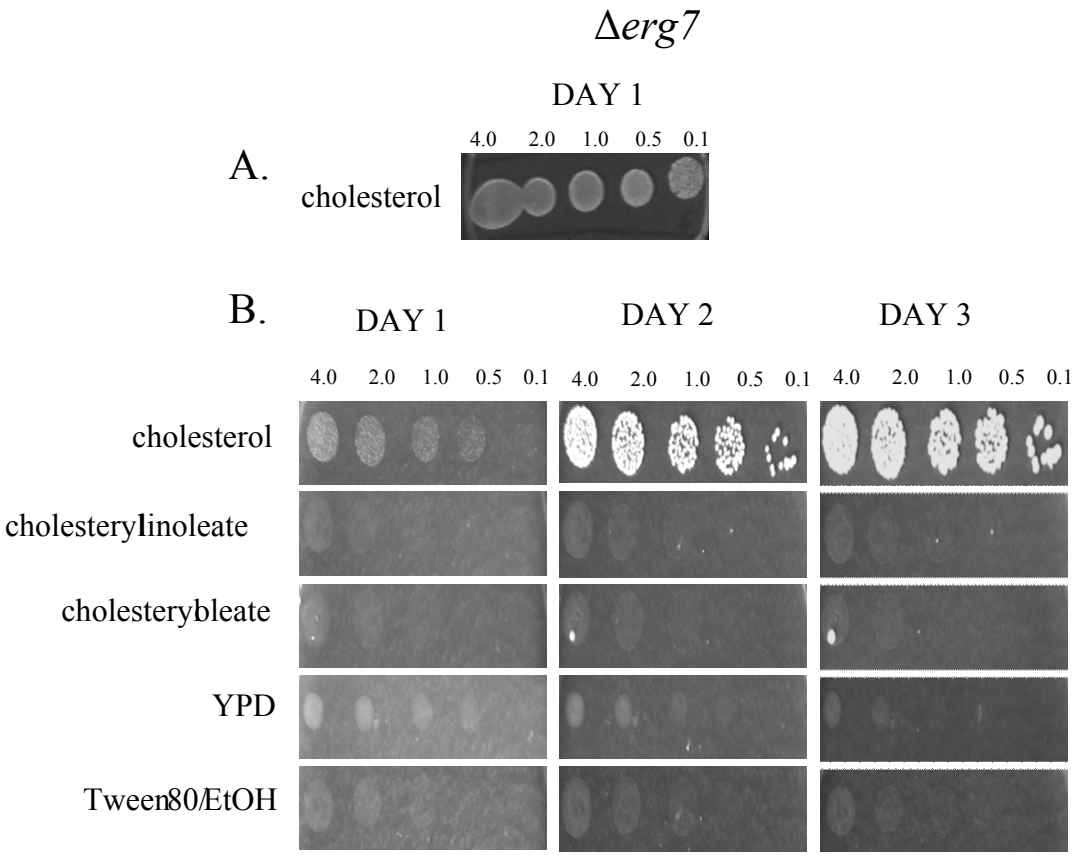

Figure 3.8 A) Δ*erg7* grown anaerobically with cholesterol. B) Spot plate analysis of Δ*erg7*. Strains grown on YPD media supplemented with 20 µg/ml sterol in Tween 80/EtOH (1:1 v/v)

Figure 3.9 shows the spot plate results for *ERG11*. YPD control plates do not have any growth; however, the YPD+Tween80 plates do show single colony growth on day three. Plates supplemented with cholesterol acquire lawn growth aerobically on day 1 that increases daily. Plates supplemented with cholesterol esters begin to acquire satellite colonies on day 3. All plates show full growth by day 1 when grown anaerobically.

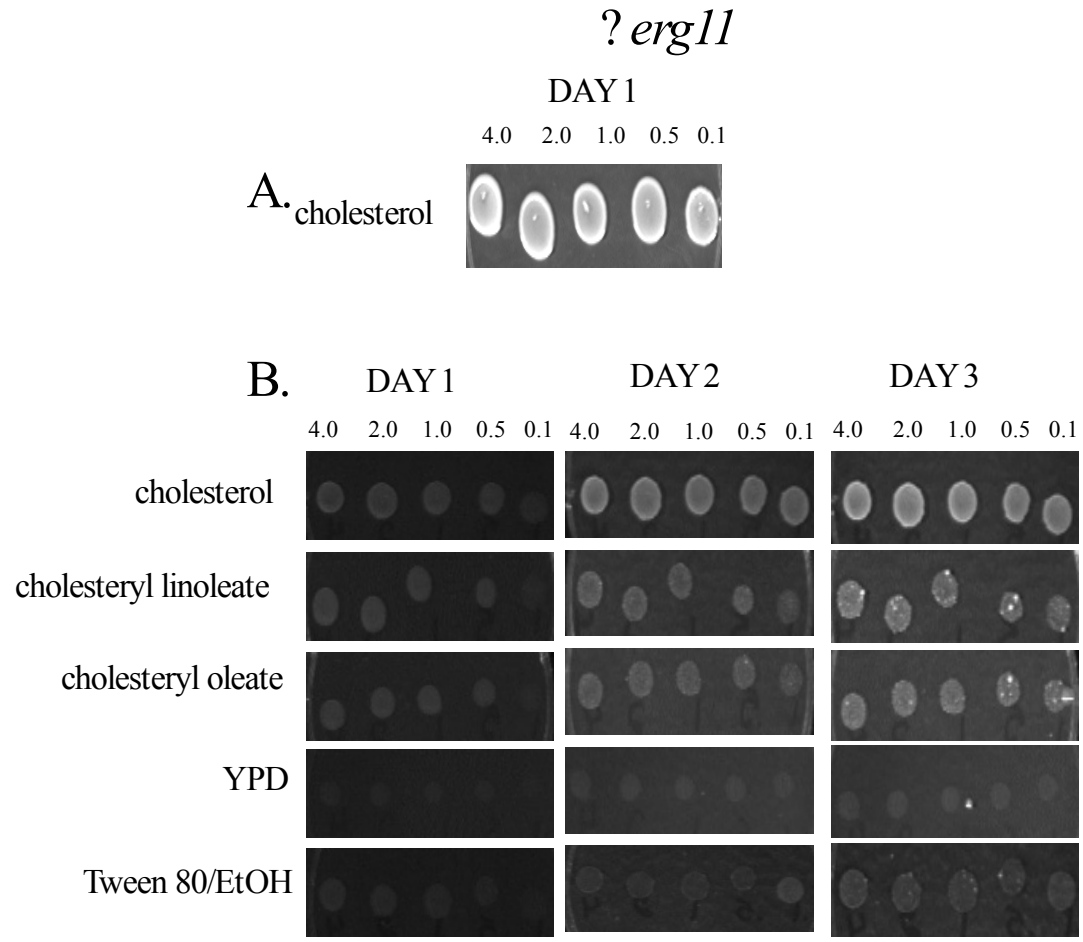

Figure 3.9 A) Δ*erg11* grown anaerobically with cholesterol. B) Spot plate analysis of Δ*erg11*. Strains grown on YPD media supplemented with 20 µg/ml sterol in Tween 80/EtOH (1:1 v/v)

Figure 3.10 shows the spot plate results for *ERG25,* which are similar to *ERG11*. YPD control plates do not have any growth; however, the YPD+Tween80 plates do show single colony growth on day three. Plates supplemented with cholesterol acquire lawn growth aerobically on day 1 that increases daily. Plates supplemented with cholesterol esters begin to acquire satellite colonies on day 1 for higher O_D values and by day 3 for lower O_D values. All plates show full growth by day 1 when grown anaerobically.

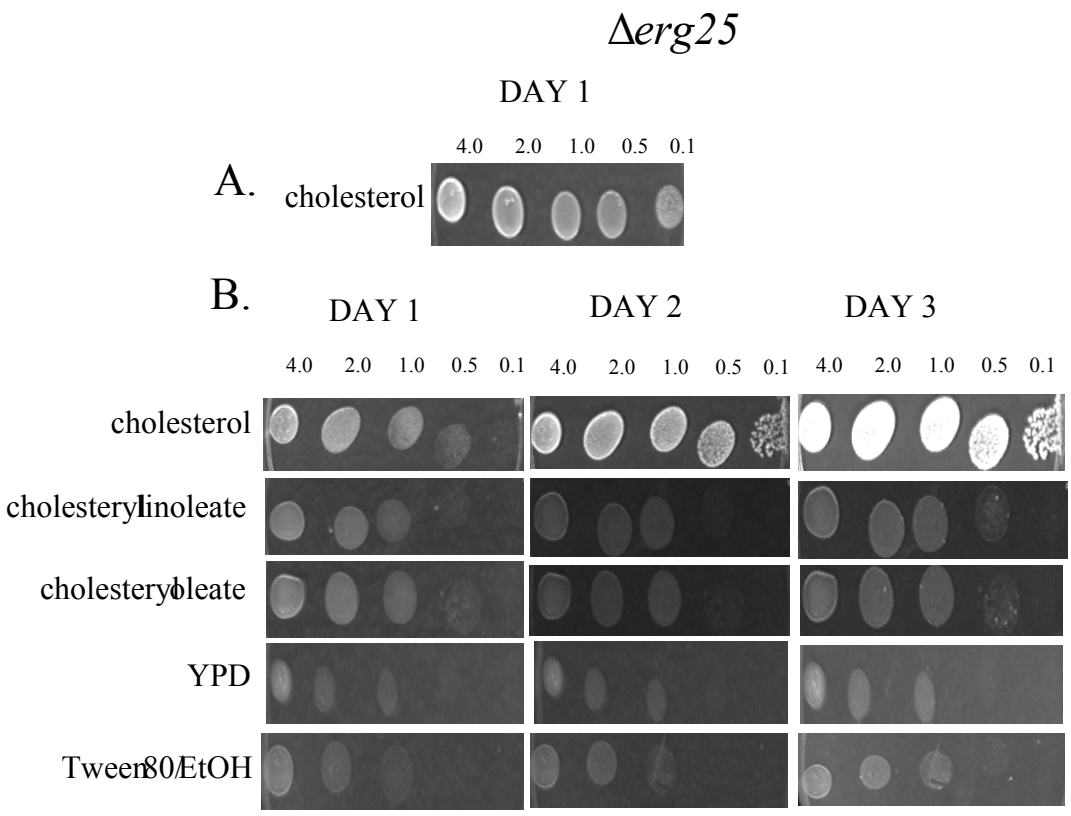

Figure 3.10 A) Δ*erg25* grown anaerobically with cholesterol. B) Spot plate analysis of Δ*erg25*. Strains grown on YPD media supplemented with 20 µg/ml sterol in Tween 80/EtOH (1:1 v/v)

Figure 3.11 shows the spot plate results for *ERG27*, which are similar to *ERG1* and *ERG7*. Control plates, YPD and YPD+Tween80, do not show any growth as expected due to the lack of sterol supplementation. Plates supplemented with cholesterol do acquire single colony growth aerobically on day 1 that increases daily. Plates supplemented with cholesterol esters, similarly to the control plates, also do not acquire any growth by day 3. All plates show full growth by day 1 when grown anaerobically.

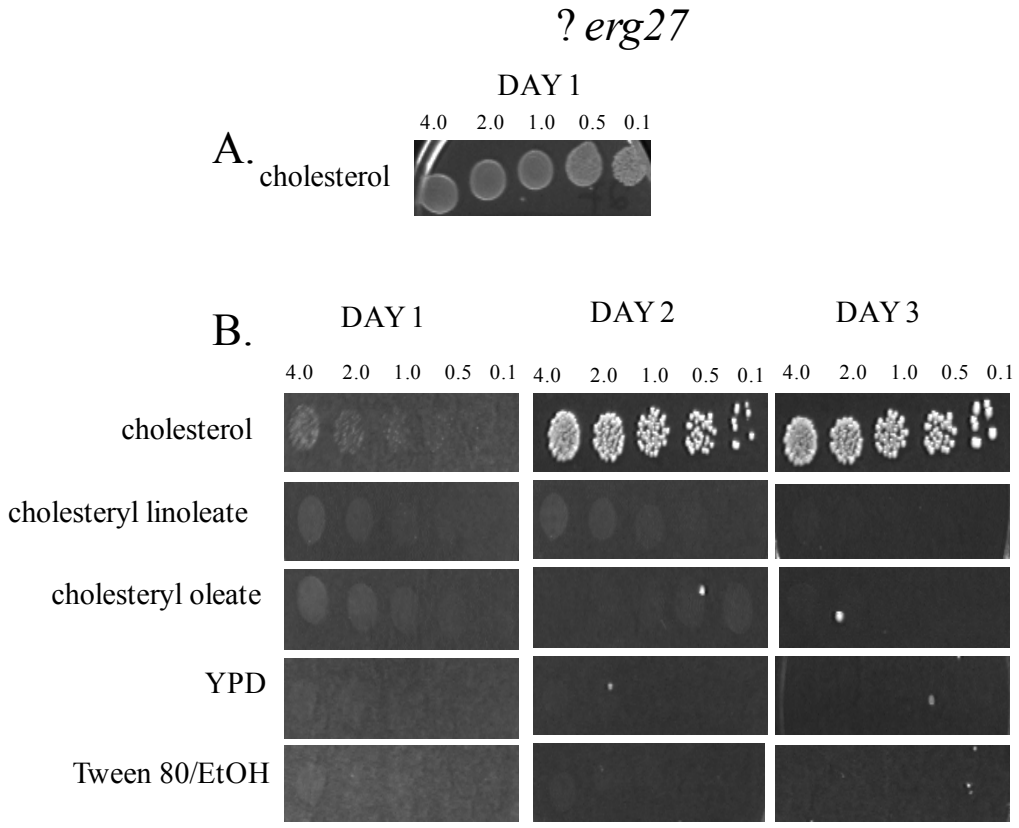

Figure 3.11 A) Δ*erg27* grown anaerobically with cholesterol. B) Spot plate analysis of Δ*erg27*. Strains grown on YPD media supplemented with 20 µg/ml sterol in Tween 80/EtOH (1:1 v/v)

It was also important to evaluate *ERG9* in *C. glabrata* particularly based on Nakayama's findings (63). These experiments compared the growth of two *C. glabrata* strains, one with the *AUS1* gene (97SQS) and one deleted for the *AUS1* gene (97SQS/Δ*AUS1*). Serial dilutions were made such that cell concentrations of 10^6, 10^5, and 10^4 in 10 µl spots were plated onto YPD+Tween 80, YPD + 1X Cholesterol (2mg/mL in 1:1 [w/v] Tween 80), YPD + 3X Cholesterol, YPD + 5% Human serum, YPD + 10% Human serum, YPD + 5% Bovine serum, and YPD + 10% Bovine serum with varying concentrations of doxycycline from 0 mg, 0.1 mg, 0.15 mg, and 0.2 mg. The expected results were noted: Doxycycline inhibits the growth of both strains because both are under the control of the tet-promoter; however 97SQS will be able to utilize exogenous cholesterol for better growth than 97SQS/Δ*AUS1*. The following seven figures (Figures 3.11, 3.12, 3.13, 3.14, 3.15, 3.16, and 3.17) display the spot plates for each type of media examined.

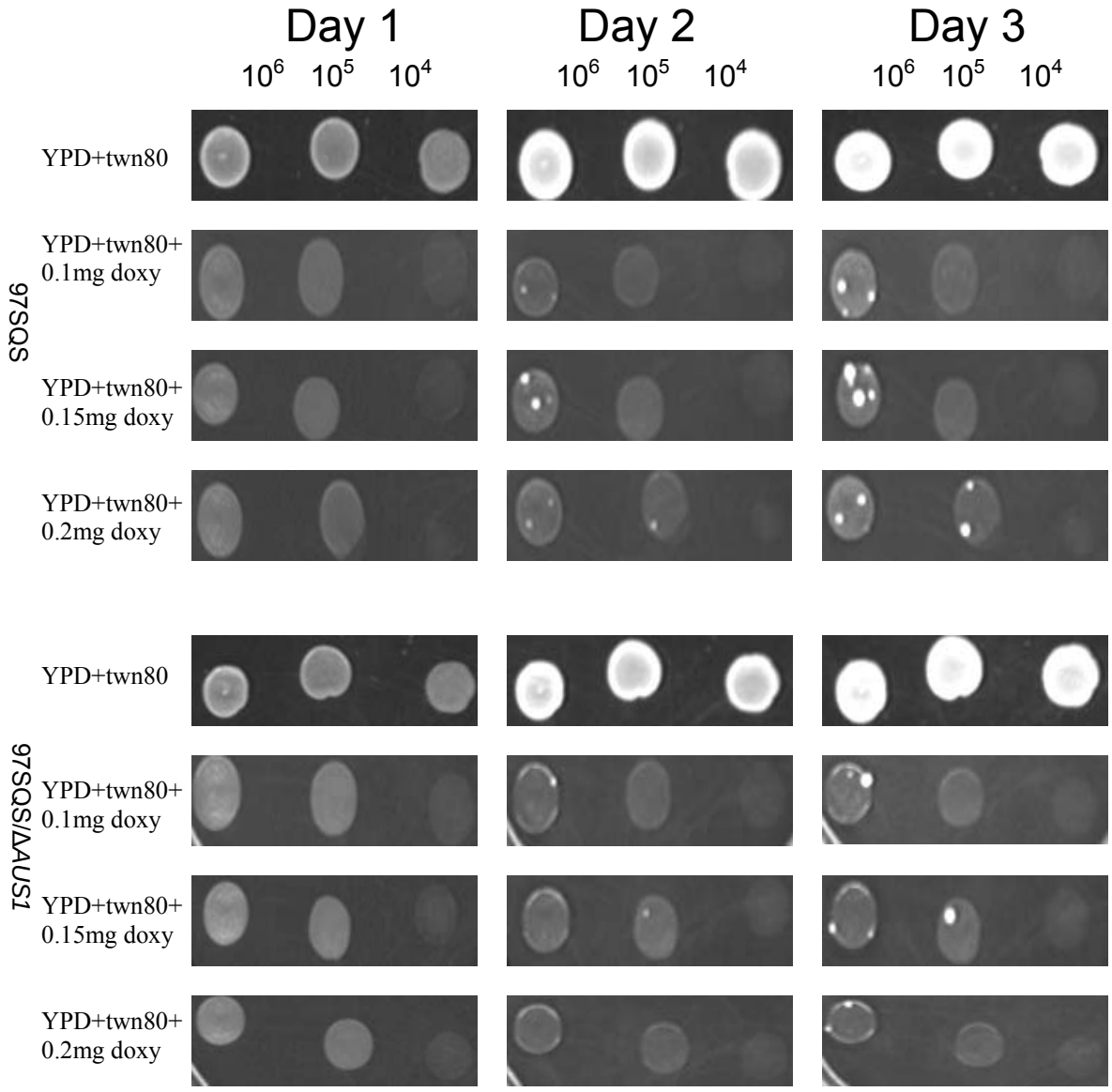

Figure 3.12 *Cg* 97SQS and 97SQS/Δ*AUS1* on YPD + Tween 80

Doxycycline obviously inhibits the growth of both strains; however more so the 97SQS/Δ*AUS1* than the 97SQS.

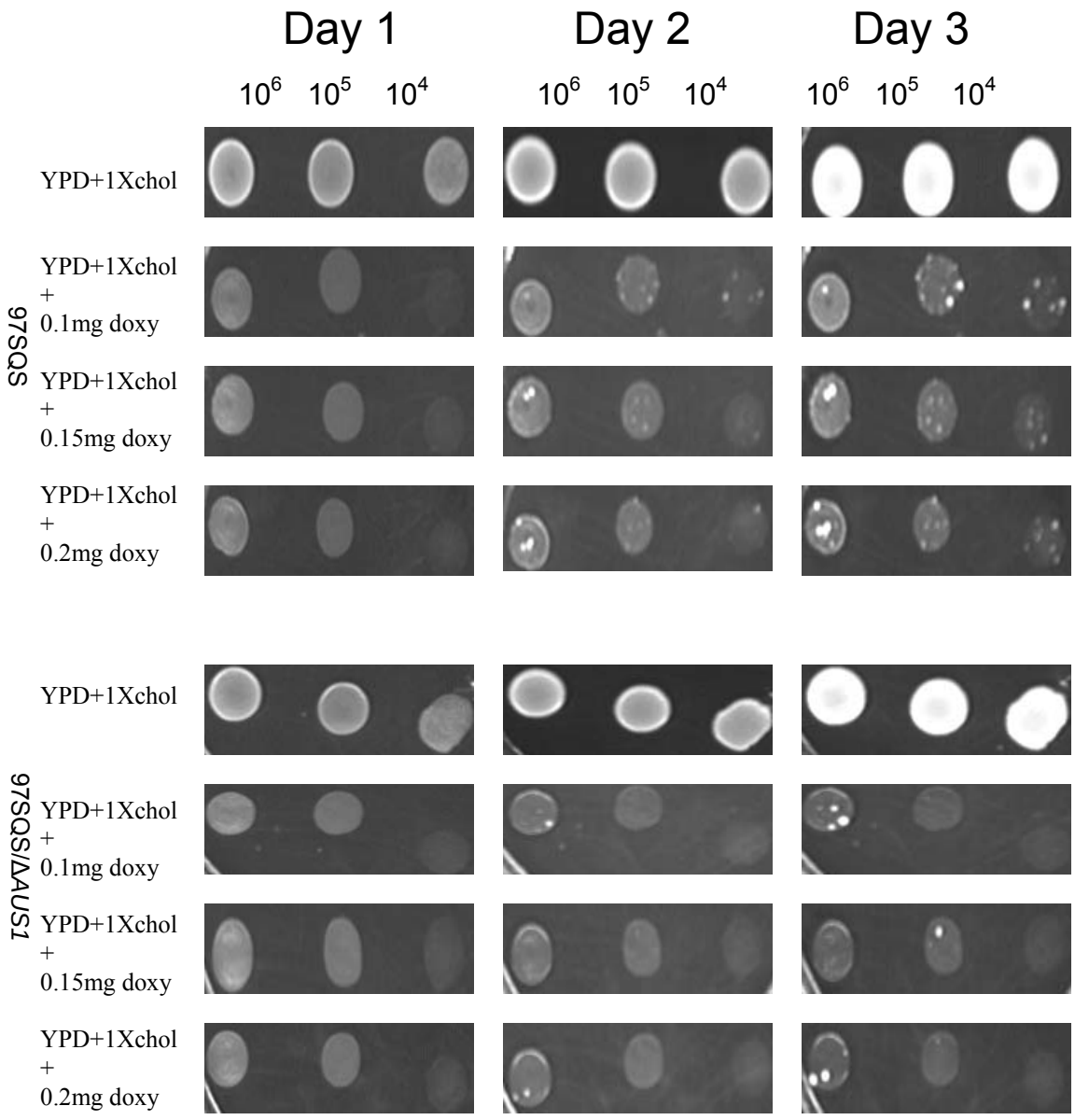

Figure 3.13 *Cg* 97SQS and 97SQS/Δ*AUS1* on YPD + 1X Cholesterol

Again, doxycycline obviously inhibits the growth of both strains; however 97SQS can accumulate more growth due to the presence of the *AUS1* gene.

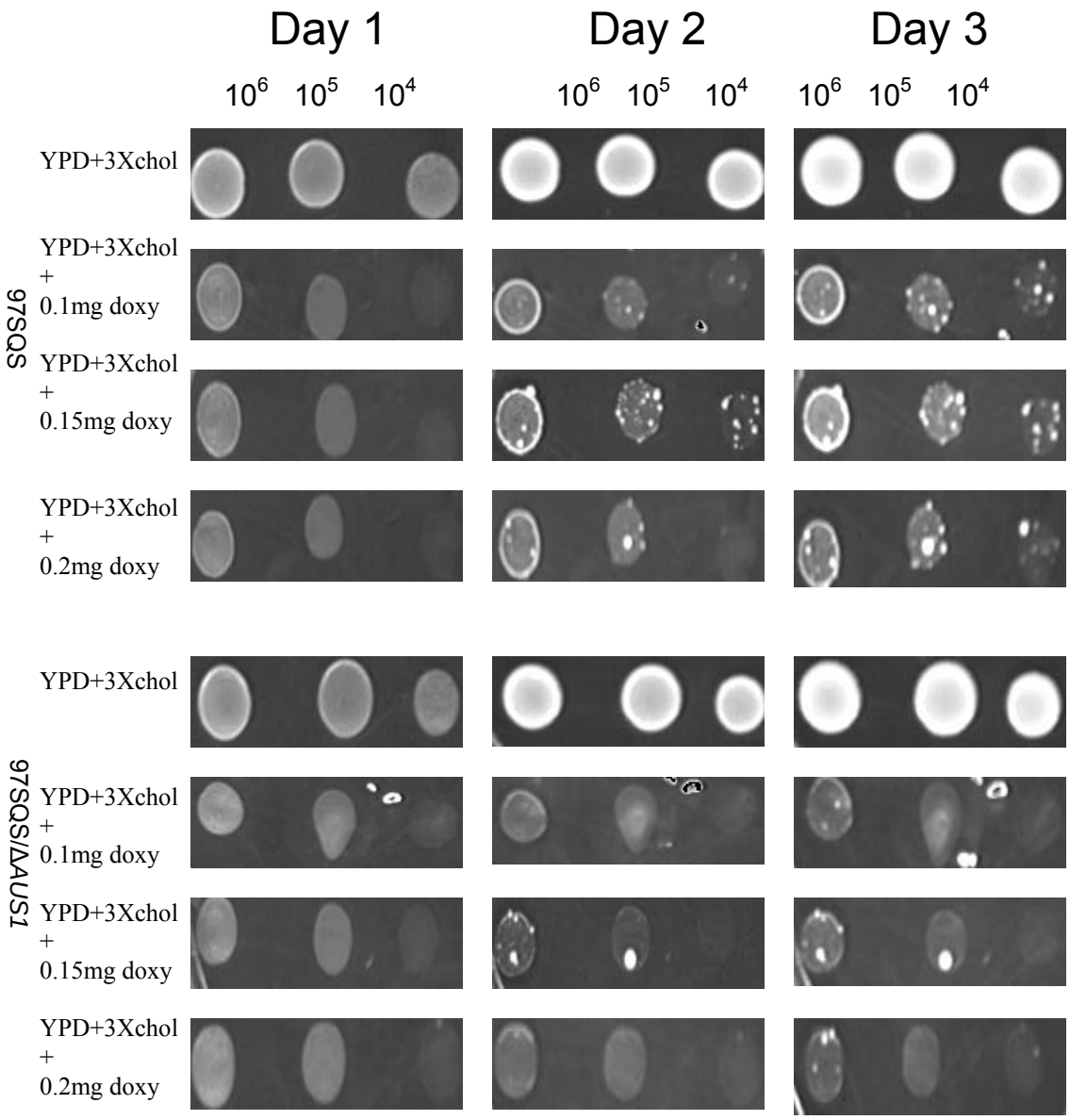

Figure 3.14 *Cg* 97SQS and 97SQS/Δ*AUS1* on YPD + 3X Cholesterol

Again, doxycycline inhibits the growth of both strains; however 97SQS can accumulate even more growth with a higher concentration of cholesterol.

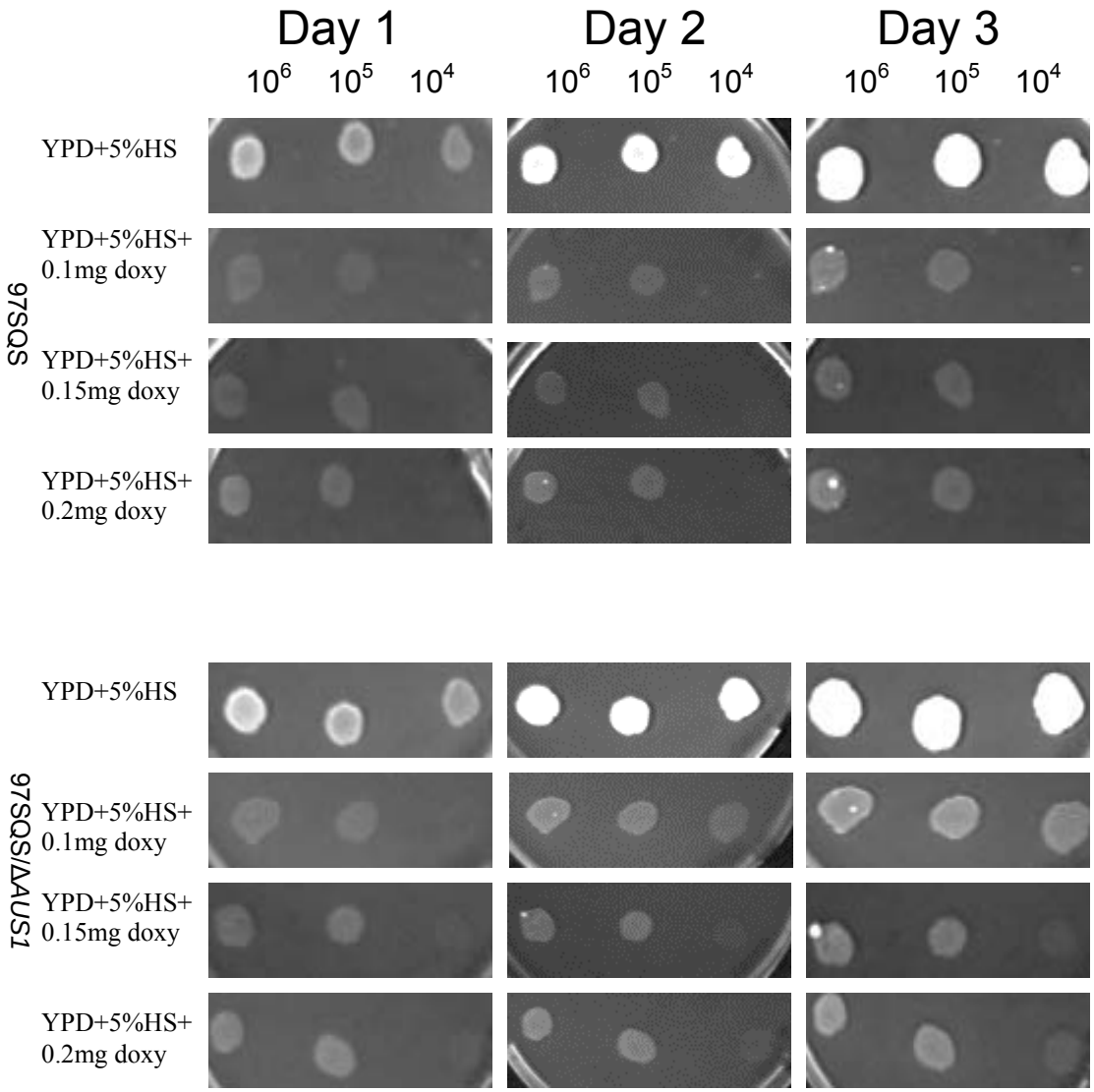

Figure 3.15 *Cg* 97SQS and 97SQS/Δ*AUS1* on YPD + 5% Human serum

Again, doxycycline obviously inhibits the growth of both strains; however 97SQS can accumulate more growth due to the presence of the *AUS1* gene.

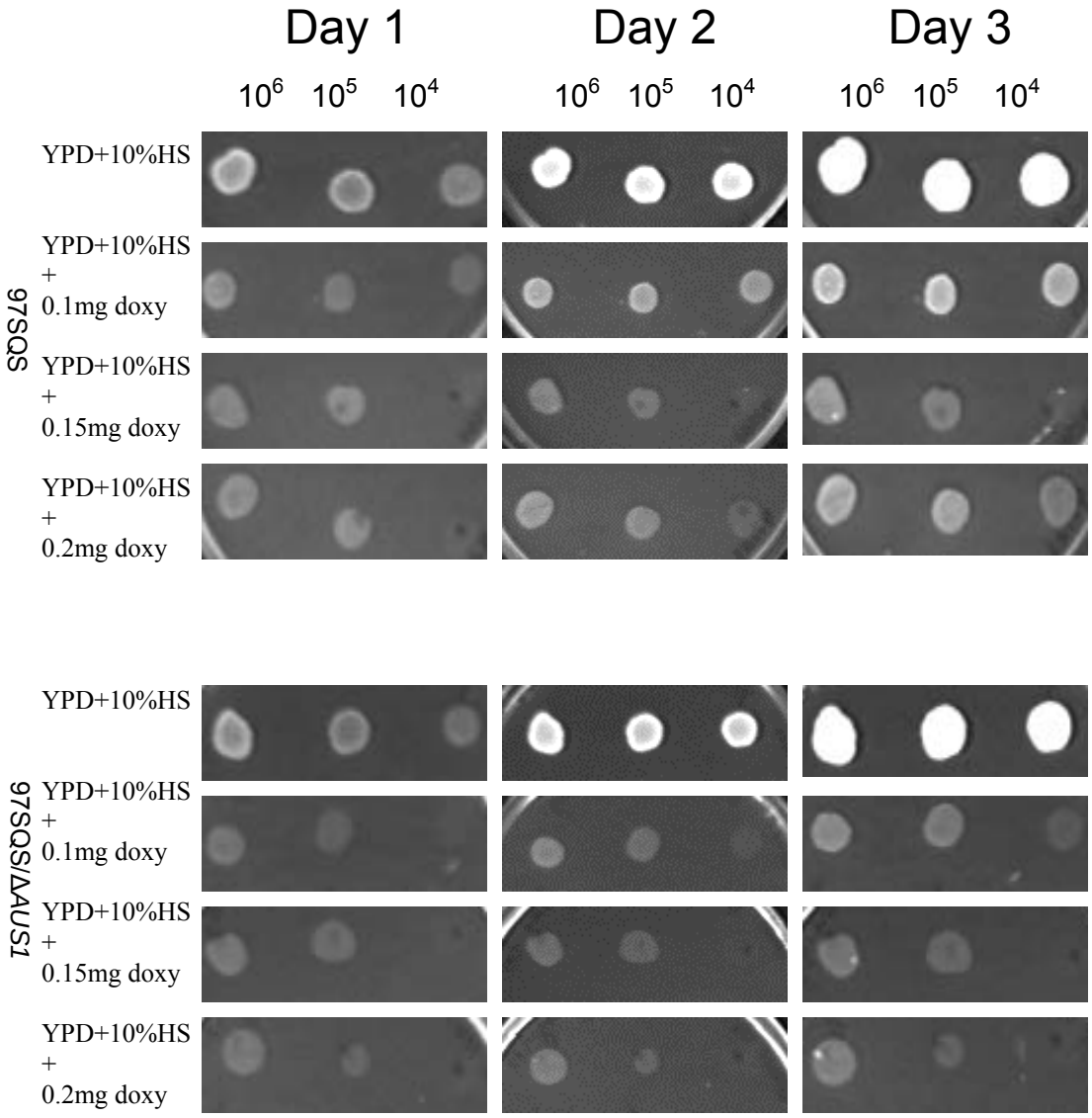

Figure 3.16 *Cg* 97SQS and 97SQS/Δ*AUS1* on YPD + 10% Human serum

Again, doxycycline inhibits the growth of both strains; however 97SQS can accumulate even more growth with a higher concentration of human serum.

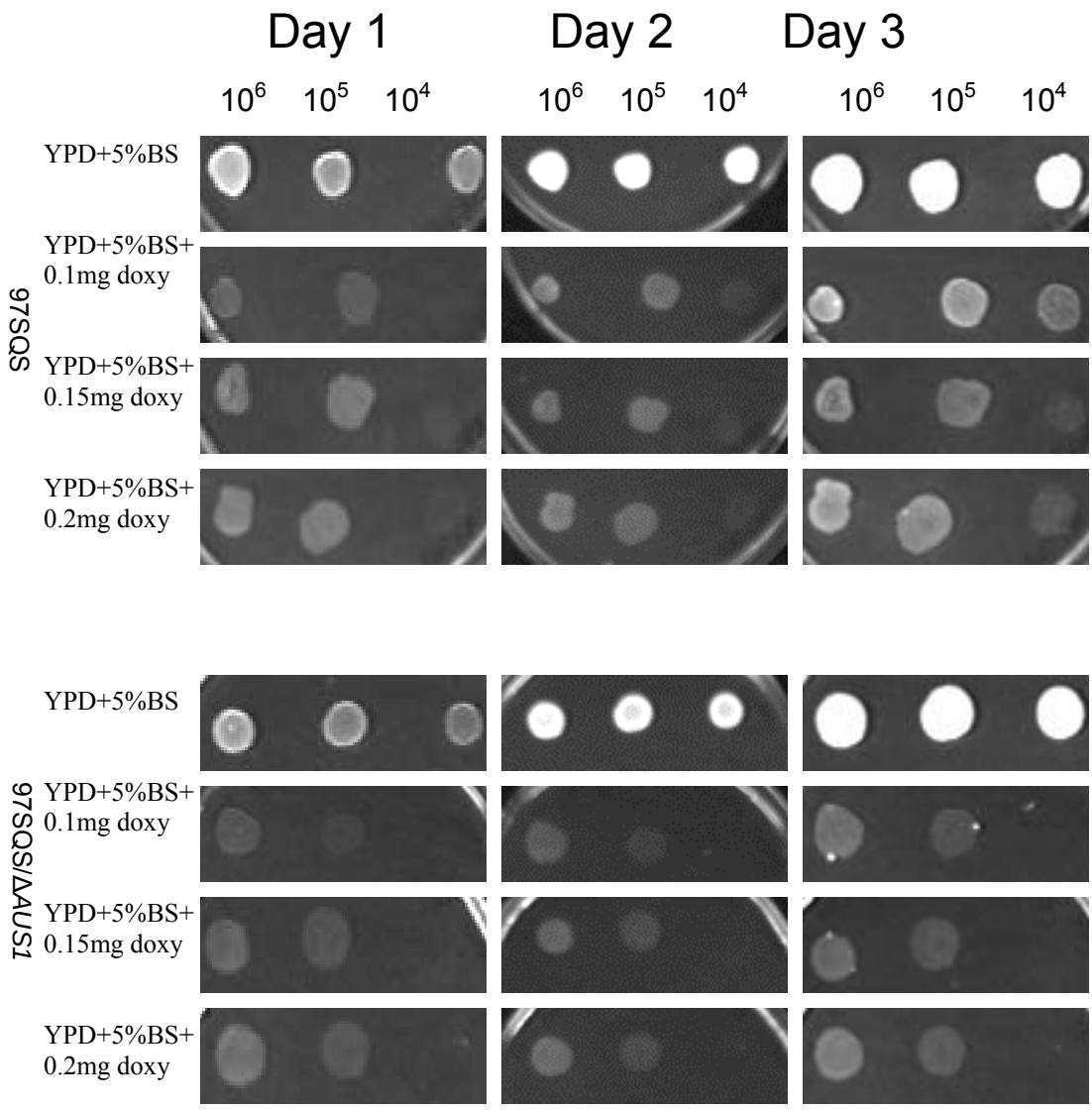

Figure 3.17 *Cg* 97SQS and 97SQS/Δ*AUS1* on YPD + 5% Bovine serum

Again, doxycycline inhibits the growth of both strains; however 97SQS can accumulate even more growth than 97SQS/Δ*AUS1* with bovine serum.

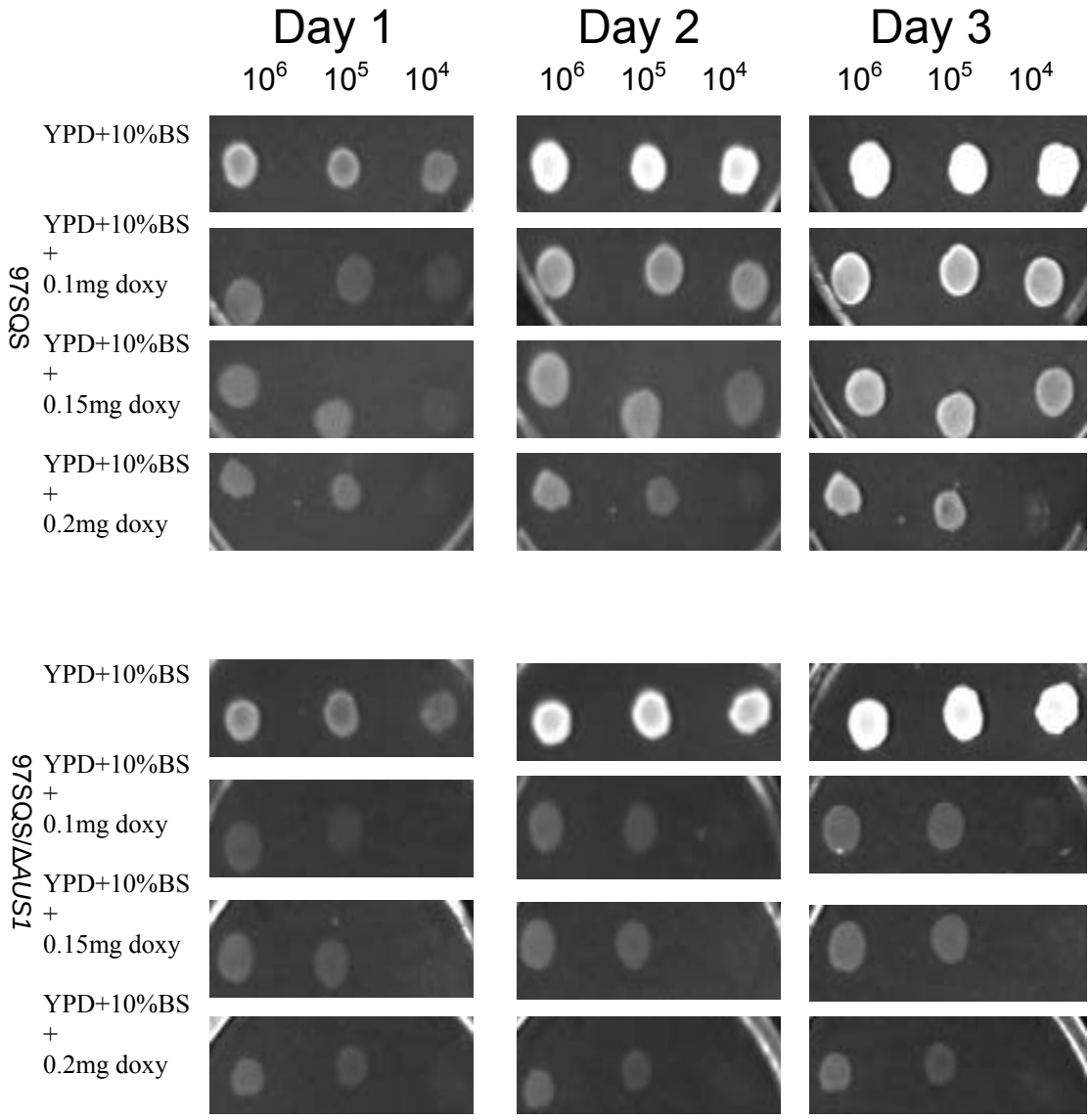
Day 1
Day 2
Day 3
10^6 10^5 10^4
10^6 10^5 10^4
10^6 10^5 10^4
YPD+10%BS
YPD+10%BS + 0.1mg doxy
YPD+10%BS + 0.15mg doxy
YPD+10%BS + 0.2mg doxy
97SQS
YPD+10%BS
YPD+10%BS + 0.1mg doxy
YPD+10%BS + 0.15mg doxy
YPD+10%BS + 0.2mg doxy
97SQS/ΔAUS1

Figure 3.18 *Cg* 97SQS and 97SQS/Δ*AUS1* on YPD + 10% Bovine serum

Again, doxycycline inhibits the growth of both strains; however 97SQS can accumulate even more growth with a higher concentration of bovine serum.

3.3 Discussion and Conclusions

Sterol auxotrophs have been isolated in *C. glabrata* clinical strains. Analyses of these strains have revealed mutations occurring in *erg1* and *erg7* or a single heme mutation. Therefore, we have decided to create knockout mutations in *C. glabrata* in *ERG1* and *ERG7* to compare these strains aerobic behavior with the clinical isolate strains able to grow aerobically. Also, we have decided to create knockout strains with the other essential genes *ERG11, ERG25*, and *ERG27*. We chose *ERG27* because of the relationship with *ERG7. ERG27* is required for *ERG7* to function (34). Disruptions in *ERG1, ERG7,* and/or *ERG27* will prevent the sterol ring structure from forming, whereas disruptions in *ERG11* and *ERG25* will have formation of the sterol ring structure. We hypothesize; the disruptions that allow a sterol ring structure to form will not take up exogenous sterol as readily as the disruptions without a sterol ring structure.

The sterol profiles obtained from gas chromatography verify that each expected gene disruption was successful. *ERG1* sterol profile indicates an accumulation of squalene as expected. *ERG7* and *ERG27* sterol profiles both show an accumulation of squalene epoxides with little lanosterol for *ERG27*. *ERG11* sterol profile has an accumulation of lanosterol and *ERG25* has an accumulation of 4,4-dimethylzymosterol. All of these sterol profiles indicated the desired ergosterol auxotrophic strains were created. All strains have stock solutions frozen and are stored at –80°C.

All five ergosterol auxotrophic strains show a uniform growth pattern anaerobically when supplemented with sterol as expected. Again, *C. glabrata* can uptake exogenous sterol under anaerobic conditions. Also, all five ergosterol auxotrophic strains begin to accumulate single colonies aerobically after two days in the presence of

exogenous cholesterol. This accumulation of aerobic sterol-uptake competent isolates implies the hypothesized secondary mutations allowing aerobic sterol-uptake are occurring in all five ergosterol auxotrophs.

As expected, *ERG1, ERG7,* and *ERG27* knockout strains do not acquire growth on the control plates without supplementation and with Tween 80, or with supplementation of cholesterol esters. However, *ERG25* and *ERG11* knock out strains do accumulate single colony growth after approximately 3 days with cholesterol esters and Tween 80 supplementation. This phenomenon was not expected, particularly with the Tween 80 plates due to a complete lack of exogenous sterol as well as a lack of sterol production.

There are several potential explanations for this phenomenon. *ERG1* and *ERG7* steps occur before any endogenous sterol intermediates are created in the ergosterol pathway. Although the *ERG27* step happens after the production of lanosterol, the first sterol precursor created in the pathway, the *ERG27* knockout will accumulate more squalene epoxides than lanosterol (34). The *ERG11* and *ERG25* steps, on the other hand, occur after endogenous sterol intermediates are created in the pathway allowing their knockout strains to accumulate such intermediates; lanosterol and 4,4-dimethylzymosterol, respectively. It is known that *C. glabrata* can utilize cholesterol in place of ergosterol due to the similarity in structure of the molecules. These auxotrophic strains could be acquiring mutations allowing them to utilize these sterol intermediates as a form of sterol supplementation for survival.

Another potential explanation for these satellite colonies accumulating aerobically with Tween 80 and cholesterol ester supplementation are suppressor mutations as

previously demonstrated by Gachotte et al. (57). This study found that *erg25* lethality could be suppressed by a combination of mutations in *erg11* and *slu1* or *slu2*. Further analysis of these satellite colonies could reveal they are *erg11* suppressor mutations. To further examine this possibility, the satellite colonies could be isolated and individually analyzed by GC/MS to verify their sterol profile. An accumulation of lanosterol and not 4,4-dimethylzymosterol would indicate an *erg11* suppressor mutation.

The ability of *C. glabrata* to uptake exogenous cholesterol aerobically has significant implications in human pathogenicity. These findings support the hypothesis of a secondary mutation occurring in ergosterol auxotrophs allowing a mechanism for *C. glabrata* aerobic sterol uptake. Further analysis of this secondary mutation could give insight into *C. glabrata's* growing resistance to current therapies and offer a potentially new route of exploration for new antifungal drug treatments.

Regarding *ERG9* spot plates, they clearly show that an increase in the amount of doxycycline in the media inhibits the growth of both 97SQS strains, however the strain with the *AUS1* gene can uptake sterol aerobically from the media for a higher survival rate. The higher the concentration of sterol in the media and the source of the cholesterol also appear have an effect on aerobic sterol uptake. Just as Nakayama showed (63), the 97SQS strain appears to have better growth with serum for cholesterol supplementation rather than cholesterol.

3.4 Future Work

Further analysis of erg*11* and *erg25* strains is required to verify the cause of growth without cholesterol supplementation and with cholesterol esters and Tween 80.

Again, the first step would be to verify the satellite colonies are not suppressor mutations. Secondly, further studies as to what mutation(s) are causing the aerobic sterol uptake would be warranted. Of particular interest, would be the analysis of the *UPC* (uptake of cholesterol) genes, which are required for the uptake of exogenous cholesterol.

CHAPTER 4

RANDOM AND SITE-DIRECTED MUTAGENESIS OF *ERG25* IN *SACCHAROMYCES CEREVISIAE*

4.1 Introduction

The *ERG25* gene encodes a sterol C-4 methyloxidase essential for sterol biosynthesis in plants, animals, and yeast (59). This gene functions in turn with *ERG26*, a sterol C-3 dehydrogenase (57), and *ERG27*, a sterol C-3 keto reductase (58), to remove two methyl groups at the C-4 position on the sterol A ring in two rounds (Figure 4.1). *ERG28* acts as a scaffold protein helping these three enzymes to work together.

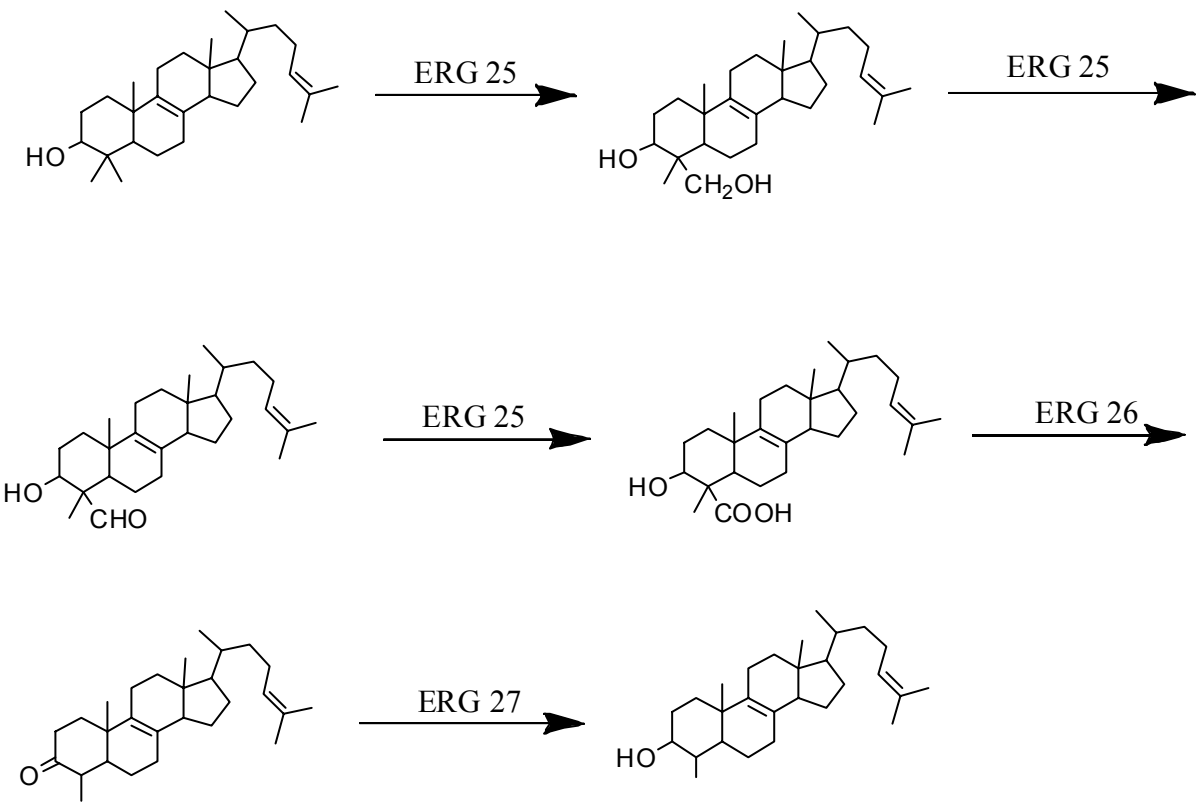

Figure 4.1 Demethylation at C-4: One round involving *Erg25, Erg26, and Erg27* shown. Two rounds are required to remove both methyl groups

This demethylation reaction at C-4 has been shown to be essential for the ergosterol pathway through several disruption studies (57, 58, 59). It has also been compared on an amino acid level to desaturases and hydroxylases (36). Shanklin (35) found three conserved histidine clusters in desaturases-hydroxylases that work as binding motifs for oxo-diiron supplying iron and oxygen for desaturase reactions. Kaplan et al. (36) proposed the *ERG3-ERG25* family of proteins have the same three putative histidine clusters plus a novel fourth cluster, which binds non-heme iron. The *ERG25* gene also has a C-terminal KKXX motif, which is a Golgi to ER retrieval motif.

Due to this interesting comparison, this study analyzed site-directed and random mutagenesis of *S. cerevisiae* using wild-type strain SCY876. Site-directed mutagenesis focused on these four histidine clusters, the KKXX C-terminal motif and other conserved amino acids among species including: *Mus musculus, Homo sapien, Arabidopsis thaliana, Candida albicans, and Saccharomyces cerevisiae*. Random mutagenesis of *ERG25* was used in an effort to find novel changes in enzyme function outside of the parameters utilized for site-directed mutagenesis.

For site-directed mutagenesis, plasmids were created carrying specific *ERG25* mutations, verified by DNA sequencing, screened for complementation in yeast, and analyzed by GC/MS. For random mutagenesis, yeast with plasmids carrying potential *ERG25* mutations were screened for complementation to restore the wild type phenotype, the plasmid extracted and sequenced, and the plasmid transformed back into yeast for GC/MS analysis. Strains that do not complement should have sterol profiles indicating the inability to synthesize ergosterol and an accumulation of 4,4-dimethylzymosterol. The four putative histidine clusters are expected to be essential for gene function by acting as non-heme iron binding ligands bringing in the oxygen required for the oxidation-reduction in the C-4 demethylation reaction. Strains that do complement should have a wild type GC profile.

4.2 *ERG25* Consensus Diagram

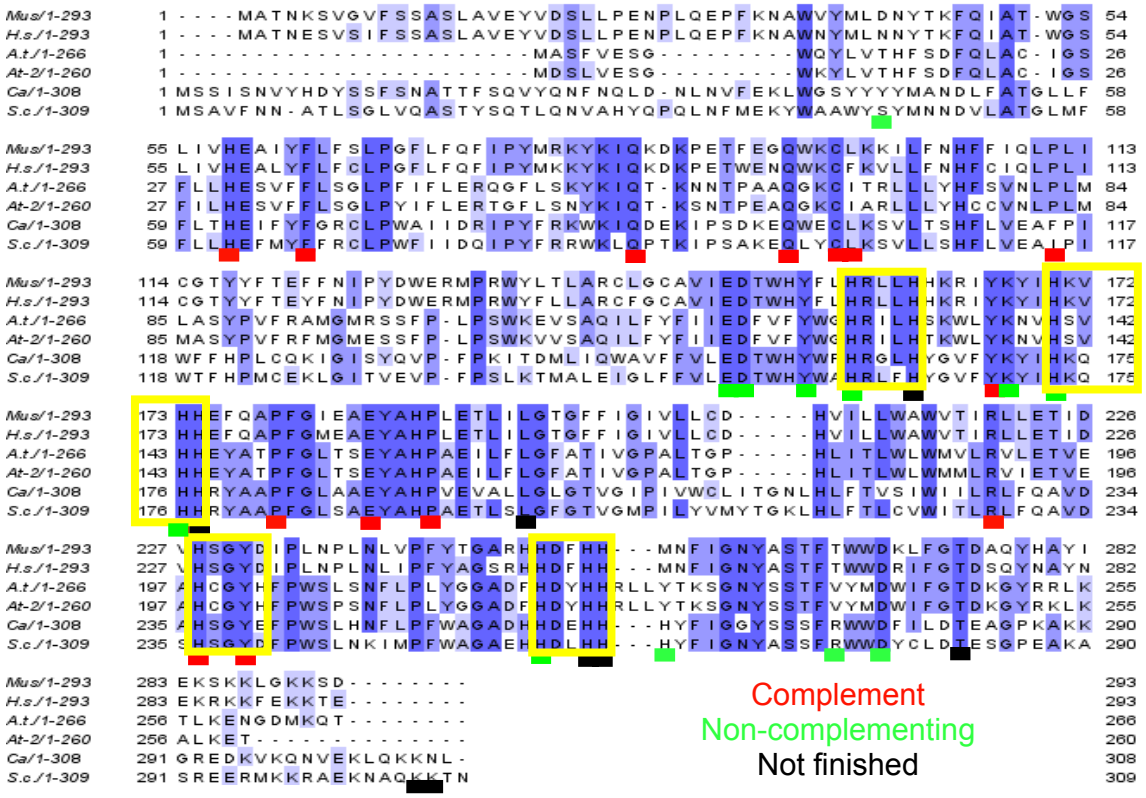

Figure 4.1 *Erg25* sequence alignment. Homology plot of: *Mus musculus* (top), *Homo sapien*, *Arabidopsis thaliana, Candida albicans*, *Saccharomyces cerevisiae* (bottom). Histidine clusters are outlined in yellow. Cluster 1 HX_3H (amino acids 160-164). Cluster 2 HX_2H_2 (amino acids 173-177). Cluster 3 HX_2H_2 (amino acids 258-262). Cluster 4 HX_2YD/H (amino acids 236-240). Amino acids changes underlined in red complement and amino acid changes underlined in green do not complement when transformed back into yeast. Amino acid changes underlined in black were attempted, but never acquired.

4.3 Results

4.3.1 Site-Directed Mutagenesis Results

Site-directed mutagenesis was accomplished using the Stratagene QuickChange Lightning Site-Directed Mutagenesis Kit. Primers were created with 1 or 2 base pair changes to create a single amino acid change to an alanine or to a phenylalanine if the original codon coded for a tyrosine. This is known as alanine change site-directed mutagenesis. A list of primers and PCR parameters are listed in Chapter 2. Table 4.1 lists all of the site-directed mutations attempted and the reasoning for each alteration.

Table 4.1 Site-directed amino acid changes

Amino Acid Change	Reason for Change
F67A	Conserved
Q88A	Conserved
Q98A	Conserved
C101A	Conserved
L102A	Conserved
I115A	Conserved
E152A	Conserved
D153A	Conserved
Y157F	Histidine cluster
H160A	Histidine cluster
H164A	Histidine cluster
Y169F	Histidine cluster
K170A	Histidine cluster
H173A	Histidine cluster
H176A	Histidine cluster
H177A	Histidine cluster
P182A	Conserved
E188A	Conserved
H191A	Conserved
L198A	Conserved
R228A	Conserved
H236A	Histidine cluster
Y239F	Histidine cluster
H258A	Histidine cluster

H261AH262A	Histidine cluster
T282A	Conserved
K306AK307A	KKXX motif

This Stratagene kit utilized a high fidelity polymerase to insert only the intended amino acid change(s) into a PCR product based on the created PCR primer (listed in Chapter 2). This PCR product was then transformed into bacteria supplied with the kit. Colonies from this bacterial transformation were prepared and sent for DNA sequencing to verify that the amino acid change occurred in the plasmid. The plasmid with the altered amino acid was then transformed into a diploid strain designated D1. Yeast transformants were screened for complementation on CSM-ura with and without ergosterol anaerobically.

Once the ability to complement was established, GC sterol profiles were analyzed. Cells were grown anaerobically in 50mL CSM-ura + cholesterol liquid cultures for two days in triplicate regardless of the ability to complement. All strains able to complement were also grown aerobically in 50mL CSM-ura liquid culture for two days in triplicate. (Non-complementing strains do not grow aerobically and therefore they were not grown under these conditions.) Cells grown aerobically and anaerobically were grown; sterols extracted, and run on GC as described in Chapter 2. Table 4.2 lists complementation and % sterol from the GC profiles for all site-directed mutants attempted.

Table 4.2 Amino Acid Changes, Complementation, and Gas Chromatography data for site-directed mutagenesis. GC values are % and samples were grown anaerobically with cholesterol supplementation

Amino Acid Δ	Complement	squalene	ergosterol	ergosterol precursors	4-methyl-lanosterol	lanosterol	4,4-dimethyl-zymosterol	Cholesterol
F67A	+	33.4±10.2	1.9±1.6	7.7±4.4	1.9±1.4	3.4±0.6	2.4±0.5	49.4±7
Q88A	+	33.6±6.9	1.2±1	8.2±1.2	0.4±0.4	2.8±0.1	2.9±0.3	51±6
Q98A	+	51.7±1.9	0.5±0.8	4.6±1.4	1.7±2	1.8±0.6	2.2±0.4	37.5±1.2
C101A	+	36.7±1.6	7.2±1	11.2±3	1.7±0.1	6.7±2.5	6.7±3.2	30±4
L102A	+	41.3±3.6	7.9±0.7	8.8±3.6	1.7±0.4	8.5±0.6	4.4±0.4	27.4±0.1
I115A	+	52.1±2.6	0	3.6±0.8	1.5±1.5	1±1.5	1.5±1.6	40.3±4.4
E152A	-	33.2±11	0	8.6±3.2	0.4±0.7	0.5±0.9	5.3±1.4	52±10
D153A	-	51.6±3.7	0	0	0	0.4±0.7	7.1±0.9	40.9±4.2
Y157F	-	25±17.7	0.8±1.4	2.8±1.7	0	0	3.9±1	33.5±20
H160A	-	49.5±2.3	0	5.6±1	4.7±4.8	0	3.6±0.4	36.6±3
H164A	not acquired							
Y169F	+	19.2±1.9	12±1.3	12.1±1.5	1.1±0.2	6.8±1.3	1.5±0.3	47.4±4.4
K170A	-	29.1±8.2	0	6.5±1.3	0	0.2±0.4	4.6±1.3	59.6±10
H176A	-	46.7±7	0	7.4±5.9	0.4±0.7	1.6±0.5	3.4±0.3	40.5±3
H177A	not acquired							
P182A	+	34.8±11.9	1.1±1	4.5±2.1	1.7±1.1	2.2±2.1	4.7±1.4	51±10.2
E188A	+	9.6±2.4	2.9±0.8	9.9±3.7	0.4±0.2	3.3±0.3	0.4±0.4	73.5±7
H191A	+	32.4±7.4	7.5±1.3	7.6±1.1	2.5±0.6	2.2±0.3	6±1.3	41.8±10.2
L198A	not acquired							
R228A	+	42.1±1.8	0	6.3±1.3	1.6±2.8	1±1	5.2±2.7	43.8±3
H236A	-	24±7.8	0	8.2±2.7	0.7±1.2	1.4±0.6	2.2±1	63.5±10
Y239F	+	10.1±1.1	52.7±1.3	16.9±2.7	4±0.5	0.6±0.9	5.4±1.1	9.7±1.1
H258A	-	35.4±3	2±0.2	0.2±0.3	0	0.3±0.5	0	60.5±4.3
H261AH262A	not acquired							
T282A	not acquired							
K306AK307A	not acquired							
RM213	wild type	35.2	1.8	0.5	0	0.7	2	60.5

Non-complementing strains, as expected, do not accumulate ergosterol. However, Y157F, a non-complementing strain, will produce small amounts of ergosterol, but not on every sample analysis of the sterol profile. Cholesterol peaks appear on each sample because cells are grown with sterol supplementation and there were difficulties in washing off all of the exogenous cholesterol in the saponification steps. Complementing strains are able to produce end product ergosterol as shown by the GC profiles. These strains can therefore be grown aerobically without the presence of cholesterol for supplementation. This data is listed in Table 4.3.

Table 4.3 Amino acid changes and gas choromatography data for site-directed mutations that are positive for complementation. GC values are % and samples were grown aerobically

Amino Acid Δ	Complement	squalene	ergosterol	ergosterol precursors	4-methyl-fecosterol	lanosterol	4,4-dimethyl-zymosterol
F67A	+	21.7 ± 2.5	33.7 ± 2.7	7.4 ± 1.2	10.8 ± 0.5	0.2 ± 0.2	26.2 ± 1.9
Q88A	+	20.8 ± 5.1	35.2 ± 2.3	6.7 ± 1.9	8.7 ± 2.3	0.33 ± 0.16	28.3 ± 0.5
Q98A	+	23 ± 5.2	37.8 ± 4.7	10.7 ± 2	7 ± 0.5	1.5 ± 1.2	20 ± 3.4
C101A	+	13.4 ± 2.8	39.4 ± 1.9	11 ± 1	8.7 ± 0.6	2.5 ± 0.05	25 ± 1
L102A	+	19 ± 2.2	34.3 ± 2.8	9.4 ± 1.5	9.5 ± 0.3	1.7 ± 0.6	27.1 ± 0.8
I115A	+	32.4 ± 1.4	28 ± 1.4	7 ± 2.1	5 ± 0.5	3.9 ± 0.5	23.7 ± 1.4
Y169F	+	2.9 ± 1	76 ± 2	17.8 ± 1.4	0.5 ± 0.3	2.2 ± 0.5	1.7 ± 0.6
P182A	+	30.5 ± 2.8	21.8 ± 0.6	1 ± 0.07	14.6 ± 0.3	0	32.2 ± 2
E188A	+	2.6 ± 0.9	65.7 ± 3	12.5 ± 7.2	1.4 ± 0.4	4.3 ± 1.2	3.3 ± 0.2
H191A	+	16.7 ± 3.7	34.1 ± 1.8	4.2 ± 1.2	10.4 ± 0.5	0.1 ± 0.1	34.5 ± 1.6
R228A	+	12.4 ± 2.6	12.5 ± 1.3	0.7 ± 0.7	13.1 ± 1.9	0	61.3 ± 4.6
H236A	+/-	4.7 ± 1.9	62.7 ± 8.3	17.6 ± 2.5	2.7 ± 1.9	2.8 ± 0.3	9.5 ± 6.5
Y239F	+	14.1 ± 2.8	38.5 ± 3.3	15.7 ± 1.5	9.8 ± 1	0.12 ± 0.1	29 ± 1.4
RM213	+	10.3 ± 2.8	44.4 ± 1.4	15.8 ± 2	4.6 ± 0.4	0	25 ± 1.4

4.3.2 Random Mutagenesis Results

Random mutagenesis was accomplished through a multi-step process known as gap repair. The first step was to create a DNA fragment called the "insert." Primers (listed in Chapter 2) were created using the pIU800 plasmid as template giving a PCR product of the *ERG25* gene with *EcoRI* and *SalI* restriction sites at the ends. Using Go Taq polymerase in the PCR reaction will result in approximately 1 base pair error in 1000 base pairs and the *ERG25* gene is approximately 937 base pairs. After the PCR reaction, the product or "insert" should contain the *ERG25* gene with one potential base pair change with the restriction enzymes, *EcoRI* and *SalI,* at the ends. Figure 4.3 is a diagram of creating the "insert."

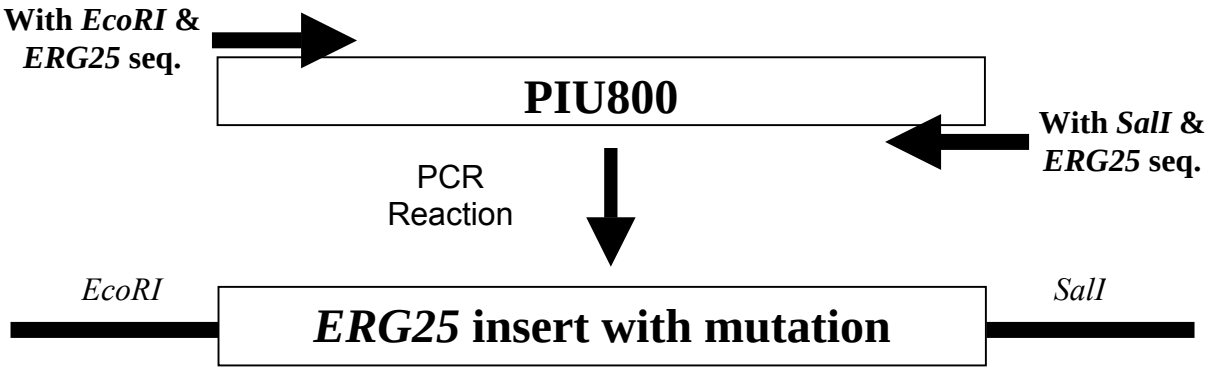

Figure 4.3 Diagrammatic scheme of creating the "insert" for random mutagenesis

A vector, also referred to as the "backbone," was also created in p426ADH by digesting the *EcoRI* and *SalI* sites. This restriction digest will linearize the plasmid vector in which the "insert," by homologous recombination, can repair the gapped vector. This will occur simultaneously as the functional plasmid (vector + insert) transforms into the

wild type yeast strain already created. Figure 4.4 is a diagram illustrating the creation of the vector “backbone” and combination with the “insert” to create the functional plasmid with a potential random mutation.

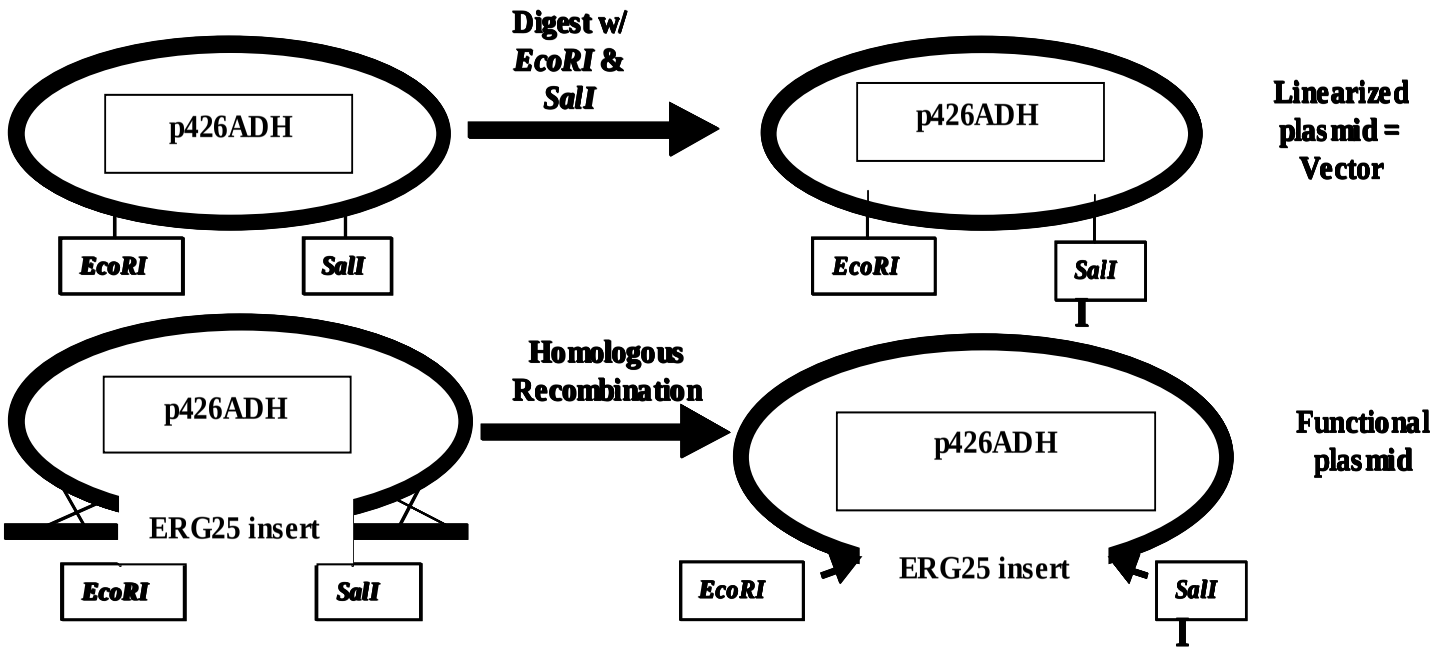

Figure 4.4 Diagrammatic scheme of creating the "vector" and functional plasmid for random mutagenesis

Once the yeast transformants in D1 became available, they were screened for complementation on CSM-ura with and without cholesterol supplementation. Strains that were negative for complementation were then yeast colony PCR'd to verify they had the insert. If the insert was noted on gel electrophoresis, the plasmid was extracted from the yeast and transformed into *E. coli*. These bacterial colonies were then prepped and sent for DNA sequencing. Table 4.4 lists the sequencing results for the random mutations created in this study.

Table 4.4 Sequence results for random mutants

Mutant	Nucleotide change(s)	Amino acid change(s)
RM213	No mutations (used as WT control)	
RM2148	No mutations (extra WT control)	
RM2118	T826C	W276R
RM21100	A820G	R274G
RM393	A133G	S45G
RM41	A788T	H263L

Once a mutation was verified, the GC profile of each was analyzed. Cells were grown anaerobically in 50mL CSM-ura + cholesterol liquid cultures for 2 days in triplicate regardless of the ability to complement. All strains able to complement were also grown aerobically in 50mL CSM-ura liquid culture for 2 days in triplicate (data listed in table 4.3). (Non-complementing strains do not grow aerobically and therefore they were not grown under these conditions.) Cells grown aerobically and anaerobically were saponified and sterols run on GC as described in Chapter 2. Table 4.5 lists all of the random mutations acquired with complementation information and % sterols.

4.5 Amino Acid Changes, Complementation, and Gas Chromatography data for random mutagenesis. GC values are % and samples were grown anaerobically with cholesterol supplementation

Amino Acid Δ	Complement	squalene	ergosterol	ergosterol precursors	4-methyl-lanosterol	lanosterol	4,4-dimethyl-zymosterol	Cholesterol
RM213	+	35.2	1.8	0.5	0	0.7	2	60.5
W276R	-	33 ± 12	0	6.1 ± 4.4	0	0.7 ± 0.6	3.2 ± 0.2	57 ± 12
R274G	-	34 ± 13	0	4.4 ± 2.1	0.8 ± 0.5	5.2 ± 8.9	5.1 ± 4.5	50.5 ± 19.7
S45G	-	47.9 ± 16.6	0	3.2 ± 3.1	0.2 ± 0.3	0	12 ± 1.3	36.7 ± 13
H263L	-	37.1 ± 9.2	0	2.6 ± 3.6	0	0.15 ± 0.2	16.8 ± 8	43.4 ± 13.3

Complementing strains are wild type and are able to produce end product ergosterol as shown by the GC profiles. Non-complementing strains, as expected, cannot produce ergosterol. Cholesterol peaks appear in each sample because cells are grown with sterol supplementation and there were difficulties in washing off all of the exogenous cholesterol in the saponification steps.

4.4 Discussion and Conclusions

All site-directed and random mutants were verified by DNA sequencing and analyzed for complementation in yeast with and without sterol supplementation. Strains positive for complementation could grow aerobically without the presence of sterol suggesting the amino acid residue change does not affect gene function. Mutant strains that do not complement only grow anaerobically in the presence of sterol suggesting these amino acid residue changes do affect gene function. Again, sterol uptake in yeast occurs under anaerobic conditions.

The sterol profile for each mutant was analyzed via gas chromatography. All strains, complementing and non-complementing, were grown anaerobically in the presence of cholesterol (Tables 4.2 and 4.5) and complementing strains were also grown aerobically without sterol (Table 4.3) for two days in liquid media and saponified. GC data is listed in Tables 4.2 and 4.5 as a percentage of sterol accumulation. The 'ergosterol precursors' include: zymosterol, fecosterol, episterol, and other ergosta-diene sterols. The key points to the sterol profile are an accumulation or lack of accumulation of ergosterol, 4-methylfecosterol, lanosterol, and 4,4-dimethylfecosterol. Complementing strains show a wildtype or nearly wildtype ergosterol profile on GC, whereas non-complementing mutant strains show an accumulation of 4,4-dimethylzymosterol.

The mutations created in the three putative histidine clusters including: H160A, H173A, H176A, H258A, and H263A were all negative for complementation and required sterol supplementation anaerobically for growth. However, mutants created in the fourth histidine cluster suggested by Kaplan (36) including H236A and Y239F do complement. H236A had particularly interesting results regarding complementation. After one day of growth, H236A does not complement. However, small, slow-growing satellite colonies were noted between day two and day three. Plating known cell concentrations onto solid media in decreasing increments further quantitated these single colonies. Based on the number of single colonies that survived compared to the number of cells plated, it was calculated that 2% of cells survived with sterol supplementation aerobically. This phenomenon is most likely due to a second spontaneous mutation occurring before these cells die allowing for aerobic growth. Site-directed mutants E152A and D153A, which are adjacent to the first histidine cluster, are also negative for complementation. All other

site-directed mutations accumulated thus far are complementing strains. The KKXX C-terminal motif (K306AK307A) was attempted along with conserved sites L198A and T282A and histidine sites in clusters H164A, H177A, and H261AH262A however were never acquired. Each had a minimum of five attempts with the Stratagene QuickChange Lightning Site-Directed Mutagenesis kit and always resulted in wild type colonies. New primers were attempted for K306AK307A and H261AH262A unfortunately with no success.

The four random mutations analyzed thus far including: S45G, H263L, R274G, and W276R, were negative for complementation and appear just before the proposed transmembrane domain (S45G) and in (H263L) or adjacent to (R274G and W276R) the last conserved histidine cluster. RM213 is a wild-type control strain, which was also created during random mutagenesis.

Mutations created thus far in and adjacent to the histidine boxes suggested by Shanklin et al. (35) have been negative for complementation and show a mutant sterol profile by GC analysis indicating that as expected these amino acid residues are essential for the *ERG25* gene to function. However, mutations created thus far in the fourth histidine cluster suggested by Kaplan et al. complement and show a wild-type profile by GC analysis indicating this putative histidine cluster is not essential for *ERG25* gene function. Several other conserved amino acids complement and do not require supplementation with sterol displaying a wild-type GC profile indicating these residues are also not essential for gene function.

4.5 Future Work

Continued work to analyze the remaining site-directed mutations would be ideal. These sites are important for finishing the evaluation of these histidine clusters as well as the KKXX C-terminal motif. Further analysis of all of these mutations by western blot will indicate whether the amino acid change results in the loss of function of the gene product or if it is causing a decrease in stability of the Erg25 protein.

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