

Searching for efflux pumps in the mitochondria of *Saccharomyces cerevisiae* by proteomics

by

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ABSTRACT:

Biological membranes such as the plasma membrane and those in mitochondria act as semi-permeable barriers that separate cells and their internal compartments from their surroundings. Transporter proteins facilitate selective entry and export of molecules, including antibiotics, across membranes. Both prokaryotic and eukaryotic cells can develop multidrug resistance through the use of membrane-bound efflux pumps. In *Saccharomyces cerevisiae*, there are pumps from the ATP-Binding Cassette (ABC) and the Major Facilitator (MFS) Superfamilies. This organism can resist high levels of mitochondria-targeting antibiotics like chloramphenicol. Our hypothesis is that there are efflux pumps in their mitochondria that remove these antimicrobials from the mitochondria or from the cells and to generate resistance. We predicted that these pumps would be overexpressed in the presence of antimicrobials. We grew cells in the presence of various concentrations of chloramphenicol, which can stop mitochondrial translation and used proteomics to help us identify the efflux pumps that were overexpressed. First, we measured the growth kinetics of the *S. cerevisiae* wild-type BY4741 to determine concentrations of chloramphenicol needed to inhibit growth. Then we isolated membrane-enriched protein fractions by sucrose gradient centrifugation followed by removal of peripheral proteins by washing at high pH. Preliminary LC-MS/MS experiments on these fractions isolated from drug-treated and drug-free cells detected pumps from various organelles. However, the approach did not enrich for mitochondrial membranes as well as expected and the washing step was not efficient. The data were insufficient for comparisons of drug-free and drug-treated cells, so a second approach was taken. Whole cell proteins were isolated from chloramphenicol-treated and drug-free cells and

subjected to proteomics analysis; this allowed biological replicate samples to be prepared at the same time. Cytosolic and mitochondrial proteins were detected. Preliminary analysis indicates that proteins like Sis1, which is a co-chaperone for misfolded proteins were upregulated. Further studies are required to identify potential chloramphenicol transporters and to confirm the role of proteins like Sis1 in protecting the cells from chloramphenicol.

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LIST OF ABBREVIATIONS:

ABC	ATP binding cassette
APS	Ammonium Persulfate
ATP	Adenosine triphosphate
CAP	Chloramphenicol
$\Delta\psi_m$	Membrane potential
DDT	Dithiothreitol
DHA1	Drug:H ⁺ antiporter type 1
DNA	Deoxyribonucleic acid
Eps	Efflux pumps
ERY	Erythromycin
EtBr	Ethidium Bromide
IAA	Iodoacetamide
LZD	Linezolid
MATE	Multidrug and toxic compound extrusion

MCPSB	Manitoba Centre for Proteomics and Systems Biology
MDR	Multidrug resistance
MFS	Major facilitator superfamily
NBD	Nucleotide binding domain
OD	Optical Density
OXPHOS	Oxidative Phosphorylation
PACE	Proteobacterial antimicrobial compound efflux
PAHs	Polycyclic aromatic hydrocarbons
RND	Resistance-nodulation-division
SDS	Sodium dodecyl sulfate
SMR	Small Multidrug Resistance
SGD	<i>Saccharomyces</i> genome database
TEMED	Tetramethylethylenediamine
TMD	Transmembrane Domain
TMS	Transmembrane Helices
TMHMM	TransMembrane Hidden Markov Model
YPD	Yeast Peptone Dextrose medium

YPG

Yeast Peptone Glycerol medium

CHAPTER 1: LITERATURE REVIEW

1. Introduction

Multi-drug resistance has become a big concern in public health. It is the resistance of various microorganisms to drugs with diverse chemical structures and mechanisms of action. Drug resistance can be conferred by several types of transporters, like those in the Adenosine triphosphate Binding Cassette (ABC) family and Major Facilitator Superfamily (MFS). These pumps transport a variety of substrates, ranging from amino acids, peptides, ions to toxins, lipids, and drugs (Catalano et al., 2022). The focus of this thesis is the search for a specific subset of transporters that provide fungi with resistance to antimicrobials that target mitochondrial translation. This chapter will outline why fungi need these transporters in their natural environments and the role of transporters in the presence of toxic compounds to allow the cell to survive.

1.1 The effect of environment on fungi

Fungi are a diverse group of microorganisms that play various roles in nature, meaning that they can be decomposers or pathogens and they can also use various strategies to degrade toxic compounds ranging from recalcitrant polycyclic aromatic hydrocarbons (PAHs) to pesticides. Many fungi can live in harsh and extreme conditions. They can survive variable conditions as they produce spores that are resistant to these conditions and germinate when the conditions are proper for them (Selbmann et al., 2005). In addition, biosurfactants made by the fungi play a paramount role in improving the availability of vital nutrients required for the development of fungi, meaning

that they are able to decrease surface tension to increase surface adsorption, and emulsification activities to degrade petroleum hydrocarbons for fungi (Mahmoud et al., 2024; Al-Hawash et al., 2019). Some ligninolytic fungi have the ability to employ a wide range of biodegradation mechanisms as they possess extracellular enzymes like laccases, which are blue multicopper oxidative enzymes that can either degrade or modify a variety of compounds by catalyzing one-electron oxidation reactions, mainly involving aromatic or phenolic compounds (Akrou et al., 2024; Shraddha et al., 2011). Secretion of laccase and lignin peroxidase plays a vital role in degrading pollutions and waste ranging from hydrocarbons to agricultural waste to ensure fungal survival (Mirgeloybayat et al., 2024). Fungi are able to absorb and accumulate heavy metals and they can tolerate toxic and heavy metals by using various strategies. They can absorb these compounds on their surface, or they can exclude them by their efflux pumps (Kumar et al., 2019). Having negative charge in fungal and yeast cell walls causes them to absorb metals with positive charges to protect themselves against heavy metals. They also typically have both enzymatic and non-enzymatic antioxidant systems for improving resistance by diverse mechanisms such as chelation (Ghosh, 2020; Kumar & Dwivedi, 2021). Fungal mycelia have an important role in transporting water, nutrient and pollutant-degrading compounds in the soil, thereby enhancing the effectiveness of bioremediation of soil co-contaminated with petroleum hydrocarbons and toxic metals (Li et al., 2020). Filamentous fungi such as *Aspergillus* can degrade polycyclic aromatic hydrocarbons, aliphatic hydrocarbons and chlorophenol as they provide carbon for *Aspergillus* as a source of energy (Li et al., 2020). Fungi also live in environments, especially soils, which have antimicrobial compounds generated by either bacteria or fungi. Moreover, fungi, by effluxing them

from the cells, respond to natural and artificial antimicrobials produced by human activity (Islam et al., 2023).

1.1.2 The influence of efflux pumps on fungal survival

Biomembranes provide barriers against all hydrophilic molecules; most of the molecules that cross the membranes either use inward transport systems or the endocytic pathway (Van Bambeke et al., 2000; Watson, 2015). One of the mechanisms that fungi can use to overcome intracellular chemical compounds as well as toxins is efflux pumps like those of the ABC and MFS families. They remove various toxic compounds by transporting with energy from ATP hydrolysis or a proton gradient. Fungal genomes encode the most transporters in comparison to other organisms (Coleman & Mylonakis, 2009).

1.2 Bacterial drug resistance mechanisms:

Mitochondria originated from α -proteobacteria that joined with eukaryotic cells, so there are similarities between mitochondrial proteins and bacterial proteins (Kurland & Andersson, 2000) and that is why we consider bacterial mechanisms. Bacterial drug resistance has been studied extensively. They use four mechanisms to develop their resistance to antibiotics: 1) changing the permeability of the cell to prevent entry of antibiotics into the cells 2) altering the molecular targets of antibiotics, 3) expressing enzymes to inactivate antibiotics, and 4) expressing efflux pumps in order to pump out antibiotics from the cell (Sharma et al., 2019).

1.3 Protein transporters:

Transporters are the group of biological membrane proteins that can facilitate the movement of water-soluble solutes through the lipid bilayer and located in the cell membrane of bacteria remove

various substrates like ions, drugs, dyes, chemicals, heavy metals, organic solvents, and toxic compounds (Huang et al., 2022). Primary active transporters utilize ATP whereas passive and secondary active transporters are dependent on existing electrochemical gradients like mediating solutes and using energy from movement of one solute to transport another solute against its gradient (Drew et al., 2021). Sodium potassium pumps are example of primary transporters and glucose transporters are secondary transporters (Clark et al., 2020).

Transporters are classified in 3 groups: uniporters, antiporters, and symporters. Uniporters, that transfer only one molecule, are classified into two major groups, channels and carriers. Channels allow fast diffusion of small molecules such as water or urea. VDAC (Voltage-Dependent Anion Channel) in the mitochondrial outer membrane is one example of a channel that is anion selective in its open state and cation selective in its partially closed state, which occurs in the presence of a membrane potential. It is involved in the movement of metabolites and chemical substrates along their concentration gradients. The lumen of a channel is accessible from both sides of the membrane and molecules can cross in both directions. In contrast, just one side of a carrier is open to transport a substrate and an energy source is used to translocate a compound against its concentration gradient in a unidirectional way (Wolfersberger, 1994). Carriers work more slowly owing to conformational changes that alternately expose the substrate binding site to each side of the membrane (Drew et al., 2021).

Symporters are transporters that employ energy in electrochemical potential gradient to move ions, molecules, and substrates in parallel ways to into the cells, while those substrates at the same time can move in same direction usually (Beckstein & Naughton, 2022; Abramson & Wright, 2009).

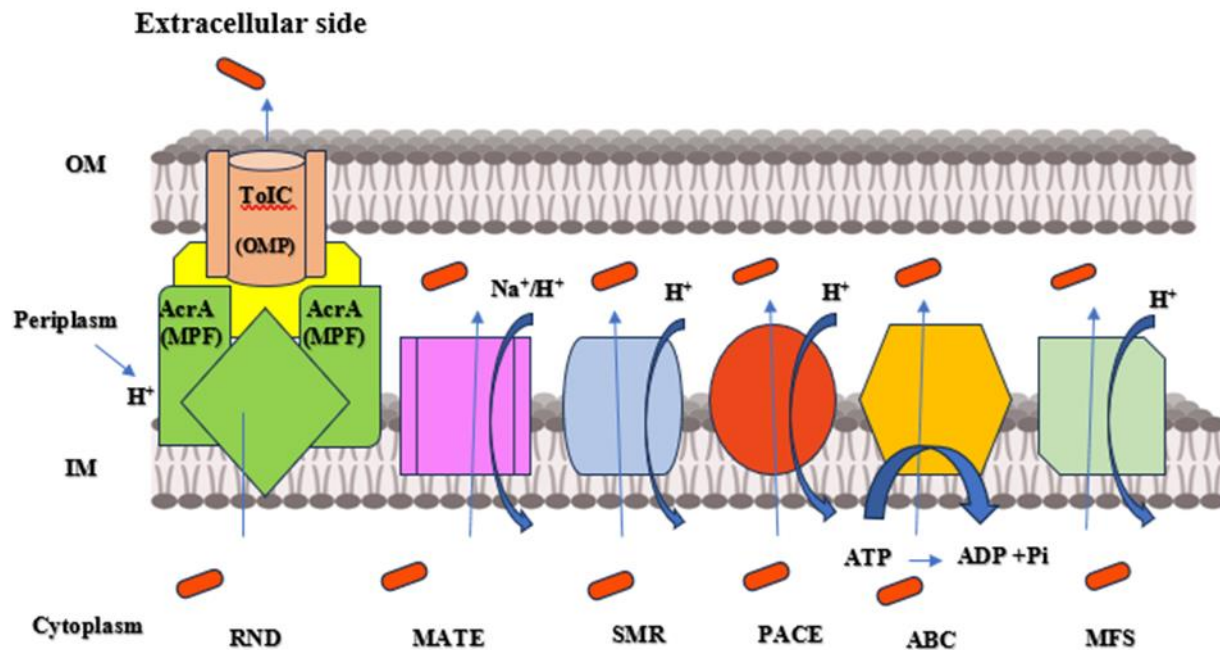
Antiporters enable exchange of two or more ions, molecules, or substrates across a membrane in opposite directions. Some use proton electrochemical gradients produced by primary pumps by linking the movement of one or more protons to the transfer of another molecule (Schuldiner, 2014). In contrast to transporters, channels do not use energy to move compounds, but they may open to response to specific environmental signals like voltage changes in membrane potential, ligand binding (interaction with some specific molecules), and various mechanical stress on membrane (Gadsby, 2009).

1.4 Efflux pumps (drug transporters):

In the early 1980s, antibiotic transporters were identified in bacteria that were resistant to tetracyclines (Saier et al., 1998). Since then, it has been shown that efflux pumps or drug transporters are important in both prokaryotic and eukaryotic cells because they can modulate the accumulation of drugs in the cell by removing them (Van Bambeke et al., 2003, Auda et al., 2020).

1.4.1 The classification of efflux pumps in bacteria:

Efflux pumps are classified into six groups in bacteria: 1) the Resistance-Nodulation-Division (RND) family, 2) the Major Facilitator Superfamily (MFS), 3) the ATP (adenosine triphosphate)-Binding Cassette (ABC) superfamily, 4) the Small Multidrug Resistance (SMR) family, 5) the Multidrug And Toxic compound Extrusion (MATE) family, and the 6) Proteobacterial Antimicrobial Compound Efflux (PACE) family (reviewed by Kornelsen et al., 2021). Fig. (1.1) summarizes the efflux pumps in bacteria.



 : Drug

Fig 1.1: A summary of bacterial efflux pumps in Gram negative bacteria. The thin arrows show the movement of the drug (red oval), and the thick arrows indicate the energy source, either the movement of ions or the hydrolysis of ATP. (Created with BioRender.com; modified from Blanco et al., 2016).

1.4.2 Efflux pump structure:

Transporter proteins include efflux pumps. MFS efflux pumps in Gram-positive bacteria typically possess monocomponent (single) proteins with 12 transmembrane domains (TM), and are located

in the cytoplasmic inner membrane. In contrast Gram-negative bacteria have multicomponent efflux systems, spanning the two membranes and the periplasmic space (Gurvic & Zachariae, 2024). RND and ABC efflux pumps can include three components for transporters: the inner membrane transporter, the outer membrane channel and the periplasmic lipoprotein.

All inner membrane components in Gram-negative bacteria do not have necessarily 12 TM domains. Some of them like SMR proteins possess about 110 residues that include four TM α -helices with short connecting loops (Poulsen & Deber, 2012) and the ABC transporter consist of four domains: two nucleotide binding domains, NBD1, NBD2 and two transmembrane domains, TMD1, TMD2 (Wilkins, 2015). RND efflux pumps possess three components. First one, located in the inner membrane, acts as an extrusion element composed of more than 1000 amino acids folding into 12 transmembrane helices. The second one is the protein in the outer membrane that creates a channel that extends through periplasmic space. The third component has a lipoprotein form that is connected to the inner membrane, and can stabilize the interactions between the other two elements (Godoy et al., 2010).

Various antibiotics can be recognized by different efflux pumps. Some classes such as tetracyclines, macrolides, fluoroquinolones, and chloramphenicol act as quasi-universal substrates, but individual drugs within these classes might interact with same or different pumps depending on the specific efflux pumps (Auda et al., 2020). Some efflux pumps can have specific substrates while some transporters pump out various antimicrobials (Hernando-Amado, et al., 2016). For example, the NorA efflux pump in *Staphylococcus aureus* that can transport fluoroquinolone antibiotics ciprofloxacin and norfloxacin by promoting their active extrusion of extrusion substrate from cells is a good example for transporting specific substrates (Palazzotti et al., 2023; Ferreira

et al., 2022). Another example is MexABOprM efflux in *Pseudomonas aeruginosa* which is responsible for transporting various chemical compounds like aminoglycosides, β -lactams, chloramphenicol, colistin, ethidium bromide, fluoroquinolones, macrolides, sodium dodecyl sulfate, sulphonamides, tetracycline, trimethoprim, and triclosan (Duffey et al., 2024).

The two main types of pumps related to this thesis are members of the ABC and MFS families and they are described below.

1.4.3 ABC

The ABC transporters are a vast family of membrane transport proteins. They comprise two types of domains: two transmembrane domains (TMD) and two nucleotide-binding domains (NBD) (Locher, 2016). (fig 1.2). The crystal structure of NBDs associated with ATP indicated that the NBD is a dimer (Wilkins, 2015). Hydrolyzing ATP in the NBD stimulates conformational changes in the TMD, causing transporters to alternate between exposing its binding sites to the inside and to the outside of the cells, which facilitates one-way transport across the lipid bilayer. ABC transporters can distinguish variety of substrates ranging from small either inorganic or organic molecules like amino acids, sugars, nucleosides, vitamins and metal clusters to large organic molecules include peptides, lipid molecules, oligonucleotides and polysaccharides. Most transporters can export toxic compounds from cells, so overexpression of these transporters can lead to multi-drug resistance (MDR) phenotypes in bacteria and mammalian cells (Wilkins, 2015). Sometimes the overexpression of efflux pumps occurs as a result of extrusion of organic compounds, co-selecting for antibiotic resistance, meaning that efflux pumps help bacteria to be resistance to toxic environments and expel antibiotics from the cell (Blair, et al., 2016). In fungi,

overexpression of ABC and MFS pumps in plasma membrane is related to MDR (Islam et al., 2023).

1.4.4 Major facilitator superfamily (MFS) efflux pumps:

Major facilitator superfamily (MFS) pumps efflux small substrates like sugars, polyols, drugs, neurotransmitters, amino acids, peptides, lipids, organic and inorganic ions, vitamins, nucleobases, nucleosides, and nucleotides using the electrochemical gradient of protons through the plasma membrane are secondary active transporters (Drew et al., 2021; Wilkens, 2015). These pumps are integral membrane proteins like ABC pumps (Drew et al., 2021; Prasad, & Rawal, M. K. 2014). Bioinformatic analysis shows that MFS transporters are in two groups, having 12 or 14 transmembrane segments (TMSs). These segments have α -helical structure and span the lipid bilayers. Based on phylogenetic studies these transporters possess two TMSs and a connecting hairpin loop after evolution which duplicated to form the more complex 12- or 14-TMS in MFS proteins (Reddy et al., 2012) (fig. 1.2).

The mechanisms of action are different for different efflux pump families in terms of their source of energy (Verchère et al., 2012). ABC efflux pumps hydrolyze ATP for energy supply; however, other efflux pumps like multidrug and toxic compound extrusion MATE, MFS, resistance nodulation cell division (RND), and small multidrug resistance (SMR) use the proton-motive force that is provided either by H^+ or electrochemical gradient of Na^+ to serve as energy (Kim et al., 2012). MFS, DHA1 contain 12 TM, and DHA2 contain 14 TM utilize cation gradient to produce energy. Some substrates like drugs depict that how energy uses for various physiological functions, and are found in eukaryotic cells, yeast, fungi, and both Gram- positive and Gram -negative bacteria (Spengler et al., 2017). The 14-spanner Drug: H^+ Antiporter family 2 (DHA2) is also

involved in multidrug resistance (MDR) and functionally similar to DHA 12 (Dias Sá-Correia, 2013). They include uniporters, symporters, and antiporters; antiporters act as drug efflux pumps (Islam, et al, 2023). These solute transporters can transport the variety of substrate depending on their specificity like antibiotics such as tetracycline, sugars like glucose by the mammalian glucose carrier and lactose permease in *E.coli* responsible for transport of lactose together with a proton (H^+) and cannot transport, phosphate esters and intermediates of the krebs cycle. (Trichez et al., 2018).

1. 4. 5 Antimicrobial Resistance in Fungi:

Antimicrobial resistance in fungi is an important problem in medicine and agriculture (Michael et al., 2014). Fungi can develop mutations in the presence of antifungal agents that create drug resistance that emerges impedes medical treatment (Michael et al., 2014). Multiple mechanisms are responsible for resistance of fungi to azoles, the most common antifungal drugs used in medicine. Azole blocks the biosynthesis of ergosterol, which is the fungal sterol in the plasma membrane (Scott Moye-Rowley, 2020). One of them is the mutation of the ERG11 gene, which codes for lanosterol 14 α -demethylase, or changes its expression. Mutation of this gene increases tolerance to toxic sterol intermediates when azoles inhibit sterol 14 α -demethylation in *Candida albicans*. Another mechanism is related to ERG11 overexpression, which increases the level of lanosterol 14 α -demethylase, declining the efficacy of azole inhibition (Urbanek et al., 2022; Liu et al., 2015). Finally, there is a direct relationship between the expression of ABC efflux pumps in fungi and azole resistance (Lamping et al., 2010).

1.5 *Saccharomyces cerevisiae*:

The focus of this thesis are efflux pumps in fungi. The model system we used is *Saccharomyces cerevisiae*, which are yeast cells with an ovoid shape and they reproduce by a budding mechanism. The cells can grow both haploid and diploid forms. Haploid cells and diploid cells have a simple mitosis life cycle, but haploid cells will die under high stress situations while diploid cells are more tolerant and enter meiosis to generate four haploid spores that are resistant to stress (Salari et al., 2017).

This research is focusing on the ability of *S. cerevisiae* to grow through both key factors respiration and fermentation. It can use glycerol as a respiratory carbon source which selects for mitochondrial

function. Therefore, it will show growth defects under conditions that impair mitochondrial functions like translation. It can also grow by fermentation of sugars like glucose. This allows mitochondrial mutants or cells exposed to high levels of mitochondria-targeting drugs to survive (Lemieux et al., 2017).

1.5.1 The classification of efflux pumps in *S. cerevisiae*:

In budding yeast, like *S. cerevisiae*, MFS transporters include 12-14 transmembrane domain proteins that provide resistance to many chemicals and drugs, toxins and fungicides. Members of this family also transport simple sugars, oligosaccharides, inositols, drugs, amino acids, and nucleosides (Chen et al., 2017; Pao et al., 1998). Therefore they participate in various cellular processes like nutrient transportation, bioenergetic processes, cell adhesion, and signal transduction (Kongpracha et al., 2022).

Efflux pumps facilitate transport of a broad of xenobiotics (Holmes et al., 2016). Fungal genomes can encode some efflux pumps from the ABC family like Pdr5 in *S. cerevisiae* which can export azoles (Wilkins, 2015) or Yor1 for exporting oligomycin and other organic compounds (Katzmann et al., 1995), and Cdr1 and Cdr2 in *C. albicans*, which are responsible for exporting azoles, allowing them to live in different environments that may include antibiotics (Coste et al., 2004; Stajich, 2017). The overexpression and mutation of *Cyp51* in *Aspergillus* species increase the resistance to azoles (Wilkins, 2015).

The antibiotic that was studied in this thesis was chloramphenicol, which targets mitochondrial ribosomes, blocking protein synthesis (Li et al., 2010). Furthermore, tetracycline inhibits mitochondrial translation while ciprofloxacin inhibits mitochondrial topoisomerase II, damaging

mtDNA replication and transcription. As a result, loss of mitochondrial DNA indirectly could reduce mitochondrial protein synthesis and mitochondrial deficiency happens. In contrast, cytosolic and nuclear translation cannot be affected (Chopra & Roberts, 2001; Hangas et al., 2018). Efflux pumps in *S. cerevisiae* are members of two groups: the ATP-binding cassette (ABC) family, and the major facilitator superfamily (MFS). The likelihood of finding transporters and drug efflux pumps in MFS is higher than for ABC transporters (Islam et al., 2023). Figure (1.1) depicts the movement of drugs via ABC and MFS efflux pumps.

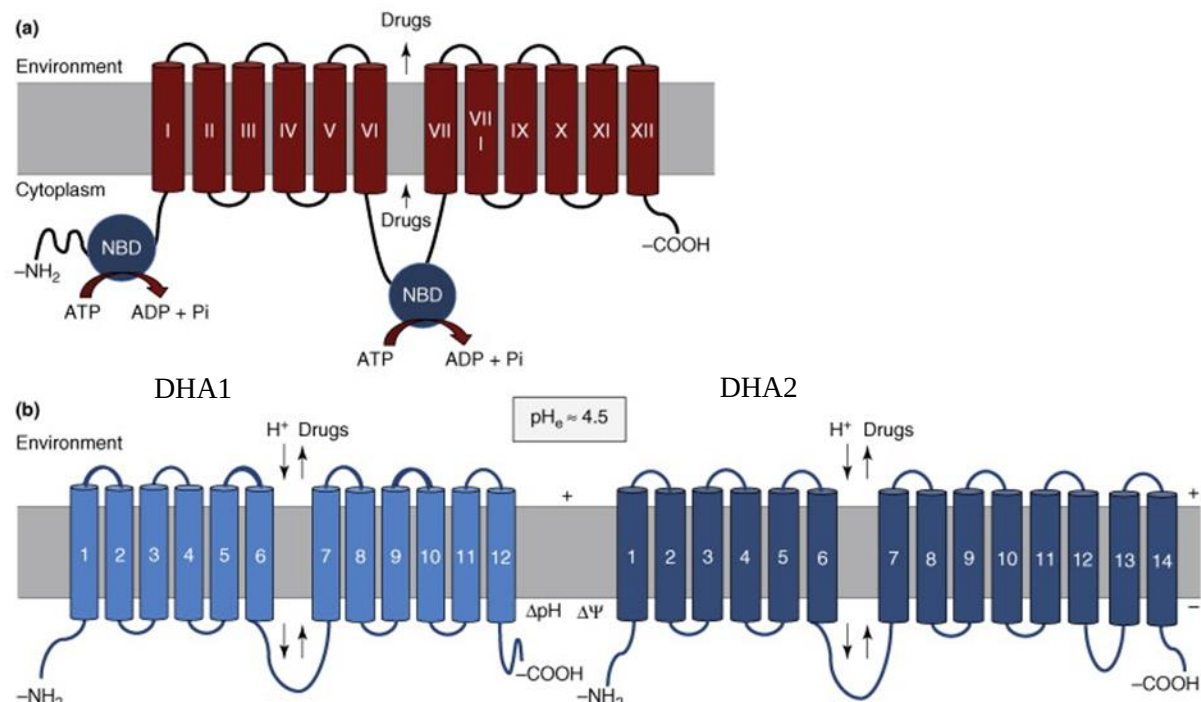


Fig 1.2 Multidrug efflux transporters in the plasma membrane of yeast. a) ABC transporters. Nucleotide binding domains (NBD) are shown as blue circles and transmembrane alpha-helices as red cylinders. b) MFS transporters. MFS-MDR transporters of both 12-TMS drug: H⁺ + antiporter

family 1 (DHA1) and 14 TMS drug: H⁺ antiporter family 2 DHA2 are shown. In both figures the movement of the drugs are shown by arrows (used with permission from Sá-Correia et al., 2009).

1.5.2 Transporters of polyamines in *S. cerevisiae*

One well-characterized group of MFS pumps in *Saccharomyces* are the transporters of polyamines (Tpo1- Tpo5) that give the cells resistance to polyamines (reviewed by M. Islam, 2021). In this yeast, there are 3 polyamines called spermine, spermidine, putrescine that are transported by a substrate/nH⁺ antiport mechanism into vacuoles. Polyamines in the cytoplasm of yeast are regulated by all Tpos, meaning that a single polyamine transport system would probably be insufficient to maintain optimal polyamine levels in the cytoplasm if the concentration of polyamines outside cells is high (Tomitori et al., 2001). Tomitori found Tpo1- Tpo4 in the vacuolar membrane of *S. cerevisiae*. Therefore, all four transporters are responsible for detoxification of polyamine (Albertsen et al., 2003). Tpo1 also is located in the plasma membrane and vacuole and is responsible for expelling spermidine. Overexpression of *TPO1* leads to a decrease in intracellular spermidine levels due to enhanced efflux, particularly under conditions where extracellular polyamine availability is limited. In contrast, deletion of *TPO1* results in spermidine accumulation, highlighting its key role in regulating polyamine homeostasis (Tomitori et al., 2001; Uemura et al., 2005). Tpo2 and Tpo3 are responsible for detoxification of spermine, while Tpo1 and Tpo4 detoxify spermidine and spermine (Tomitori et al., 2001) and Tpo5 exports polyamines through post-Golgi secretory vesicle membranes. In addition, Tpo2 and Tpo3 also serve resistance to both short-chain acetic and propionic acids and Tpo4 confers resistance to quinidine (Islam, 2021).

The overexpression of Tpos can lead to decreasing spermidine levels in the cell by efflux enhancement, promoting their export (Tomitori et al., 2001). However, some studies have reported that overexpression of some *TPO* genes like *TPO1*, can increase sensitivity to polyamines during stress situations in the cell and *TPO1* deletion causes spermidine accumulation (Krüger et al., 2013; Matta et al., 2001; Albertsen et al., 2003). This discrepancy probably reflects differences in substrate specificity, the concentration of polyamine, and cellular context containing the risk of over-exporting vital polyamines or disrupting ion homeostasis. The Tpo family of proteins are relevant to this research because some of them are located in mitochondria. Vögtle *et al.* (2017) identified proteins in purified mitochondria of *S. cerevisiae*, including Tpo1 and Tpo3, using a quantitative mass spectrometry method. Preliminary results showed that Tpo1 and Tpo3 may be involved in defending yeast cells from chloramphenicol (CAP) and Erythromycin (ERY). Islam indicated that $\Delta TPO1$ and $\Delta TPO3$ mutants were more sensitive to various concentrations of chloramphenicol than WT (Islam, 2021).

In *S. cerevisiae*, Tpo1, which provides resistance to a range of toxic compounds is associated with multidrug resistance proteins and is regulated by Pdr1 and Pdr3, which are multidrug efflux pump gene regulators (Albertsen et al., 2003). Pdr1 and Pdr3 are transcription factors that regulate PDR in *S. cerevisiae* through the PDR responsive elements (PDREs) to influence on gene expression.(Buechel & Pinkett, 2024). Activation of the PDR network strongly induces the expression of ABC and MFS transporters, which play vital roles in exporting diverse toxic substances and there is no doubt that PDR roles are significantly important. However, differentiation of the roles of Pdr1 and Pdr3 is still unknown (Buechel & Pinkett, 2024). Pdr5 transports various chemicals like azoles, ionophores, antibiotics and xenobiotics while Tpo1

located in plasma membrane of *S. cerevisiae* can involve in the detoxification of excess spermidine in the yeast cell (Albertsen et al., 2003).

In summary, there is evidence that transporters in mitochondria are associated with drug resistance, therefore we will focus on mitochondria in this research.

1.6 Mitochondria:

Mitochondria are found in the majority of eukaryotic cells and they are descendants of bacteria (Prokisch et al., 2004). Mitochondria possess their own genetic machinery, contain multicopy genomes and two membranes like Gram-negative bacteria as they have endosymbiotic origins (Malina et al., 2018). They produce energy for the cell by converting substrates into ATP via oxidative phosphorylation (OXPHOS) (Casanova et al., 2023). Ribosome structure is shared by mitochondria and bacteria due to their common ancestral α -proteobacteria, so some antibiotics can inhibit mitochondrial function. Some antibiotics can affect mitochondria by inhibiting their translation like chloramphenicol (CAP) and oxazolidinones, including linezolid (LZD) (Table 1.1). Mitochondrial ribosomes are more closely related to bacterial ribosomes than cytoplasmic ribosomes. They are composed of a small subunit (mt-SSU) and a large subunit (mt-LSU) that are similar to the bacterial 30S and 50S subunits. Some antibacterial antibiotics bind to the ribosomal peptidyl transfer center (PTC) of the mitochondrial ribosome to inhibit mitochondrial translation (Table 1.1). This binding blocks tRNA substrate from binding to the A site to disrupt peptide bond formation, thus these antibiotics impair elongation and termination of translation and stop mitochondrial protein synthesis (Bibel et al., 2024). Similarly, erythromycin family drugs can bind near the A-site and hinder aminoacyl-tRNA from binding. However, tetracycline binds the mt-SSU near A-site, to impede translation (Suárez-Rivero et al., 2021).

Table 1.1: Mitochondria-targeting antibiotics and their mechanisms of action

List of antibiotics	Target	Mechanisms
Chloramphenicol	mt-LSU*	Inhibits peptidyl transferase activity (Yu & Zeng, 2024).
Erythromycin	mt-LSU	Prevent protein synthesis and inhibit ribosome to stop elongation of peptides (Seefeldt et al., 2021).
Linezolid	mt-LSU	Prevents initiation complex formation (Swaney et al., 1998).
Clindamycin	mt-LSU	Inhibits peptidyl transferase (Fitzhugh, 1998).
Josamycin	mt-LSU	Inhibits the formation of second and third peptide bond (Lovmar et al., 2004).
Tiamulin	mt-LSU	Inhibits peptidyl bond formation (Schlünzen et al., 2004).
Tetracycline (Doxycycline)	mt-SSU*	Inhibits amino-acyl tRNA binding to A-site (Schlünzen et al., 2004).
Minocycline (semi-synthetic tetracycline)	mt-SSU	Inhibit mitochondria and has a role in apoptosis and prevent protein synthesis in 30S subunit (Garrido-Mesa et al., 2013).

Neomycin	mt-SSU	Targets translation fidelity (Waldsich et al., 1998).
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* mt-LSU, mito-ribosome large subunit; mt-SSU, mito-ribosome small subunit

1.6.1 The role of mitochondria membrane in activating the Pleiotropic Drug Resistance Pathway (PDR):

In the yeast *S. cerevisiae*, multidrug resistance (MDR) is regulated by a network referred to as the PDR (pleiotropic drug resistance). Most of these genes encode ABC transporters, such as Pdr5, Pdr15 and Snq2 in plasma membrane, and Ycf1 in vacuole (Paumi et al., 2009). These pumps export diverse toxic compounds out of the cell using ATP energy. Moreover, the MDR system also includes drug transporters from the MFS such as Atr1 (Kanazawa et al., 1988) and Sge1 located in plasma membrane (Moreira et al., 2012; Moreira et al., 2012), which efflux drugs using energy from the proton motive force. The expression of these transporters is actively regulated by different transcription factors like Pdr1 and Pdr3, which are involved in regulating *PDR* genes.

Other transcriptional regulators like Yap1 and Yap2 can also affect multidrug resistance via regulation of multidrug resistance at the level of transcription of genes in the situation of oxidative stress and for detoxification (Balzi & Goffeau, 1995). Mitochondria play a vital role not only in producing energy but also in regulating cellular signaling pathways, like retrograde signaling (RTG). Mitochondria connect to nucleus by this signaling pathway to respond to changes in mitochondrial function ranging from stress to dysfunction. After impairing mitochondrial function, the RTG pathway is activated, leading to activation and nuclear translocation of the transcription

factors Rtg1 and Rtg3, which can regulate the expression of some specific genes in the nucleus involved in both metabolic adaptation and stress responses. Mitochondrial RTG can impact the expression of genes which are associated with PDR. During this pathway, the cell can upregulate PDR transporters to protect the cell from toxic compounds and enhance drug efflux in the cell. Therefore, the role of mitochondria in modulating drug resistance through transcriptional factors should not be neglected and MDR happens due to the presence of many efflux pumps and these pumps have wide substrate specificity, which allow them to transport various drugs (Giannattasio et al., 2005). *PDR3* and the *RTG* genes probably act together to respond to mitochondrial defects (Hallstrom et al., 2000).

1.6.2 Evidence of mitochondrial drug efflux in *Neurospora crassa*:

There is evidence for the activity of efflux pumps in mammalian mitochondria. For instance; the overexpression of ABC transporters like P-glycoprotein (Pgp), breast cancer resistance protein (BCRP) and multidrug resistance-associated protein (MRP) cause the efficacy of chemotherapy drugs to decrease as these pumps, located in plasma membrane, extrude drugs out of the cells (Dartier et al., 2017). In contrast, there is very little direct evidence for efflux of drugs from fungal mitochondria, although *S. cerevisiae* can carry out mitochondrial translation in concentrations of CAP that are over 100x of the concentrations of CAP that block bacterial translation (Islam et al., 2023). Preliminary evidence was obtained using the drug mimic ethidium bromide (EtBr), which is used in bacterial efflux assays (Islam 2021). In an EtBr efflux assay using intact mitochondria, an increase in fluorescence is indicative of DNA intercalation by EtBr, whereas the decrease in fluorescence suggests the active efflux of the dye out of mitochondria. This phenomenon was observed with mitochondria from the fungus *N. crassa*, and was dependent on the establishment

of a membrane potential ($\Delta\Psi_m$) across the mitochondrial membrane, suggesting that an MFS pump was involved. In one experiment, mitochondria of *N. crassa* were extracted. First, mitochondria without membrane potential were incubated with ethidium bromide (EtBr) to bind with DNA, which led to increasing fluorescence. Next, succinate was added and a membrane potential was generated, which activated efflux pumps, causing a decrease in fluorescence, suggesting an MFS-type pump was involved. Because ADP was not added to the buffer, ATP was not produced, which indicates that this activity is not reliant on ATP and the pump is not likely of the ABC family. After disrupting of membrane potential, uncoupling agent CCCP (carbonyl cyanide m-chlorophenyl hydrazone) was used, and led to increasing fluorescence again, indicating that EtBr was not being expelled (Islam et al., 2023).

The pumps working in *N. crassa* are not known, so we decided to search for them in *S. cerevisiae*, because it is easier to carry out mutations and grow these cells. Islam conducted a thorough examination of potential MFS encoding genes in both *N. crassa* and *S. cerevisiae*, being model organisms utilized in the laboratory (Islam 2023). However, from their sequences, we cannot predict which MFS transporters are in mitochondria, so we chose a proteomics approach.

1.7 Hypothesis:

There are some efflux pumps in the plasma and mitochondrial inner membranes of *S. cerevisiae* which pump out mitochondria-targeting antibiotics to exports them out of the cell or out of the mitochondrion, resulting antibiotic resistance. These pumps are overexpressed when the cells are exposed to drugs.

1.8 Objectives:

- A) To evaluate the efficiency of isolation of plasma membrane and mitochondrial membrane fractions using sucrose gradient centrifugation.
- B) To use proteomics to identify membrane pumps that are overexpressed in *S. cerevisiae* cells in isolated membranes, or whole cells.
- C) To predict the location of the potential drug pumps based on localization data.

CHAPTER 2: MATERIALS AND METHODOLOGY

2.1 Strains:

In this study, the wild-type (WT) *S. cerevisiae* strain BY4741 with the genotype (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was used (Branchmann et al., 1998).

2.2 Analysis of the growing the yeast on the media in the presence of drugs:

2.2.1 Selection media:

Two media were chosen for the growth of the yeast. Yeast extract peptone dextrose (YPD), which is vital for fermentation and yeast extract peptone glycerol (YPG), which is important for respiration and requires the yeast to have functional mitochondria.

All media components were bought from Thermo Fisher Scientific company (Winnipeg, Canada). For plates, components, including agar (1.5% w/v), were dissolved in Milli-Q water and autoclaved at 121° C for 15 min and poured in petri dishes after cooling. YPD liquid medium contains 20 g glucose, 20 g Bacto™ peptone and 10 g yeast extract per 1000 ml Milli-Q water. YPG liquid media contain 30 ml glycerol, 20 g Bacto™ peptone and 10 g yeast extract per 1000 ml Milli-Q water. YPEG medium also contains 3% ethanol (v/v).

2.2.2 Chemicals:

All chemical compounds and reagents were bought from Thermo Fisher Scientific (Winnipeg, MB), Sigma-Aldrich Canada (Oakville, ON), or BioShop Canada (Burlington, ON). A stock solution of chloramphenicol with the concentration of 50 mg/ml was used and contained 2.5 gram of chloramphenicol mixed with 50 ml of 95 % ethanol.

2.2.3 Antibiotic susceptibility test in 96-well plates:

Glycerol stocks of wild type and *S. cerevisiae* were streaked on YPD and YPG plates and incubated for 2-3 days (YPD) or 4-6 days (YPG) at 30° C. The growth of the yeast on YPG is slower than on YPD. A single colony was used to inoculate 25 ml of YPG or YPD in 50 ml flasks at 30° C in shaking water bath incubator for 2 days. YPD was used for fermentation while YPG was used for respiration of mitochondria. The concentration of cells was estimated by optical density (OD) 600 nm and the sample's OD was measured every hour. When the OD/ml was 0.05 to 0.2, they were transferred into the 96-well plate. In one microfuge tube, 900 µl of media (YPG or YPD) were mixed with 100 µl of culture and 27 µl of 95% ethanol or the desired concentration of chloramphenicol (final concentration 1.5 mg/ml) in 95% ethanol. 95% ethanol was added as a solvent control (Olivares-Marin et al., 2018). 180 µl of each mixture was added to a well in a 96-well plate and the plate was incubated in a plate reader, with shaking, at 30° C for 48 hours. Growth was followed by measuring OD 600 nm in a Biotek Synergy HMX reader or 630 nm in an Agilent BioTek 800 TS. The growth kinetics of wild type BY4741 and were measured in the presence or absence of chloramphenicol.

2.2.4 Large scale production of yeast for protein extraction

To determine the timing of the maximum growth rate for protein extraction, five 25 ml flasks of YPG were cultured with WT BY4741 *S. cerevisiae* and when the OD was appropriate (0.2 OD/ml), various amounts of 50 mg/ml chloramphenicol in ethanol were added to them and additional ethanol was added to make sure there are the concentration of ethanol was the same in all samples

- 1) 750 μ l Ethanol + 0 μ l Chloramphenicol $\rightarrow$ 0 mg/ml CAP
- 2) 500 μ l Ethanol + 250 μ l 50 mg/ml Chloramphenicol $\rightarrow$ 0.5 mg/ml CAP
- 3) 375 μ l Ethanol + 375 μ l 50 mg/ml Chloramphenicol $\rightarrow$ 0.75 mg/ml CAP
- 4) 250 μ l Ethanol + 500 μ l 50 mg/ml Chloramphenicol $\rightarrow$ 1.0 mg/ml CAP
- 5) 0 μ l Ethanol + 750 μ l of 50 mg/ml Chloramphenicol $\rightarrow$ 1.5 mg/ml CAP
- 6) *Chloramphenicol was solved as a 50 mg/mL stock solution in ethanol and equivalent volumes of ethanol were added as a solvent control.

The OD of the cultures was measured every hour manually.

To generate enough cells for extraction of proteins, 2 flasks were chosen (without drug and with drug) and proteins were isolated based on method of Wei Xiao (Xiao, 2006). First 100 ml of cells were cultured in YPG in four 250-ml baffled flasks and incubated at $28 \pm 2^\circ\text{C}$ for 24 hr. After measuring their OD, the cells were transferred into 500 ml flasks and 150 ml of YPG was added to the flasks and they were incubated for 12 hr. At that time, an additional 250 ml of YPG was added to the 1000 ml flasks and cells were grown for 2 days and when their OD₆₀₀ reached 0.3 OD/ml drug was added to them. For 500 ml of YPG, 15 ml of ethanol or 50 mg/ml chloramphenicol in ethanol was added to flasks. The cells were incubated again and every hour, their OD₆₀₀ was

measured. After 6-7 hr, they reached maximum growth rate, and extraction of proteins was started. Next, the concentration of proteins was done by Bradford assay (see section 2.4) and some parts of samples were run on the SDS-PAGE gel (for method see section 2.4). For an initial test, some samples of 100 µg were sent to Manitoba Centre for Proteomics and System Biology to identify transporters.

2.3 Isolation of yeast membranes:

Yeast membranes were isolated by the method of (Xiao, 2006), which utilizes flotation gradients to enrich membranes. 25 ml of YPG was inoculated and incubated at 28 ± 2 °C and after 1 day 125 ml of YPG was added to them and incubated for 36 hr. Then 850 ml of YPG was added to 125 ml culture in a 2000 ml flask to have a large scale and after reaching to maximum growth (6-7 hr), 1 liter of wild-type cells in YPG broth was collected in Beckman 250 ml centrifuge tubes and centrifuged at 5700 rpm with a Sorvall 1500 centrifuge rotor for 5 min. Next, the cell suspensions were divided into two 50-ml polycarbonate tubes and centrifuged for 10 min at 6400 rpm in a Beckman JA-20 rotor. The supernatant was removed and twice the pellet volume of acid-washed glass beads was added. Then, twice the combined volume of the pellet and glass beads of buffer A (0.4 M sucrose, 25 mM imidazole, (pH = 7)) was added and vortexed for 2 min and kept on ice for 30 s. This step was repeated 3 times. The samples were diluted 3 times in buffer A and centrifuged at 2100 rpm for 20 min in the JA10 rotor to pellet cells and to remove glass beads. The supernatant was centrifuged at 2100 rpm for 5 min to ensure there were no beads left, and then the supernatant was centrifuged from last step at 13500 rpm for 30 min to collect a pellet containing membranes. The pellet from the last step was resuspended in 2 ml buffer A by vortexing gently for 30 seconds. Then, discontinuous sucrose gradients were prepared by overlaying three 12-ml

layers of 2.25, 1.65, and 1.10 M sucrose (in 25 mM imidazole buffer) in 14 x 89 mm Beckman (Fullerton, CA) Ultra Clear tubes (Cat. No. 344058). 2-ml aliquots of resuspended membrane were added onto the top of the sucrose gradient and centrifuged in a Beckman SW32 rotor at 20400 rpm overnight. Finally, membranes were collected at the interfaces of the 2.25 and 1.65 M sucrose (bottom fraction) and the 1.65 and 1.10 M sucrose layers (top fraction) of the gradient, with a pipette, and diluted it once, with four volumes of buffer A. The suspension was centrifuged at 15800 rpm for 40 min JA-20 (Xiao, 2006). The pellet was resuspended in buffer B (25 mM imidazole, 50% (v/v glycerol)), and stored at -20°C.

2.3.1 Washing membrane proteins to remove the non-integral proteins:

10 µl of protein sample (~ 5 µg) was mixed with 10 µl of 6 M urea or 0.2 M sodium carbonate and kept on the ice for 10-20 min and centrifuged at 4 C° for 20 min at high speed in a microfuge (Kongprach et al., 2022). The supernatant (S) contained the proteins that were removed and it was collected, leaving the pellet (P) that contained the washed membranes.

2.4 Protein comparisons

The top and bottom fractions of the sucrose gradients from the three biological replicates of drug-free and chloramphenicol-treated cells did not contain unique sets of proteins and could not be analyzed separately (see results). Therefore, the protein data obtained from top and bottom fractions were combined for each condition and biological replicate, and intensities of corresponding top and bottom samples were summed to yield 3 no drug and 3 plus drug intensities per protein. All proteins that were not quantified in all 3 replicates of either condition were removed, as were all proteins with less than 2 unique spectral counts. Next, percent Coefficients of Variation (%CVs) were calculated for the plus drug and no drug replicates and all proteins

having a %CV greater than 30% in one of 2 sets (max %CV) were removed. The resulting reproducibly quantified proteins were analyzed, log2 differences and ratios were determined, as well as p-values. All proteins surviving a false discovery rate 10% upon multiple testing correction were marked as significant (i.e. 2 out of the 24 proteins are statistically assigned "significant" by chance). Two more proteins were quantified in all 3 no-drug but not detected in single plus drug sample. These were also noted.

2.5 Whole cell protein extraction with Trizol:

The second approach was to isolate whole cell proteins using Trizol (Invitrogen). Wild-type BY4741 *S. cerevisiae* was streaked on a YPG plate, which selected for mitochondrial respiration. Isolated colonies were used to inoculate 2 25-ml flasks of YPEG, which were incubated for 15-17 hr at 30° C in a shaker incubator. The yeast were grown in the presence or absence of 1.5 mg/ml chloramphenicol; one flask received the drug and the other an equivalent volume of ethanol without the drug as a control and after 17 hr 180 µl of each culture, and of control media, were put into the wells of a 96 well plate and put in 96 well reader at 30° C. OD_{630nm} was measured and cells from 33 wells were collected from the 96 well-plate during maximum growth after 8-10 hrs. The remaining samples were incubated for 48 hours and OD_{630nm} was recorded. The cells collected from each condition were pooled and centrifuged at 300 g for 5 min. The supernatants were removed and then, 1 ml of Trizol and 50 µl of glass beads were added to samples and they were vortexed for 10-15 min, and centrifuged 12000 g at 4° C for 15 min. Next, the supernatant was transferred to new tubes and 200 µl of chloroform were added and agitated vigorously. After that, samples were put at room temperature for 30 min to 1 hr and centrifuged 12000 g for 15 min at 4° C to separate the aqueous phase containing RNA; after removing RNA, 300 µl of 100% ethanol

were added and mixed by repeated inversion and left 2-3 min at room temperature. DNA was sedimented by centrifuge at 14000 rpm at 4° C. Next, protein was precipitated with 1.5 ml of isopropanol for each tube and left for 10 min at room temperature (RT). Samples were centrifuged at 12000 g for 10 min at 4° C. After removing supernatant and washing pellet, 2 ml of acetone were added to samples. Having been vortexed at least 1 min, samples were centrifuged at 10000 rpm for 10 min. Then, the protein pellets were vortexed with 2 ml of ethanol 100 % and centrifuged 16000 xg for 10 min. Supernatants were removed and the pellets were dried by leaving the tubes open at room temperature for 10 min. Finally, samples were dissolved in 300 µl of 1% SDS and put into a heat block for 15 min at 55° C and then centrifuged at 10000 rpm for 10 min at 4° C (Hummon et al., 2007). The supernatants were transferred to new tubes and put at -20° C to be run on the gel or precipitated for proteomics.

These samples were sent to the University of Regina for LC-MS/MS analyses by Dr. Tzu-Chiao Chao. The samples had to be precipitated to remove SDS. To do this, 200 µl of sample was transferred in a 2 ml tube, followed by the addition of 800 µl methanol, 200 µl chloroform, and 600 µl water and were mixed. The samples were centrifuged at 14,000 g for 5 min, and the supernatant was discarded. Then, 600 µl ethanol was added to the pellet and mixed. The samples were centrifuged again at 14,000 g for 10 min, and the pellets were dried (Yang et al., 2020). All peptides were resuspended in the buffer of LC-sample (1% (vol/vol) acetonitrile, 0.1% (vol/vol) formic acid) supplemented with 40 fmol/mL phosphorylase B Hi3 (Waters Corporation, Milford, MA) as an internal standard. Each sample was analyzed by a Waters Acquity UPLC M-Class system coupled to a Waters Synapt XS HDMS. Then, samples were loaded into a Waters Acquity UPLC Symmetry C18 trap column (180 µm x 20 mm, particle size 5 µm) in order to desalt and

chromatographically focus peptides for 3 min (5 μ L/min flow rate) prior to elution onto the Waters Acquity UPLC M-Class HSST3 analytical column (75 μ m x 200 mm, particle size 1.8 μ m). Peptides were separated via a 120-min gradient from 3% acetonitrile/ 0.1% formic acid to 45% acetonitrile/0.1% formic acid) at a flow rate of 0.35 μ L/min and again analyzed in positive HDMSE resolution mode with 0.9 second scan time. In low-energy MS mode, all data were collected at a constant collision energy of 4 eV; however, high-energy collision was ramped between 18 and 42 V. The resulting raw spectra were processed with the ProteinLynx Global Server (PLGS) v. 3.02 with Identity (Waters) and compared. Next, against a *S. cerevisiae* (S288c) proteome sequence (Uniprot, downloaded 2024). Protein abundance was measured with PLGS using the Hi3 method. Data were provided in form of list of proteins, PLGS Score, Peptides, Theoretical Peptides, Coverage (%), Precursor RMS Mass Error (ppm), Products, Digest Peptides, Modified Peptides, Products RMS Mass Error (ppm), Products RMS RT Error (min), Amount (fmol) and Amount (ngrams) (Appendix 2; yeast samples from Regina ; Silva et al., 2006).

The location of transmembrane proteins was found by the *Saccharomyces* Genome Database (SGD) (Chervitz et al., 1999) and transporter database (<https://www.membranetransport.org/transportDB2/index.html>) was used to identify transporters (Islam., 2023). The intracellular location of proteins also was determined by comparing them to datasets for mitochondria (Vögtle et al., 2017), endoplasmic reticulum (Szopinska et al., 2011), nucleus (Huh et al., 2003) and vacuoles (Huh et al., 2003). The overexpression or downregulation of proteins in the presence of chloramphenicol were considered by measuring fold change and using a t-test to calculate the p value of 3 technical replicates.

2.6 Other methods

2.6.1 Bradford protein assay:

To generate the standard curve for protein concentration, 100 μ l Milli-Q water and 900 μ l of Bradford Dye Reagent (Thermo Fisher Scientific) was added to 8 1.5-ml cuvettes. Sample 1 contained no added protein and was the blank. 2 μ l of 0.25, 0.5, 1.0, and 2.0 mg/ml of BSA were added to tubes 2, 3, 4, and 5, respectively and 1 μ l, 2 μ l, and 4 μ l of the membrane protein sample were added to tubes 6, 7 and 8, respectively. Concentrations were determined from the standard curve (Kruger, 2009). The concentration of protein in the membrane fractions ranged from 1.5 to 4.0 mg/ml.

2.6.2 Loading samples to the gel:

20 μ g of protein were loaded into wells of Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels to be separated based on their weights.

Based on the concentration of proteins, samples were loaded into the gel. 5 μ l of marker (Thermo Scientific 26610) was loaded into the first well. Protein samples with total volumes of 20 μ l were generated by adding 10 μ l of 2x Laemmli Sample Buffer (Bio Rad) loading buffer to 10- μ l mixtures that contained varying ratios of protein sample and buffer (Kielkopf et al., 2021).

2.6.3 Making gel for separating proteins by SDS- PAGE:

A 15 % acrylamide gel was prepared by mixing 10 ml distilled H₂O, 360 μ l 10% SDS, 150 μ l Ammonium Persulfate (APS 10 %), 7.5 ml of 1.5 M Tris (pH 8.8) 11.3 ml 30% acrylamide (BioRad Cat. No. 1610154), and 15 μ l Tetramethylethylenediamine (TEMED) in a 50-ml Falcon tube. This volume was used to pour 6 casts, and 500 μ l isopropanol was added to the surface of

the separating gel to remove the bubbles. After 30 min, the isopropanol was removed and the stacking gel was added. To make these, 6 ml Milli-Q water, 2.25 ml 0.5 M Tris (pH 6.8), 100 μ l 10% SDS, 1.33 ml 30% acrylamide, 10 μ l TEMED, 50 μ l APS 10 % were mixed and poured in the cast and the comb was added. After 20 min when they became solidified, the gels were stored at 4° C until used. This method was adopted from (Jember, 2021).

Gels were run in standard running buffer (25 mM Tris, 19.2 mM Glycine, 0.1% SDS, pH 8.3), for 40 min at 150 V (Kielkopf et al., 2021) . After running, gels were stained in a solution of Coomassie Blue (0.25 % w/v), 50% (v/v) methanol and 10% (v/v) acetic acid, followed by destaining in 60% (v/v) methanol, 10% (v/v) acetic acid (Motojima, 2018).

CHAPTER 3: RESULTS

3.0 Objectives:

To identify membrane protein pumps in *S. cerevisiae* that mitigate the impacts of mitochondrial translation inhibitor to describe their overexpression or downregulation, focusing on proteins from the ABC and MFS families in whole membrane of the *S. cerevisiae* cell by proteomics.

3.1 Effect of chloramphenicol on the growth of the yeast on YPG media:

According to growth kinetics graph of the wild type BY4741 yeast in the presence of diverse concentrations of chloramphenicol in small scale (fig 3.1), the growth of the yeast without chloramphenicol was higher than the samples containing chloramphenicol. The slowest growth was for the sample with 1.5 mg/ml chloramphenicol, which was the highest concentration tested, so chloramphenicol decreased the growth rate of the yeast and the maximum OD reached on YPG media. The sample with the concentration of 0.75 mg/ml showed intermediate growth and was chosen for further study.

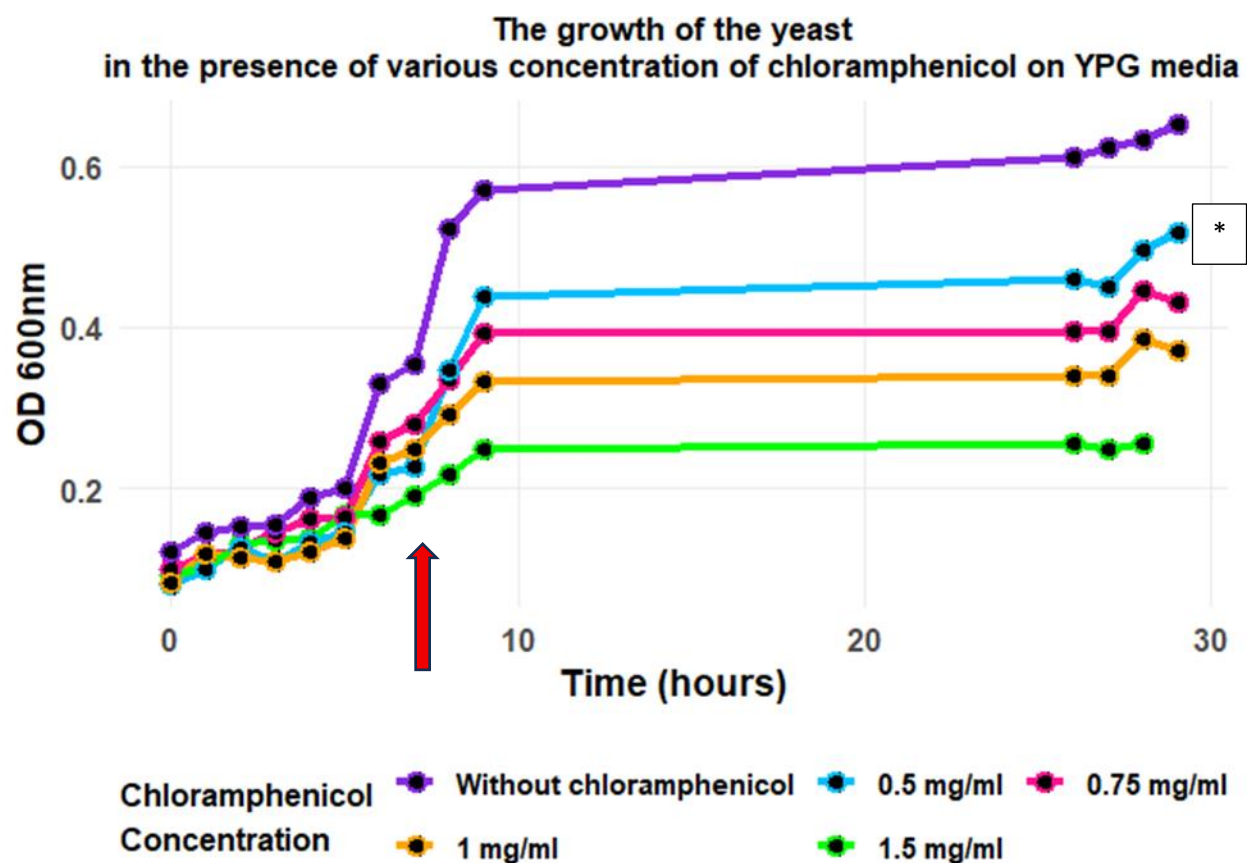


Fig 3.1: The growth of *S. cerevisiae* in the presence of various concentrations of CAP in YPG media. Various concentration of chloramphenicol decreases the growth of the yeast and cell were collected after 8 hr and after extraction were sent to Manitoba Centre for Proteomics and Systems Biology (MCPSB). The line graph depicts how different concentrations of drug affect the growth of the yeast and the arrow indicates when samples were taken. First, the average of 3 technical replicates was considered, then standard deviation of samples for 7 hours when samples were in maximum growth was calculated. The standard deviation of sample 1 without drug was 0.2, sample

2 with 0.5 mg/ml = 0.169, sample 3 with 0.75 mg/ml = 0.178, sample 4 with 1 mg/ml = 0.169, and sample 5 with the highest concentration of chloramphenicol (1.5 mg/ml) was 0.2. Intermediate concentration showed that yeast could grow in the presence of 0.75 mg/ml of CAP but the growth was slower than in the absence of CAP. The intermediate concentration showed that yeast could grow in the presence of 0.75 mg/ml of CAP but the growth was slower than in the absence of CAP based on the slope and the t-test. Three biological and three technical replicates were considered and the average of them was calculated for making the graph. The protein samples were collected after 7 hours as red arrow can show it and the star shows that which sample was sent to MCPSB.

Table 3.1: Slopes of samples grown in the presence of different concentrations of chloramphenicol.

Slopes were determined from the region of maximal growth on the curves shown in Fig. (3.1).

Concentration of chloramphenicol	Slopes (OD/hr)	p-value from t-test comparing to samples without CAP	p-value from t-test comparing to samples with 0.75 mg/ml CAP
0 mg.ml	0.07	n/a	0.33
0.5 mg/ml	0.04	0.12	0.44
0.75 mg/ml	0.04	0.33	n/a
1.0 mg/ml	0.04	0.21	0.68
1.5 mg/ml	0.02	0.03	0.11

According to the t-test results for the maximum growth rates of samples with different concentrations of chloramphenicol, comparisons between all samples and both the control sample and the 0.75 mg/mL chloramphenicol condition indicated no statistically significant differences. In addition, slopes show that sample with the highest concentration of CAP had the lowest slope as the sample in the absence of chloramphenicol had more slope in comparison with samples

having various concentrations of chloramphenicol, so the best concentration was 1.5 mg/ml of chloramphenicol to decrease the growth of the yeast.

Table 3.2. Final OD of samples in grown in the presence of chloramphenicol. Final OD were determined from the curves shown in Fig. (3.1).

Sample numbers with their concentration of chloramphenicol	Final OD (630 nm)	p-value from t-test comparing to samples without CAP	p-value from t-test comparing to samples with 0.75 mg/ml CAP
0 mg.ml	0.65	n/a ¹	0.03
0.5 mg/ml	0.37	0.03	0.6
0.75 mg/ml	0.43	0.03	n/a
1.0 mg/ml	0.51	0.06	0.3
1.5 mg/ml	0.26	0.006	0.13

The t-test results for the final OD show that all concentrations of chloramphenicol (CAP) have a significant difference compared to the 0 mg/ml control, except for the sample with 1 mg/ml concentration which was not significant. In contrast, when comparing the final OD values of all CAP concentrations against the 0.75 mg/ml group, only sample with 0.75 mg/ml concentration

shows a significant and other samples were not significant. Moreover, the highest OD was for the samples without chloramphenicol and low concentration of chloramphenicol.

3.2 Test proteomics run of membrane fractions

To determine the efficiency of our procedures, we extracted membrane proteins from cells grown in the absence of chloramphenicol and a sample from the bottom layer of the gradient were sent to the MCPSB to identify proteins in the membranes and potentially efflux pumps in various organelles. A sucrose gradient can be used to separate membrane proteins and we expected that the bottom layer contained plasma membrane and top layer contained both mitochondrial and plasma membranes together (Bermejo et al., 2014).

3.2.1 Sucrose gradient fractions:

The fractions from the bottom layer and the top layer of the resulting sucrose gradients (fig. 3.2) were run on an SDS-PAGE gel and both bottom layer and top layer were similar (fig. 3.3), suggesting that the sucrose gradient did not separate the different membranes well.

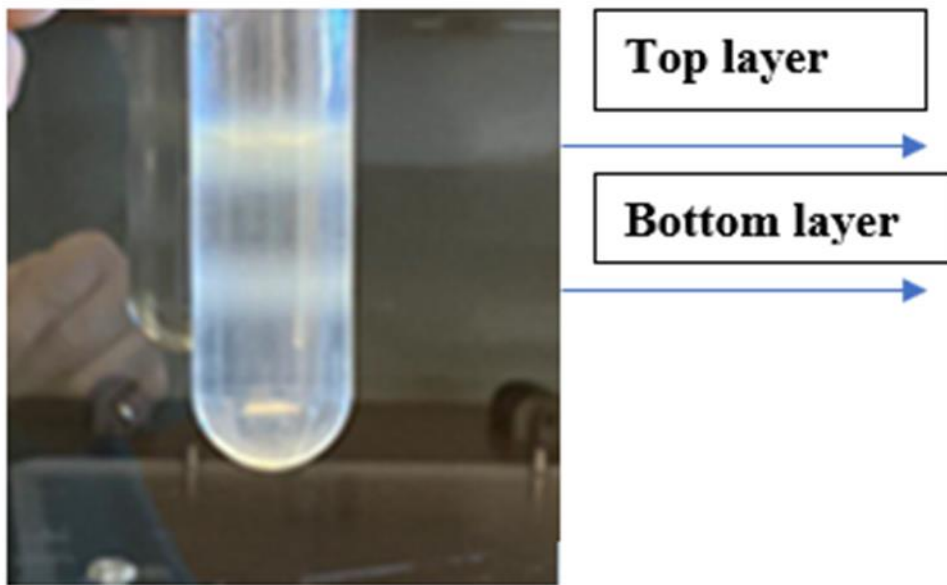


Fig. 3.2: The separation of yeast membranes by sucrose gradient. The gradient was run as described in Chapter 2. The top layer was expected to contain mitochondrial and plasma membrane proteins, and the bottom layer was expected to be highly enriched for plasma membrane proteins.

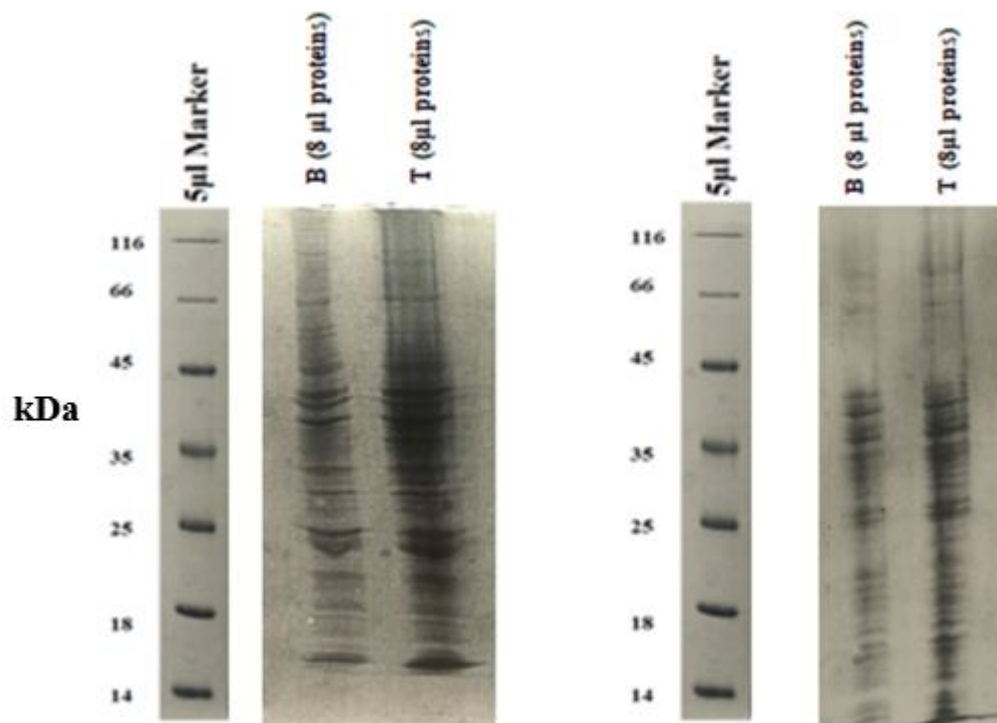


Fig 3.3: SDS-PAGE analysis of proteins in the top and bottom fractions of the sucrose gradient on the gel. 4 µg of protein were added to each lane and while the left side shows the protein samples from cells in the absence of chloramphenicol, on the right-side, protein samples were collected in the presence of chloramphenicol. The molecular weights of standard proteins are shown to the right of each panel (in kDa).

3.3 Proteomics analysis of the test protein sample from the bottom layer:

The LC-MS/MS steps and the bioinformatics analyses were carried out in the Manitoba Center for Proteomics and System Biology (MCPBS) by Tenny Lao and Dr. Rene Zahedi.

The total number of proteins detected by LC-MS/MS was 1474. About 25% of the total proteins predicted to be found in yeast *S. cerevisiae* were detected. The intracellular locations of proteins in various organelles were predicted from information in the *Saccharomyces* genome database (SGD, Hu et al. 2003) and searching other researcher's lists of proteins (see fig (3.4)). For instance, the number of proteins in nucleus, Golgi, and vacuole was compared with data from Huh et al., 2003 and the number of proteins in ER as well as plasma membrane was compared with Szopinska et al., 2011. Mitochondrial proteins were compared with the research of Vögtle et al., 2017 who identified the detailed locations of the proteins in mitochondria (fig. 3.4). Transporters also were found by Transmembrane Hidden Markov Model (TMHMM) and 10 transporters were identified in the bottom layer and 8 transporters in the top layer and 5 transporters were same in both layers. Surprisingly, most of the proteins detected in our study were cytosolic rather than membrane associated (fig. 3.5), and after the cytosol, most proteins were in mitochondria membranes.

The number of detected proteins by LC-MS/MS in various organelles

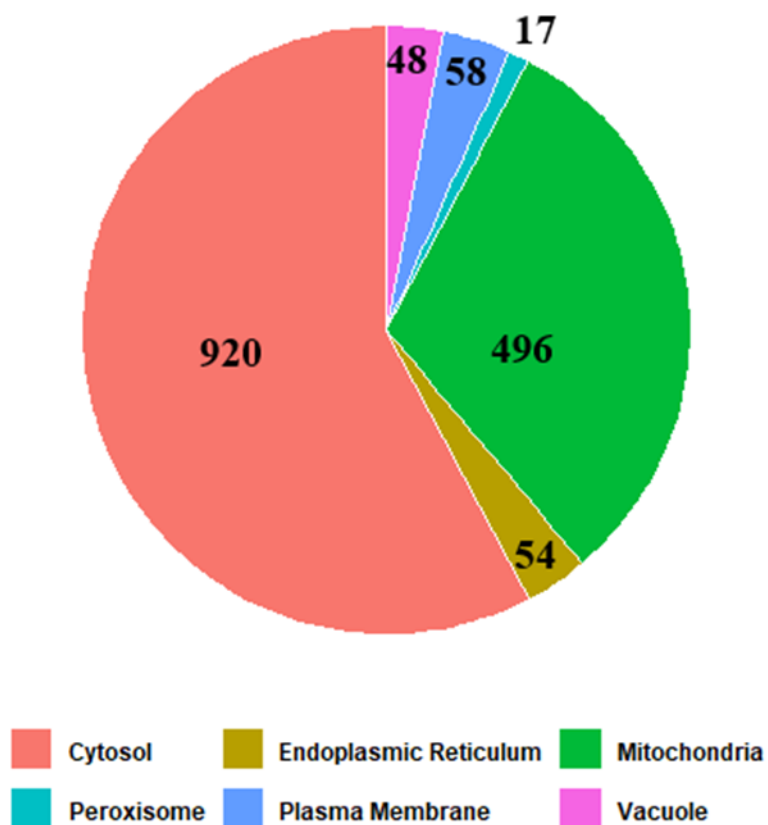


Fig. 3.4: The number of proteins in the test sample. Proteins were isolated from cells grown without chloramphenicol and not treated by washing by sodium carbonate. The pie chart shows how many proteins were detected from various organelles of the cell. Among 1474 proteins, 920 were in the cytosol, 496 proteins were in mitochondria, 54 were in endoplasmic reticulum, and the rest of them were in vacuole, plasma membrane, and peroxisome with 48, 58, 17 respectively. References for comparisons are provided in the text.

The Number of Proteins in the Mitochondria

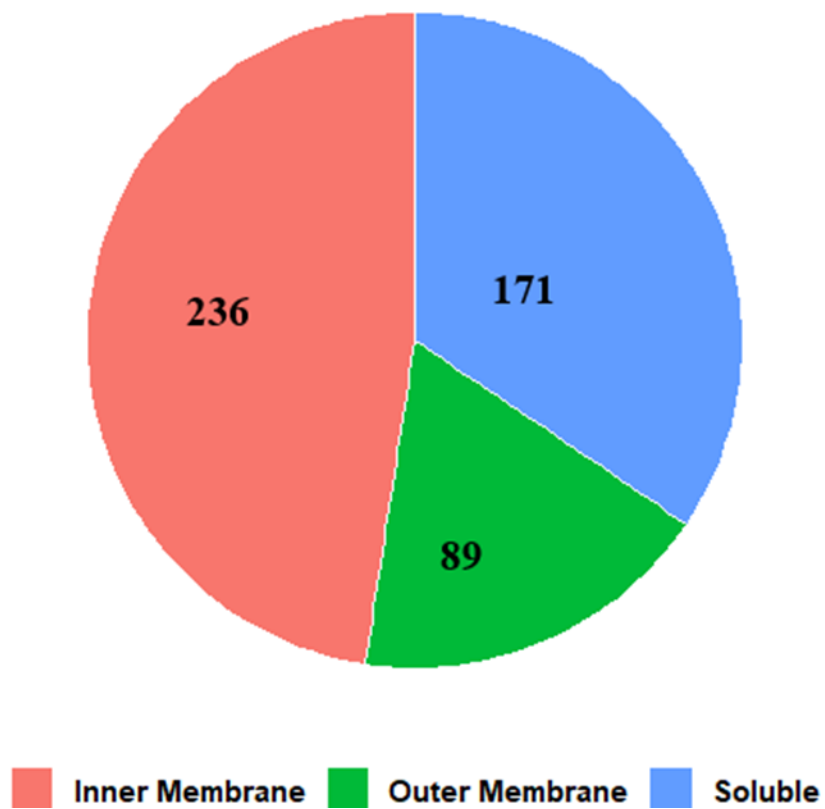


Fig 3.5: The location of mitochondrial proteins in the test cells that were grown without chloramphenicol and without washing. The pie chart shows that among 496 proteins, the highest number of proteins was in the inner membrane with 236, and the lowest number of proteins was in the outer membrane with 89, and 171 were soluble. The number of proteins were compared with the data of Vögtle et al., 2017.

3.4 Analysis of proteomes from cells grown in the presence and absence of CAP after washing

Based on the success of the trial run, an experiment was run to compare proteomes of cells treated with CAP, with those of untreated cells. Cell lysates were prepared, and membrane fractions were isolated by sucrose gradient centrifugation. Samples from the top and bottom layers of the gradient were washed with 0.1 M sodium carbonate to try to remove peripheral proteins and enrich for membrane proteins.

3.4.1 The results of protein samples:

Proteins from the bottom and top fractions of cells grown with and without chloramphenicol were run on SDS-PAGE gels (fig (3.6)). After washing membrane fractions with sodium carbonate solution, the membranes were collected in pellet, and the solubilized proteins were found in the supernatant. Most of the proteins were found in the pellets and the supernatants contained low amounts of proteins.

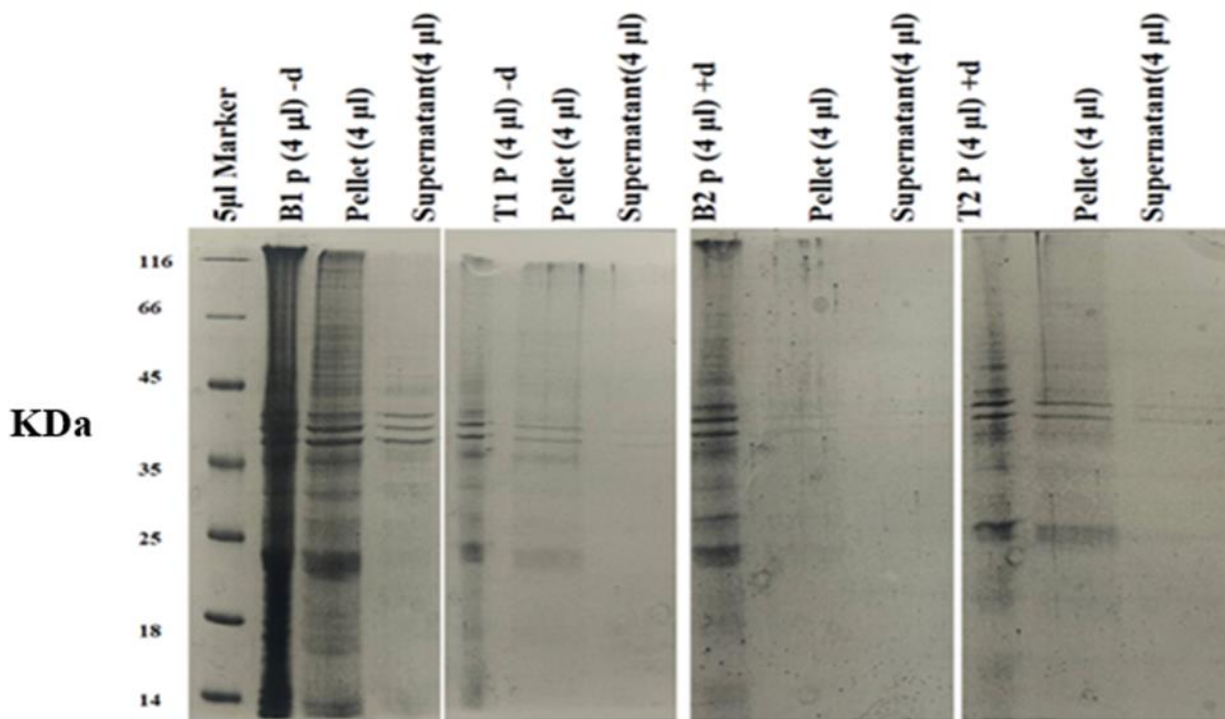


Fig 3.6: Proteins bottom layer (B) and top layer (T) pellets, and supernatant after washing with sodium carbonate, were run on the SDS-PAGE gel. On the left hand side B1 and T1 are proteins in the top and bottom fractions isolated from cells grown without chloramphenicol. Samples washed with carbonate are on the right hand side (pellet and supernatant). B2 and T2 are proteins from cells grown with chloramphenicol, after washing with carbonate. Two biological replicates are shown.

3.5 Analyzing membrane proteins by LC- MS/MS:

We extracted washed protein samples from cells treated with chloramphenicol 1.5 mg/ml or without chloramphenicol and sent them to MCPSB to identify proteins in the membranes and

potentially efflux pumps (Wang & Liang, 2012; Li et al., 2017). Three biological replicates of each condition were sent.

3.5.1 Proteomics analysis of carbonate-washed samples from cells treated with or without CAP:

The datasets from the 12 samples were compared to confirm whether the biological replicates of each condition were similar. Unfortunately, the samples with or without CAP, and those of the top and bottom layers did not cluster with each other (fig. (3.7)). Statistical analysis of the top and bottom fraction data (fig. (3.8B)) did not reveal any statistically significant differences among the samples. Together these results suggest that the biological replicates of each condition were not sufficiently similar for comparisons.

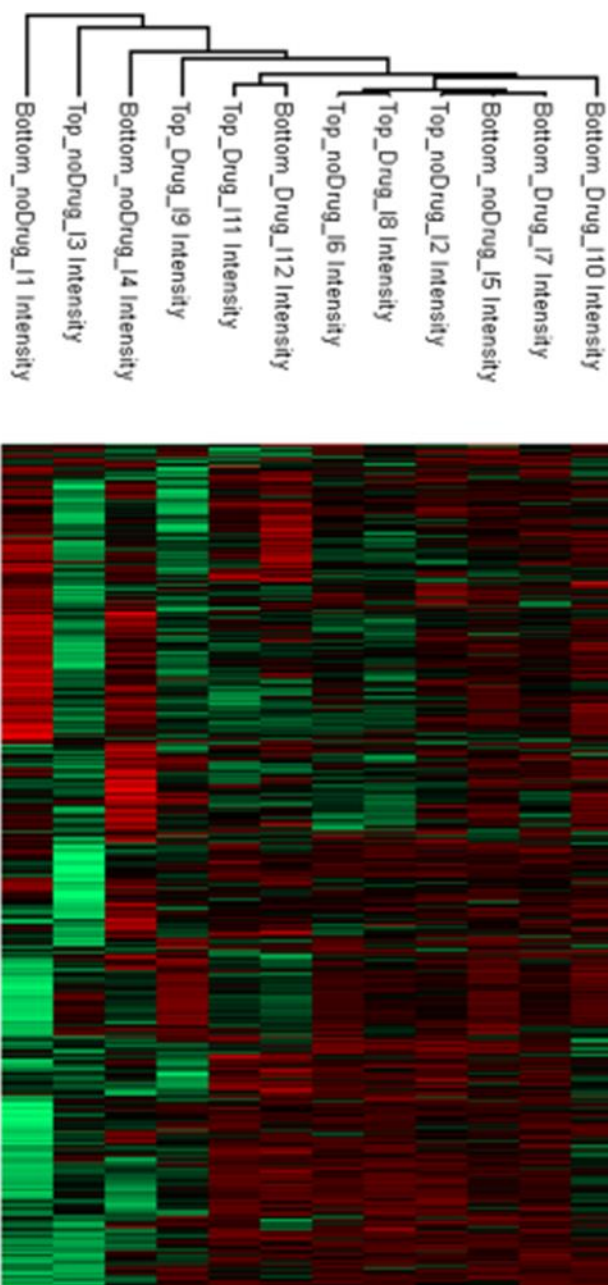


Fig. 3.7: Unsupervised clustering of protein intensities from the 12 samples analyzed. Note that the samples with and without drug do not cluster together, nor do the top and bottom bands of the sucrose gradient. Figure provided by R. Zahedi, MCPSB.

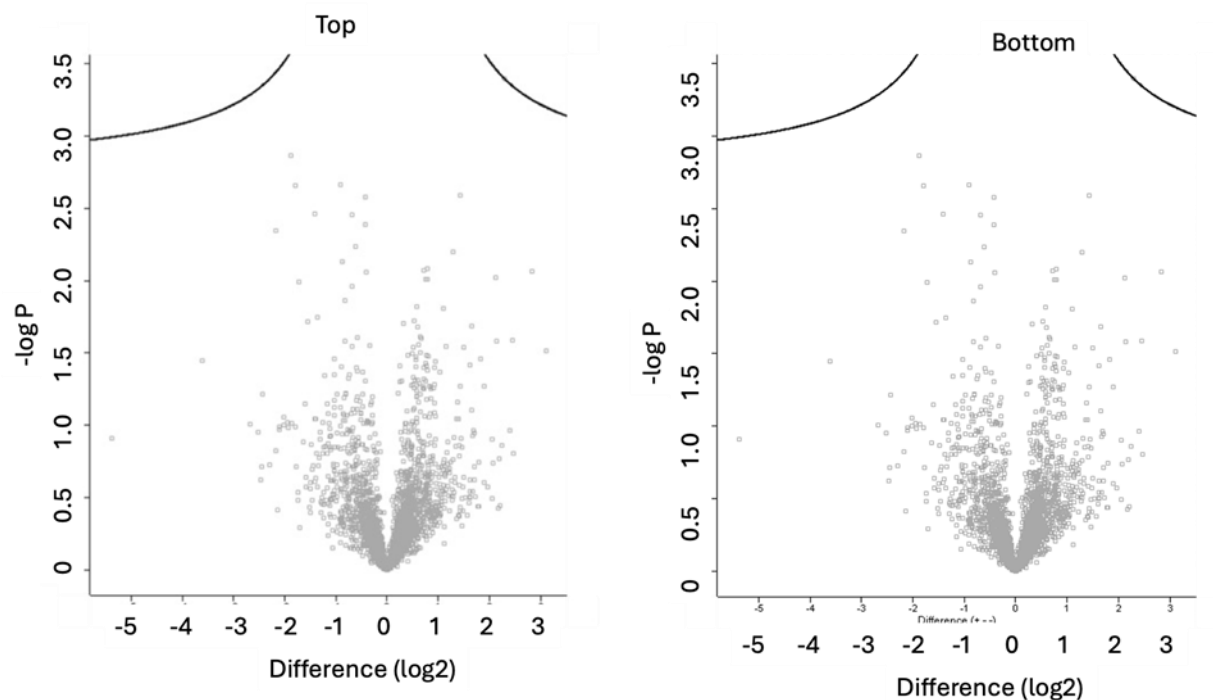


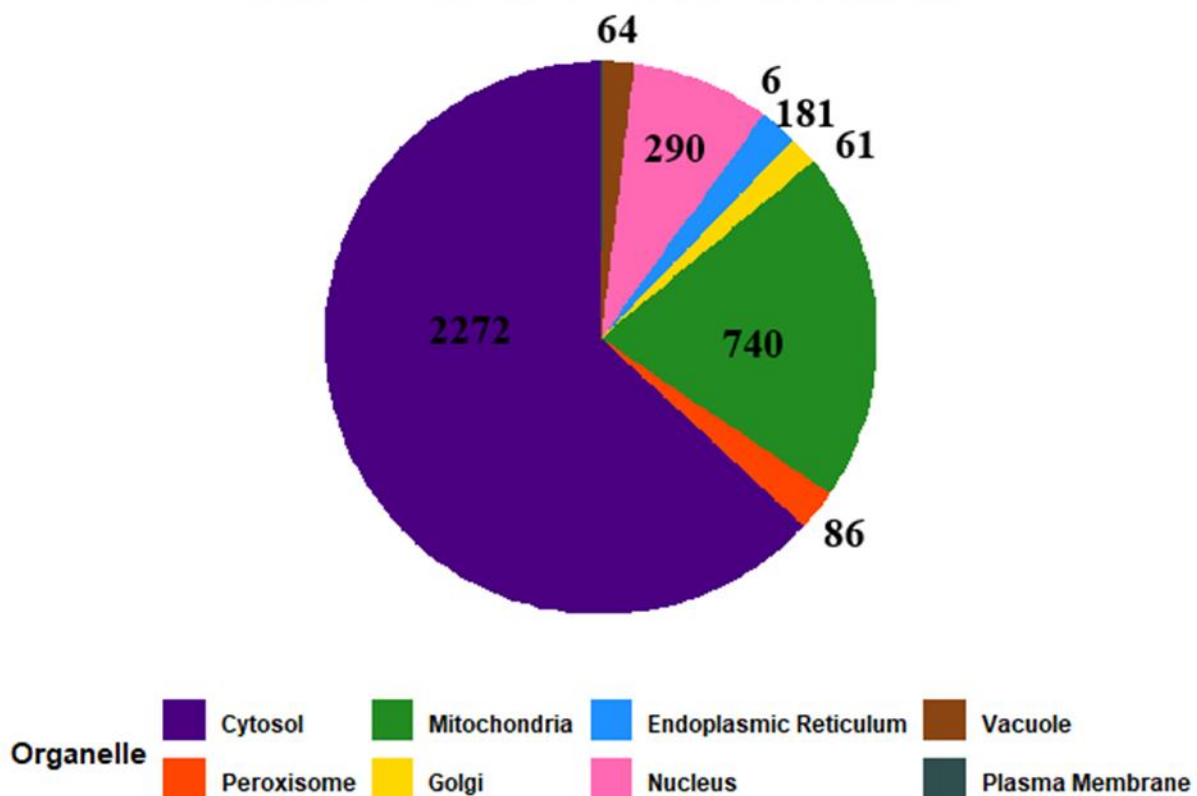
Fig. 3.8: Volcano plots from the combined samples. The left and right panels contain the data from the combined top and bottom layers, respectively. The black line depicts the cutoff for significant differences. It should be considered none of the proteins were present in statistically different amounts in the presence and absence of drug. Figure provided by R. Zahedi, MCPSB.

As a second approach, the proteomics data from the top and bottom fractions from all samples were pooled to assess the efficiency of membrane protein extraction and enrichment. The total number of proteins detected by LC-MS/MS in the combined top layers (with and without chloramphenicol) were 3355, and 8 predicted transporters were predicted based on TMHMM, and their locations were predicted from information based on GFP tagging stored in the *Saccharomyces*

Genome Database (SGD). While in the bottom layers, with chloramphenicol and without chloramphenicol, from a total 3700 proteins, 10 were putative transporters. Fig. (3.9) represents the number of both bottom and top layer proteins detected from in various organelles. Based on the data, most proteins in both bottom and top layer were from the cytosol, mitochondria and nucleus, while the lowest number of them were in other organelles, especially plasma membrane.

A

The number of proteins detected by LC-MS/MS in various organelles (Bottom layer)



B)

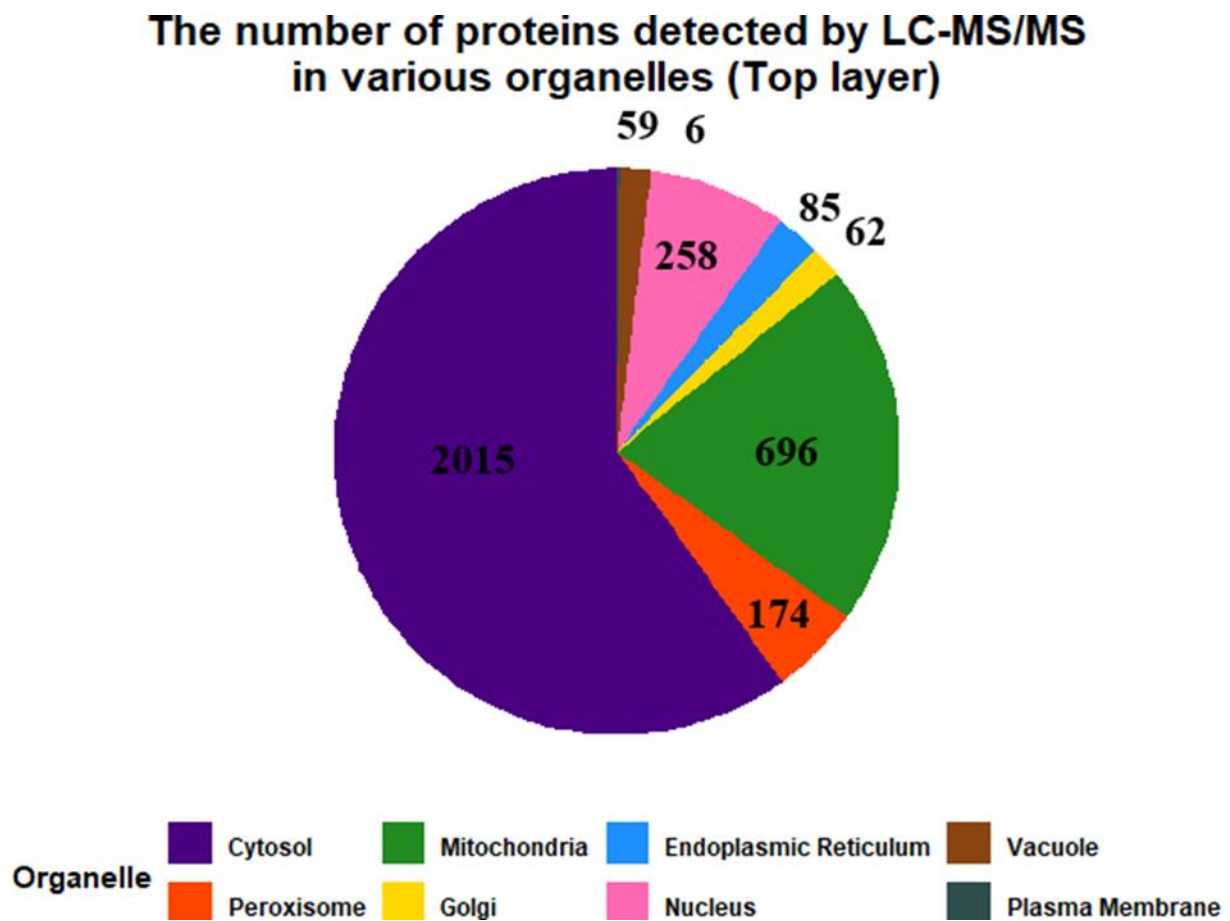
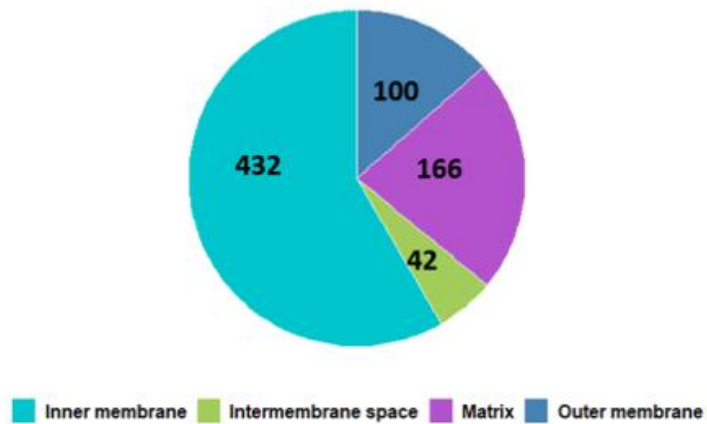


Fig 3.9 (A, B). The number of proteins in various organelles detected by LC-MS/MS from top and bottom layer. The pie charts demonstrate that how many proteins are in various organelles detected by LC-MS/MS from both with and without chloramphenicol after washing with sodium carbonate. Among total proteins, we found 258 in nucleus in top layer (B) and 290 in bottom layer (A) and

64 proteins were in vacuole in bottom layer and 59 in top layer. In bottom layer and top layer both there were 6 proteins. In Golgi, 61 proteins were in bottom layer and 62 in top layer and 85 were in peroxisome in bottom layer, and 86 in top layer and 181 proteins were ER in bottom layer and 174 in top layer. Moreover, we found 740 proteins in mitochondria in bottom layer and 696 in top layer and the remaining proteins were in cytosol. As the number of proteins was low, R studio could not show the number of plasma membrane proteins (6) on the pie chart.

We are interested in mitochondrial proteins, because the drug chloramphenicol acts on mitochondrial ribosomes. Therefore, we examined the location of the mitochondrial proteins in more detail (fig. (3.10)). Among all proteins in mitochondria most proteins were in the inner membrane and the lowest number of proteins detected were in intermembrane space, so most of them were located in inner membrane in both bottom and top layer. In summary, there were not big differences between the protein content of the top and bottom layer and most proteins identified were from the cytosol.

The Number of Proteins in Various Parts of Mitochondria (Bottom Layer)



The Number of Proteins in Various Parts of Mitochondria (Top Layer)

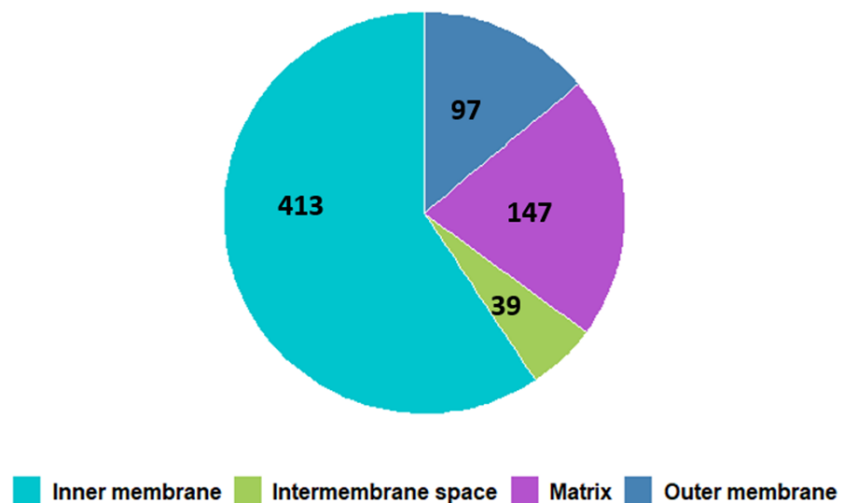


Fig 3.10 (A, B). The number of proteins in various parts of mitochondria identified in the bottom (B) and top (T) layer. The pie charts show that the highest number of proteins from total proteins 696 from top layer were in IM with 413, however, the lowest amount was for IMS with 39. In addition, 97, 147 proteins were in OM and matrix respectively. However, the highest number of proteins from bottom layer from total 740 mitochondrial proteins in IM was 423, the lowest amount was for IMS was 42, and 100, 166 were in matrix and OM respectively. Data from one biological replicate is shown.

Table 3.3: The number of transporters in both top and bottom and their function layer from the sucrose gradient experiment in the presence of chloramphenicol.

Transporters	Bottom layer	Top layer	Function
Agp2	Y		Plasma membrane regulator of polyamine and carnitine transport; has similarity to transporters but lacks transport activity; may act as a sensor that transduces environmental signals; has a positive or negative regulatory effect on transcription of many transporter genes
Avt1	Y	Y	Vacuolar transporter; imports large neutral amino acids into the vacuole; member of a family of seven <i>S. cerevisiae</i> genes (<i>AVT1-7</i>) related to vesicular GABA-glycine transporters
Avt3	Y	Y	Vacuolar transporter.
Avt4	Y		Vacuolar transporter; exports large neutral amino acids from the vacuole; member of a family of seven <i>S. cerevisiae</i> genes (<i>AVT1-7</i>) related to vesicular GABA-glycine transporters; localizes to sites of contact between the vacuole and mitochondria (vCLAMPs)
Avt7	Y	Y	Vacuolar transporter; exports large neutral amino acids from the vacuole; member of a family of seven <i>S. cerevisiae</i>

			genes (<i>AVT1-7</i>) related to vesicular GABA- glycine transporters
Mep1	Y		Ammonium permease; belongs to Mep-Amt-Rh family of well-conserved ammonium (NH_4^+) transporters that includes the human Rh factors; expression is under the nitrogen catabolite repression
			regulation; activity regulated by TORC1 effectors, Npr1p and Par32p; human homolog RHCG complements yeast null mutant; mutations in human homolog RHCG implicated in metabolic acidosis
Pho89	Y	Y	Plasma membrane Na^+/Pi cotransporter; active in early growth phase; similar to phosphate transporters of <i>Neurospora crassa</i> ; transcription regulated by inorganic phosphate concentrations and Pho4p.
Pxa1	Y	Y	Subunit of heterodimeric peroxisomal ABC transport complex, with Pxa2p; required for import of long-chain fatty acids into peroxisomes
Si1	Y		Ferrioxamine B transporter; member of the ARN family of transporters that specifically recognize siderophore-iron

			chelates; transcription is induced during iron deprivation and diauxic shift; potentially phosphorylated by Cdc28p
Ygl11w	Y		Putative protein of unknown function; predicted member of the oligopeptide transporter (OPT)
Ybt1		Y	Transporter of the ATP-binding cassette (ABC) family; involved in bile acid transport; negative regulator of vacuole fusion; regulates release of luminal Ca ²⁺
Ypt1	Y		Transporter of the ATP-binding cassette (ABC) family; involved in bile acid transport; negative regulator of vacuole fusion; regulates release of luminal Ca ²⁺ stores;

3.6 Whole cell proteomics:

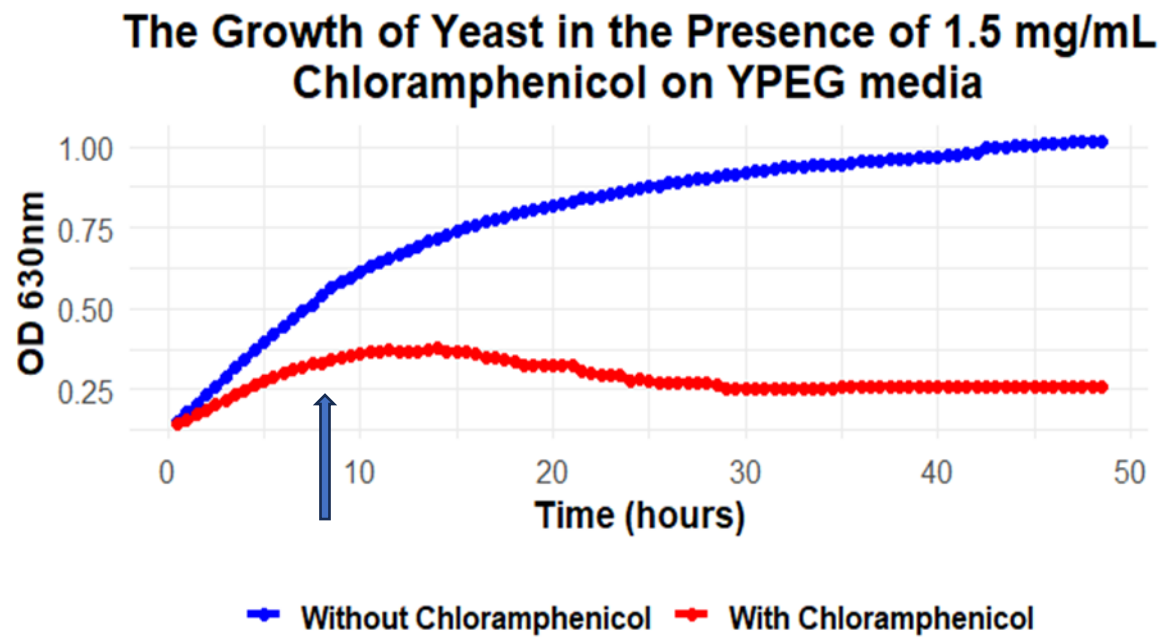
Due to the difficulties in the previous approach, we decided to use whole cell protein extracts. Proteins were extracted by Trizol solution to save the time and expense. We also decided to grow cells in a 96-well plate, so that the growth phase could be followed better. The Trizol method requires a smaller sample of cells, so this was possible. The medium was changed to YPEG, which contains 3% ethanol and increases the growth of yeast (Zhang et al., 2019).

Wild-type BY4741 *S. cerevisiae* was grown from a yeast extract peptone glycerol plate (YPG), which selects for mitochondrial respiration and was cultured in 2 25-ml flasks of YPEG for 15-17

hr at 30° C in a shaker incubator. One flask contained 1.5 mg/ml of chloramphenicol, and the other received an equivalent volume of ethanol without the drug as a control.

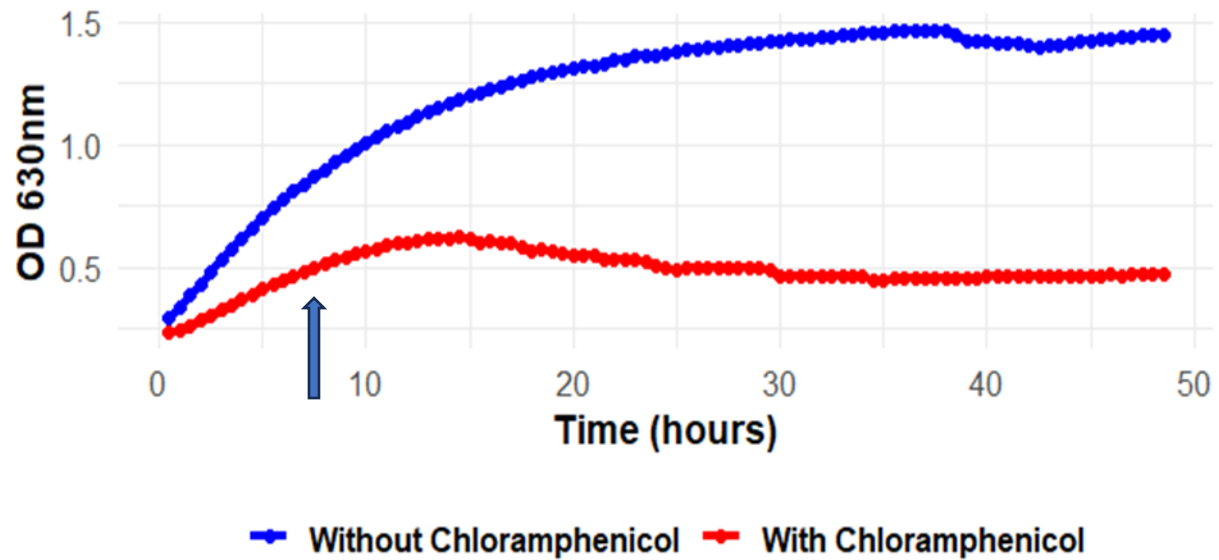
The cultures were then distributed to 88 wells in a 96-well plate, so their growth could be measured every 30 min (fig. (3.11)). The cells were grown at 30 ° C and after 8 hr they were collected for protein extraction when the difference in growth pattern was clear between the samples with and without chloramphenicol.

A



B

The Growth of Yeast in the Presence of 1.5 mg/mL Chloramphenicol on YPEG media



C

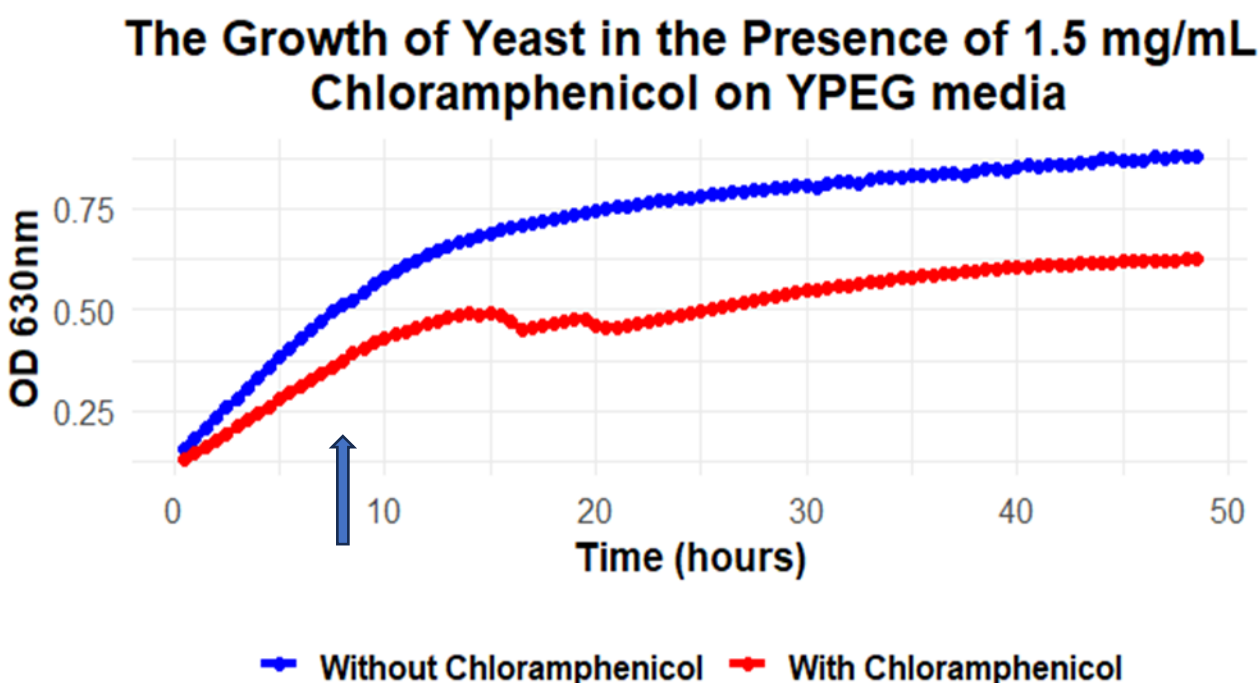


Fig 3.11. The growth of the wild-type BY4741 in the presence (red line) or absence (blue line) of 1.5 mg/ml chloramphenicol on YPEG media. Each panel (A, B, C) shows a different biological replicate and the average of 3 technical replicates is plotted on each graph. The results of T-test for final OD showed that just graph B is significant. After 8 hr cells were collected for protein extraction in all 3 experiments. Chloramphenicol decreased the growth of the yeast in all replicates. Slopes of samples grown in the presence of 1.5 mg/ml concentrations of chloramphenicol. Slopes were determined from the region of maximal growth on the curves shown in Fig. (3.1).

Table 3.4. Shows samples' final OD with the concentrations of 1.5 mg/ml of chloramphenicol

	Slopes (OD/hr)	p-value from t-test comparing to samples without CAP (A)
Conc. CAP (0 mg/ml) Graph A	0.01	n/a ¹
Conc. CAP (1.5 mg/ml) Graph A	0.05	1.41×10^{-4}
Conc. CAP Graph B (0 mg/ml)	0.01	n/a
Conc. CAP Graph B (1.5 mg/ml)	0.06	1.97×10^{-11}
Conc. CAP Graph C (0 mg/ml)	0.05	n/a
Conc. CAP Graph C (1.5 mg/ml)	0.03	1.33×10^{-7}

According to the results of t-test of maximum's growth, samples are significant statistically.

Table 3.5. Shows samples' final OD with the concentrations of 1.5 mg/ml of chloramphenicol.

	Slopes (OD/hr)	p-value from t-test comparing to samples without CAP (A)
Conc. CAP (0 mg/ml) Graph A	0.06	n.a ¹
Conc. CAP (1.5 mg/ml) Graph A	0.03	$10^{-0.6}$
Conc. CAP Graph B (0 mg/ml)	0.1	n/a
Conc. CAP Graph B (1.5 mg/ml)	0.05	7×10^{-4}
Conc. CAP Graph C (0 mg/ml)	0.5	n/a
Conc. CAP Graph C (1.5 mg/ml)	0.04	$1.32 \times 10^{-0.5}$

3.7 The results of protein extraction by Trizol kit:

Proteins were isolated from cells collected after 8 hours growth with or without CAP and analyzed by SDS-PAGE (fig (3.12)). High molecular weight proteins were detected in all samples, indicating that intact proteins were isolated.

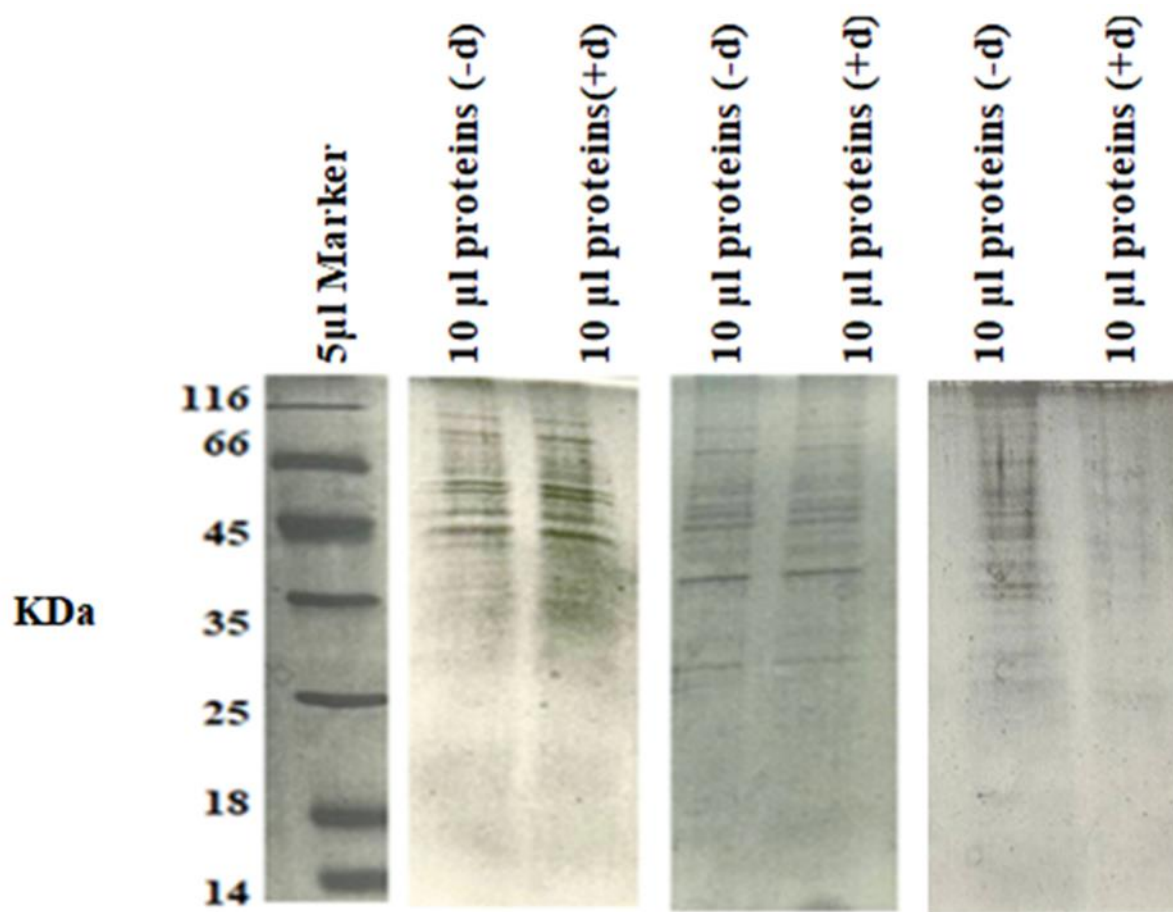


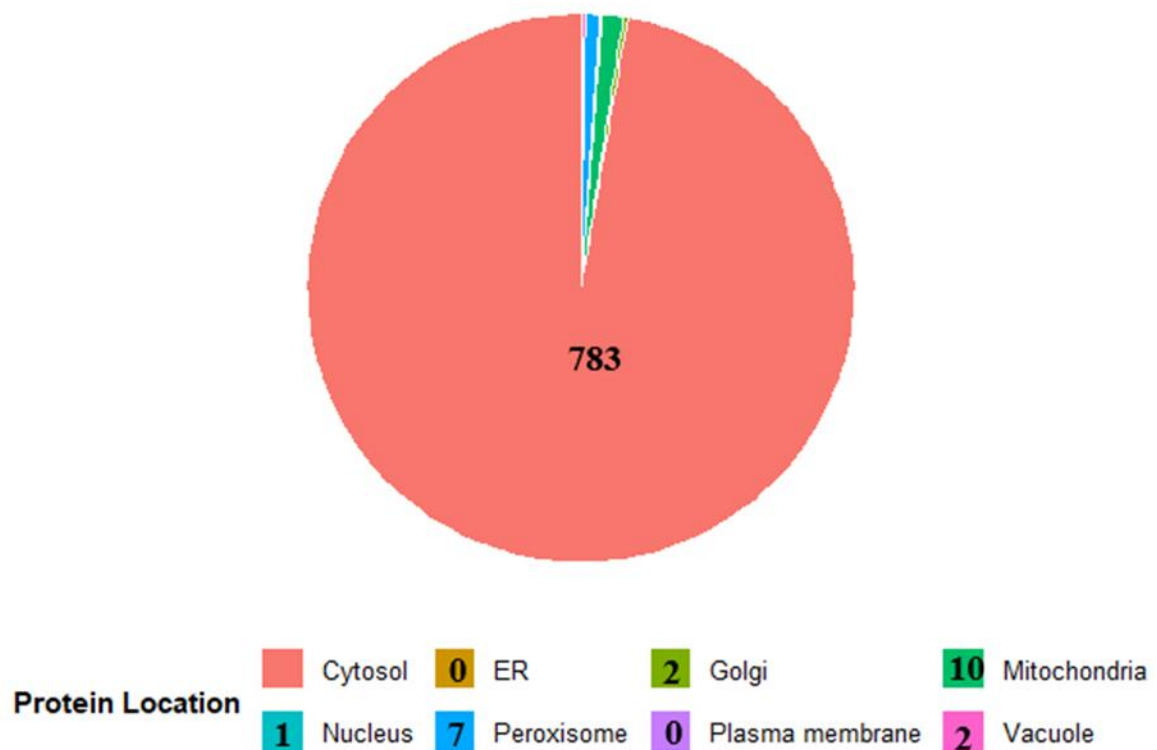
Fig 3.12. SDS-PAGE analysis of protein samples. Proteins without (-d) and with (+d) chloramphenicol were run on the SDS-PAGE gel after extraction by Trizol. 3 biological replicates are shown.

3.8 Results of proteomics data of extracted proteins by Trizol:

Samples were sent to the University of Regina for proteomics analysis. Many cytosolic proteins were in samples with and without chloramphenicol and a few of them were from mitochondria and other organelles. Fig (3.13) depicts the number of proteins in various organelles.

A

**Number of extracted proteins with chloramphenicol by TRIZOL
Detected by LC-MS/MS**



B

**Number of extracted proteins without chloramphenicol by TRIzol
Detected by LC-MS/MS**

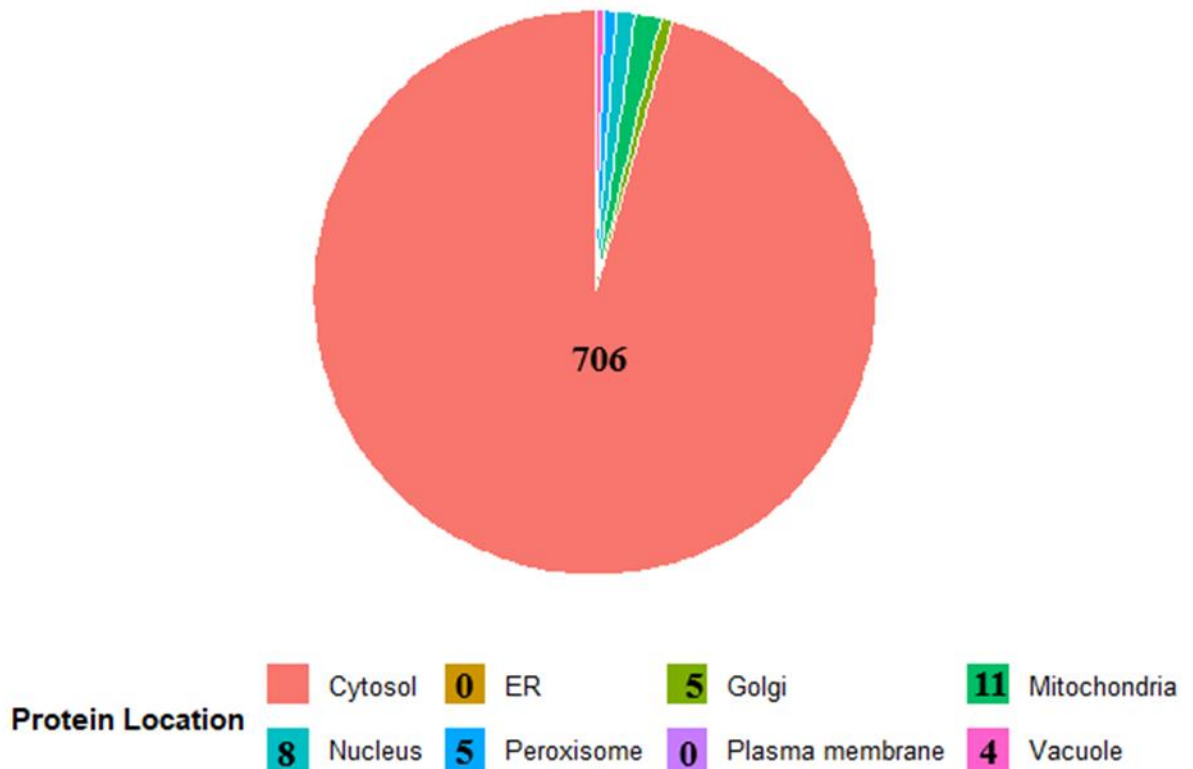
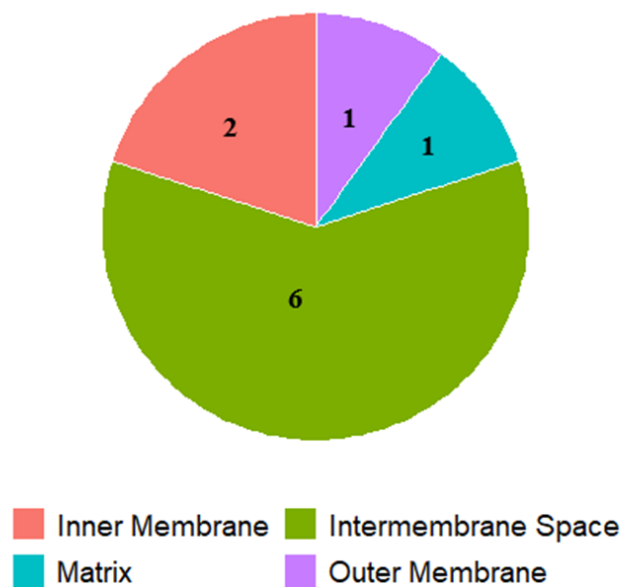


Fig 3.13. The location of proteins, from cells grown in the presence (A) and absence (B) of chloramphenicol, detected by LC-MS/MS. The highest number of proteins were in cytosol in both samples 706 in without chloramphenicol, 783 in sample with chloramphenicol. Proteins in mitochondria were 11 and 10 for cells grown with or without chloramphenicol. Moreover no proteins were found in some organelles or the ER.

3.9 The number of proteins detected in various parts of mitochondria:

Further analysis showed that some proteins were in diverse parts of mitochondria (fig. (3.14)).

A **Number of proteins in various parts of mitochondria
in the presence of chloramphenicol**



B

**Number of proteins in various parts of mitochondria
in the absence of chloramphenicol**

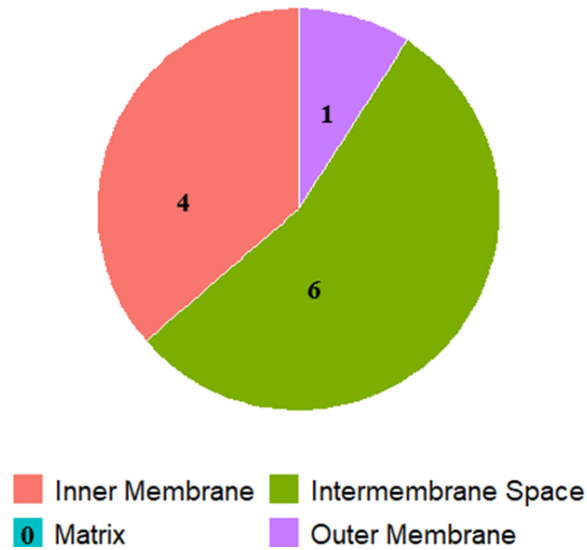


Fig 3.14: The number of proteins detected in various parts of mitochondria. (A) among the 10 proteins in samples grown in the presence of chloramphenicol, 6 of them were in the intermembrane space, 1 was in each of the matrix and the outer membrane, and 2 were in the inner membrane. (B) shows that among 11 proteins in samples grown in the absence of chloramphenicol, 6 were in intermembrane space, 1 in the outer membrane, 4 in the inner membrane and no proteins from the matrix were detected.

3.10 The lists of transporters in bottom and top layer:

The number of transporters and their functions were determined based on a published list of yeast transporters (Islam et al., 2023). In fact, proteins with more than 4 putative transmembrane domains, were considered as transporters based on Islam et al. 2023.

3.11 Analysis of proteomics data of extracted proteins by Trizol:

The file of detected proteins by LC-MS/MS was received from the University of Regina and 3 technical replicates of 1 biological set (with and without chloramphenicol) were considered for analysis as they had a sufficient number of proteins. We had a total of 6 samples, including 3 with chloramphenicol and 3 without chloramphenicol. Only proteins found in all 6 samples were considered. The data were normalized by dividing the mass of each protein (nanograms) divided by the total nanograms of protein detected. Next, their p value, fold changes, log p and log fold all were calculated to depict on a volcano plot (Fig. (3.16)).

3.11.1 Overexpression and underexpression of some proteins in the presence of chloramphenicol:

The analysis shows that most proteins were from the cytosol and a small number of proteins were overexpressed and others were downregulated (Table 3.6). While these observations could suggest that they have a role in drug resistance or survival, the reasons for their altered regulation are not obvious from the known functions of these proteins. YH11B, Sis1, Pur7, AtpF became overexpressed. YH11B is a cytosolic protein encoded by a transposon and encodes a capsid protein (CA) that forms the shell of Ty1 virus-like particles and helps RNA dimerization, primer

annealing, and initiation of reverse transcription. After that the reverse transcriptase/RNase H converts the RNA genome into LTR-flanked double-stranded DNA for integration. Sis1 is a cytosolic Hsp40 co-chaperone that works with Hsp70 to enhance proper protein folding, inhibit aggregation, and keep prion propagation. It is vital for cell viability and stress tolerance. Pur7, which is a cytosolic enzyme, can catalyze the reaction between 4-carboxy-5-aminoimidazole ribotide (CAIR) and aspartic acid to create N-succinyl-5-amino-imidazole-4-carboxamide ribotide (SAICAR) in the pathway of purine biosynthesis. AtpF is a subunit of the mitochondrial ATP synthase (Complex V, F1F0 ATPase) that generates ATP from ADP and uses the proton gradient across the inner membrane. The F1 domain includes the catalytic core for the synthesis of ATP, although the F0 domain produces the proton channel; a central stalk transfer energy via proton movement, but the peripheral stalk keeps the stability of the complex.

Other proteins like Gal1, Hsp73, and ImdH3 became downregulated, and the reasons for this remain unclear. Gal1 which is Galactokinase participate in galactose metabolism that changes alpha-D-galactose to galactose-1-phosphate and can also promote transcription of yeast *GAL* genes when the sole carbon source is galactose. Hsp70 assists in protein folding as well as quality control. IMDH3 which is an enzyme responsible for catalyzing the rate-limiting conversion of inosine 5'-phosphate (IMP) to xanthosine 5'-phosphate (XMP), an essential step in guanine nucleotide synthesis that regulates cell growth (<https://www.uniprot.org>).

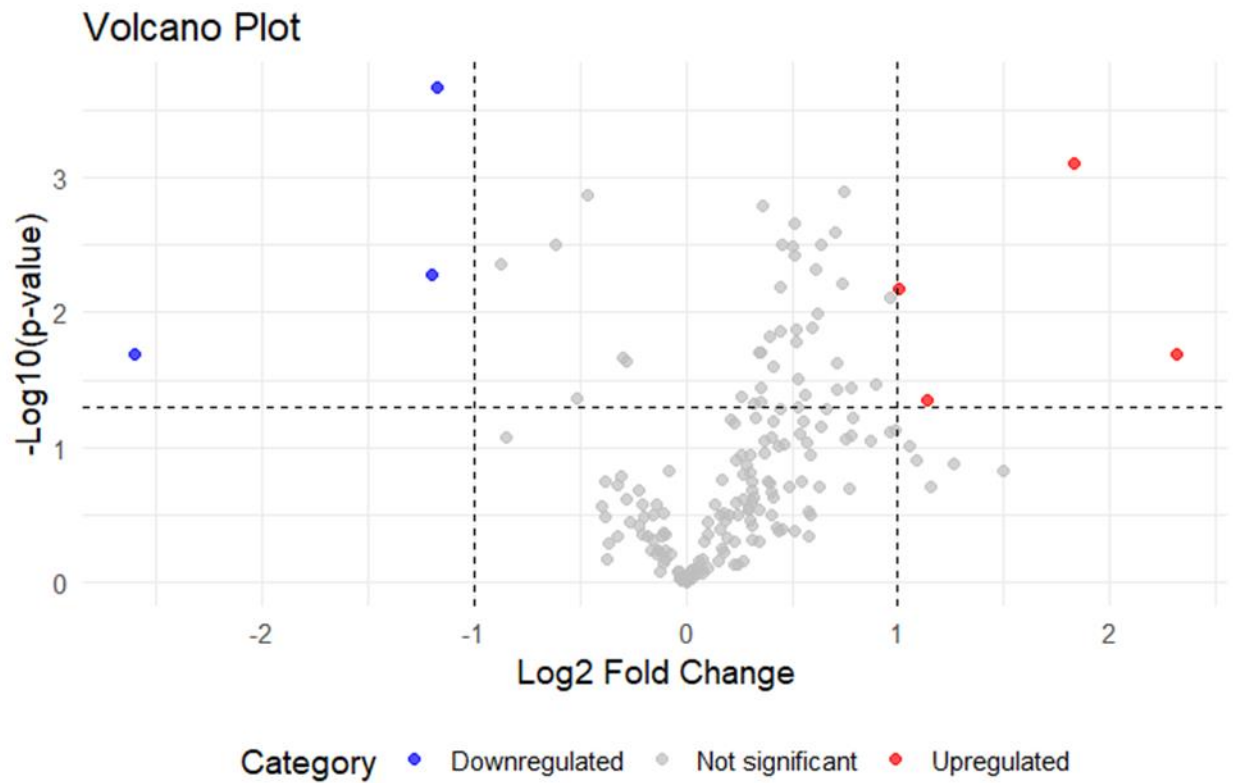


Fig 3.15: The overexpression and downregulation of some proteins in the presence of chloramphenicol. On the left hand side, there are some proteins downregulated and on the right hand side there are some proteins overexpressed in the presence of chloramphenicol.

Table 3.6: The number of upregulated and downregulated proteins in the presence of chloramphenicol from Trizol sample detected by LC-MS/MS:

Upregulated	Function
YH11B	“Capsid protein (CA) is the structural component of the virus-like particle (VLP), forming the shell that encapsulates the retrotransposons dimeric RNA genome. The particles are assembled from trimer-clustered units and there are holes in the capsid shells that allow for the diffusion of macromolecules. CA also has nucleocapsid-like chaperone activity, promoting primer tRNA(i)-Met annealing to the multipartite primer-binding site (PBS), dimerization of Ty1 RNA and initiation of reverse transcription/ Reverse transcriptase/ribonuclease H is a multifunctional enzyme that catalyzes the conversion of the retro-elements RNA genome into dsDNA within the VLP. The enzyme displays a DNA polymerase activity that can copy either DNA or RNA templates, and a ribonuclease H (RNase H) activity that cleaves the RNA strand of RNA-DNA heteroduplexes during plus-strand synthesis and hydrolyzes RNA primers. The conversion leads to a linear dsDNA copy of the retrotransposon that includes long terminal repeats (LTRs) at both ends”(https://www.uniprot.org/)

SIS1	“Type II HSP40 co-chaperone that interacts with the HSP70 protein Ssa1p; shuttles between cytosol and nucleus; mediates delivery of misfolded proteins into the nucleus for degradation; involved in proteasomal degradation of misfolded cytosolic proteins” https://www.yeastgenome.org/ “Required for nuclear migration during mitosis. It is required for the normal initiation of translation. Might mediate the dissociation of a specific protein complex of the translation machinery. Essential for viability”(https://www.uniprot.org/)
PUR7	“Catalyzes the reaction of 4-carboxy-5-aminoimidazole ribotide (CAIR) and aspartic acid with the formation of N-succinyl-5-amino-imidazole-4-carboxamide ribotide (SAICAR) in the purine biosynthesis pathway”(https://www.uniprot.org/)
ATPF	Mitochondrial membrane ATP synthase (F1F0 ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain. F-type ATPases consist of two structural domains, F1 - containing the extramembraneous catalytic core, and F0 - containing the membrane proton channel, linked together by a central stalk and a peripheral stalk. During catalysis, ATP synthesis in the catalytic domain of F1 is coupled via a rotary mechanism of the central stalk subunits to proton translocation. Part of the complex F0 domain and the peripheric stalk, which acts as a stator to hold the catalytic alpha3beta3

	subcomplex and subunit a/ATP6 static relative to the rotary elements (https://www.uniprot.org/).
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Downregulated	Function
Gal 1	“Galactokinase is a key enzyme in the galactose metabolism where it catalyzes the conversion of alpha-D-galactose to galactose 1-phosphate. It can also induce the transcription of the yeast GAL genes in response to the organism being challenged with galactose as the sole source of carbon. It's striking amino acid sequence similarity to GAL3 might explain its GAL3-like induction activity” Galactokinase is a key enzyme in the galactose metabolism where it catalyzes the conversion of alpha-D-galactose to galactose 1-phosphate. Can also induce the transcription of the yeast GAL genes in response to the organism being challenged with galactose as the sole source of carbon (https://www.uniprot.org/).
Hsp73	“As a member of the heat shock protein 70 family and a chaperone protein, it facilitates the proper folding of newly translated and misfolded proteins, as well as stabilize or degrade mutant protein” (https://www.uniprot.org/).

IMDH3	“Catalyzes the conversion of inosine 5'-phosphate (IMP) to xanthosine 5'-phosphate (XMP), the first committed and rate-limiting step in the de novo synthesis of guanine nucleotides, and therefore plays an important role in the regulation of cell growth” (https://www.uniprot.org/).
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3.11.2: The number of transporters identified by LC-MS/MS in Trizol experiment:

Just 2 transporters, called Hxt6 which is hexose transporter in sample with chloramphenicol (Perez et al., 2005) and Gal1 which is responsible for transporting galactose were identified in sample without chloramphenicol (Mirisola et al., 2007).

CHAPTER 4: DISCUSSION

In this study, we were searching for efflux pumps in the mitochondria of the yeast. Mitochondria have a common ancestor with some bacteria and some antibiotics like chloramphenicol can affect their activities. The concentration of chloramphenicol for inhibiting *E. coli* is 6.3 mg/ml and for inhibiting mitochondria translation in isolated organelles is 9.7 mg/ml, while for respiratory growth of whole cells 4000 mg/ml is required to completely prevent respiratory growth (Islam et al., 2023). In this research, we used 1.5 mg/ml chloramphenicol, which decreased the growth of the yeast. Samples were taken when the cells were still growing, to ensure that we were examining differences in the presence of chloramphenicol, not differences between living and dead cells. We predicted that under these conditions, efflux pumps in the mitochondrial membranes could protect the ribosomes within the mitochondrial matrix.

To determine a sampling time for proteomics, the growth of the yeast on YPG and YPEG media was measured in the presence and absence of the chloramphenicol. YPG as well as YPEG media were chosen as they are vital for mitochondria respiration of the yeast (O'Rourke et al., 2002). However, the growth of the yeast on YPEG was faster than YPG as YPEG has ethanol which can increase the growth of the yeast and improve respiration non-fermentable carbon source than glycerol alone (Benning et al., 2025). Chloramphenicol decreased the growth of the yeast wild type BY4741 of *S. cerevisiae* on both YPG and YPEG media. Proteins were extracted at the 8 hours time point (fig. (3.12)), when we observed significant differences between growth without chloramphenicol and with chloramphenicol. This is similar to the effects reported by Islam (2021)

with the wild-type strain. He studied *TPO* mutants in *S. cerevisiae*, which lack pumps that provide resistance to a range of toxic compounds.

The Tpo1 protein is regulated by Pdr1 and Pdr3. Islam (2021) indicated that among *ΔTPO1-4* strains, *ΔTPO1* and *ΔTPO3* were more sensitive to various concentrations of chloramphenicol than WT (Islam, 2021). Subsequent experiments in the lab did not replicate this data (data not shown).

To measure the growth kinetics to find the best time to compare strains in the presence or absence of CAP, large scale flasks were considered; however, this method is time-consuming and it is difficult to analyze multiple replicates on the same day. Therefore 96-well plates replaced the large scale flasks. The accuracy of this method is better than for large flasks since the reader can measure the OD every hour and it is controlled automatically.

Sucrose gradients were used at first to isolate membrane proteins, but the plasma membranes were not well-separated from the other membranes as expected (Table 4.1). It is possible that the crude membrane suspension was not resuspended sufficiently before the sucrose gradient step, and therefore clumps of mixed membranes were put in the gradient and did not separate well. In addition, the membrane washing process did not remove non-integral proteins totally as many cytosolic proteins were isolated (Fig. (3.9)). This could also be the result of poorly suspended crude membranes. In order to improve these steps, either vortexing longer, or centrifuging at a lower speed to generate a looser pellet might help us to isolate crude membranes that resuspend more easily.

Handling integral membrane proteins is difficult since they are hydrophobic and the number of them is low; also, non-membrane proteins stick to membranes. Thus for better identification and

quantification, they have to be enriched by sodium carbonate washing which can remove peripheral proteins that are loosely attached to the surface and then just integral membrane proteins that are inside of the membrane in lipid layer remain (Kongpracha et al., 2022).

For the first experiment, proteins were isolated from untreated cells and unwashed proteins from the bottom layer of a sucrose gradient were sent to MCPSB. Many membrane proteins were detected, indicating that the method was able to detect non-soluble proteins. Therefore, to remove the non-membrane proteins in future experiments, the crude membrane pellet was washed in either sodium carbonate (pH 10) or 6 M urea.

In the second experiment, protein samples from cells grown with and without 1.5 mg/ml chloramphenicol were purified on sucrose gradients. Bottom layer and top layer samples were washed with sodium carbonate. After washing, three biological replicates were sent to the MCPSB to search for proteins that were overexpressed in the presence of chloramphenicol. Before sending to MCPSB, the protein samples were run on the SDS-PAGE gel (fig.(3.6)) and both layers had similar patterns of highly expressed proteins, suggesting that the sucrose gradient may not have separated the plasma membranes from other membranes as well as expected. A higher number of proteins were detected from our washed samples; there were over 3000 detected. 3355 proteins were detected by LC-MS/MS in top layer samples grown in chloramphenicol were 3355, and of these about 2181 were cytosolic or found in the mitochondrial matrix (65%). Similarly, about 65% of the 3694 proteins found in the combined bottom layer samples were soluble. Thus, the washing step appears to have removed a small fraction of the soluble proteins in the membrane samples.

Table 4.1: The number of protein detected by LC-MS/MS by sucrose gradient method associated with the cytosol, as well as different organelles and membranes

Fraction	Number of known proteins (reference)	Number of proteins detected (%)
Plasma membrane	113 (Szopinska et al. 2011)	Top: 6 (5%) Bottom: 6 (5%)
Peroxisome	115 (Yifrach et al. 2023)	Top: 86 (75%) Bottom: 85 (74%)
Nucleus	465 (Hu 2003)	Top: 258 (55%) Bottom: 290 (62%)
Mitochondrial inner membrane	483 (Vögtele 2017)	Top: 432 (89 %) Bottom: 413 (86%)
Mitochondrial matrix	193 (Vögtele 2017)	Top: 147 (76%) Bottom: 166 (86%)
Mitochondrial outer membrane	95 (Vögtele 2017)	Top: 100 (~100%) Bottom: 97 (~100%)
Inter membrane space	49 (Vögtele 2017)	Top: 39 (80%) Bottom: 42 (86%)
Vacuole	95 (Hu et al. 2003)	Top: 59 (62%) Bottom: 64 (67%)
ER	226 (Hu et al. 2003)	Top: 174 (77%)

		Bottom: 181 (80%)
Golgi	70 (Hu et al. 2003)	Top: 62 (88%) Bottom: 61 (87%)
Cytosol (all other proteins)	4459*	Top: 2015 (45%) Bottom: 2272 (51%)

*All proteins were counted in various organelles and decreased from total amounts of proteins in cytosol.

As many proteins from the cytosol were detected, this method did not wash away most of the peripheral proteins either. Also, we could not get our different replicates on the same day and we could not get reliable growth curves. Therefore, the sucrose gradient purification of proteins was replaced by protein extraction by Trizol, and in this method we focused on whole proteins of the cell not just membrane proteins. The results of proteins on the SDS-PAGE (fig. (3.13)) indicates that, although the amount of cells was not high in this method, the extraction of the cells worked well and we do not need to employ the large scale preparation for protein extraction. The Trizol method would be a cost and time effective way.

For these experiments, only one set of replicates had sufficient proteins for analysis. We collected LC-MS/MS data in for three technical replicates to improve our confidence in the analysis of this sample. We were able to compare about 300 proteins that were detected in all replicates. The results showed several proteins that were in different relative amounts in cells grown with and without CAP (Table 3.2). The reasons for these changes are not clear. YH11B, is a capsid protein

(CA) forms and acts as chaperone and forms parts of the shell of virus-like particles (VLPs) that are common in *Saccharomyces* (Herring & Bevan, 1974). The capsid surrounds the dimeric RNA genome of retrotransposon elements and possess trimeric units with pores for diffusion of macromolecules. This chaperone promotes tRNA(i)-Met annealing, dimerization of Ty1 RNA, and initiation of reverse transcription. The activation of the Ty1 retrotransposon in *S. cerevisiae* can be increased significantly under conditions of mitochondrial dysfunction. In mitochondrial dysfunction condition, RTG is activated, which in turn stimulates Ty1 transcription (Morillon et al., 2000). Therefore, this activation happens via specific transcription factors and the modifications of chromatin when mitochondria are under stress. Therefore, the increased level of YH11B may be a result of general stress in the presence of chloramphenicol, rather than a direct result of the drug.

Sis1 levels are also higher. Sis1 is a chaperone and mediates delivery of misfolded proteins into the nucleus for degradation and it is involved in proteasomal degradation of misfolded cytosolic proteins. Sis1 protein abundance increases in response to DNA replication stress, when members of heat shock protein families (HSPs) are upregulated and the concentrations of aggregation proteins increase remarkably (Malinowska et al., 2012). In addition, Sis1 is upregulated in the presence of arsenic stress since it would be a key chaperone that protect the proteins from misfolding and proteotoxicity (Rodrigues et al., 2023). If protein misfolding results from the chloramphenicol treatment, Sis1 may be involved in protecting the cells.

Pur7 became upregulated. Pur7 catalyzes the 4-carboxy-5-aminoimidazole ribotide (CAIR) reaction and aspartic acid N-succinyl-5-amino-imidazole-4-carboxamide ribotide (SAICAR) formation in the purine biosynthesis pathway. De novo purine biosynthesis contributes to tolerance

of *S. cerevisiae* BY4741 under acid stress situations, which may increase the demand for nucleotide or repair function and therefore some genes involved in the synthesis of purines; (Zhang et al., 2019). However, the role of purine synthesis as a stress response to blocking mitochondrial translation is unknown.

AtpF is upregulated and is a component of the mitochondrial membrane ATP synthase, which generates ATP from ADP in the presence of a proton gradient across the membrane produced by electron transport complexes of the respiratory chain (Ghaemmaghami et al., 2003). Increased production of ATP synthase subunits in the presence of CAP may occur in order to increase ATP synthesis.

Some proteins like Gal1, became downregulated. Expression of the *GAL* genes in *S. cerevisiae* is associated with sugar galactose metabolism that is regulated by the carbon source and this process is mediated by the products of two regulatory genes and at least 2 pathways are needed (Flick & Johnston, 1990). Thus utilizing galactose is energy consuming for the cells and in the presence of chloramphenicol cells may try to survive by reducing energy and some genes are downregulated (Flick & Johnston, 1990).

Hsp73, which is a member of the heat shock protein 70 family and a chaperone protein, facilitates the proper folding of newly translated and refolding of misfolded proteins, became downregulated. Rotenone, which is mitochondrial complex I inhibitor, also decreases heat shock protein Hsc70 expression at both transcriptional and protein levels; however, the reason of this is unknown (Sala et al., 2016).

Sis1, also a heat shock protein, was upregulated in the presence of chloramphenicol, and it is unknown why one heat shock protein became downregulated and another one was upregulated. *IMDH3* (also called *IMD3*) was downregulated. It encodes inosine monophosphate dehydrogenase involved in GTP synthesis. It may be lower in order to save the energy and help the cells survive; thus may not be specifically related to drug resistance. Some drugs like mycophenolic acid (MPA) and 6-azauracil (6AU) can inhibit IMPDH and GTP levels decrease significantly (Shaw et al., 2001).

Only two transporters were identified in this experiment. One transporter called Hxt6 responsible for hexose transportation was found in the sample from chloramphenicol treated cells (Llopis-Torregrosa et al., 2016), and 1 transporter called Gal2, which is responsible for transporting galactose, was found in the sample without chloramphenicol (Swaffer et al., 2023). Some factors like stress are the most reason for upregulation and downregulation of proteins in the cell cause that some proteins react differently to defend the cell.

In some cases, investigation of proteins produced under one condition, such as the presence of CAP, but not under the control condition, can provide insights into proteins that are normally expressed at very low levels, and are highly upregulated under certain conditions (Fu et al., 2015). In our Trizol experiments, we found over 300 proteins in samples treated with CAP that were not detected in the control conditions. Similarly, there were over 300 proteins in the control samples that were not detected in the CAP-treated cell samples. Therefore, our data could not be examined in this way.

In summary, the individual up- or down-regulated proteins as groups did not represent any particular pathway or process. Furthermore, direct links between the upregulated or downregulated genes and the effects of chloramphenicol were not found. This may not be surprising because the number of proteins analyzed was small (300/6000) and most proteins were cytosolic. Therefore, larger data sets with more replicates need to be analyzed.

CHAPTER 5: FUTURE WORK

To search for chloramphenicol transporters, it is important to be able to separate membrane proteins more effectively. To do this the following could be tried:

First, the growth phase of the cells is important. Choosing and optimizing the best media containing glycerol for respiratory of mitochondria is also vital. Earlier time points could be selected in case the transporters are expressed early in the response to CAP. For all experiments, the cells with and without chloramphenicol should be grown at the same time. This is challenging in sucrose gradient experiments.

In the sucrose gradient method, the pellet should be broken better, to separate membranes and clumps of cells. One way to do this would be by using a vortex genie and by glass beads to have a well-disrupted membrane sample to be run on the ultracentrifuge. Alternatively, a sonicator could be used as a physical way for disrupting cell membranes. In addition, the time and speed centrifugation used to collect membranes after the sucrose gradient should be lower to collect pellets that resuspend better. This will increase the yield of proteins.

For the experiments using Trizol, more cells need to be used to increase the yield of protein. Two-ml 96-well plates could be used. A method that does not require 1% SDS to resuspend the final protein pellet is needed, because most of the protein was lost at this step. Overall, this method may be best for cytosolic proteins, and might be suitable for looking for transcription factors that might regulate drug efflux pumps, like Pdr1 and Pdr3 (Buechel & Pinkett, 2024).

In summary, the methods need to be refined to allow careful measurement of cell growth during exposure to chloramphenicol, and to allow extraction of sufficient proteins. Improved samples will allow more thorough examination of the proteome that will survive statistical testing.

CHAPTER 6: REFERENCES

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CHAPTER 7: APPENDIX:

Buffers: Two buffers called A and B were used for isolating membranes.

Buffer A: 2.25 M sucrose buffer:

Molecular weight of imidazole: 68.077 g/mol

Molecular weight of sucrose: 342.39 g/mol

Sucrose 77.0175g/l

Imidazole 0.1701 g/ml

We need 0.025 g imidazole

Per 50 ml of sucrose buffer 2.25 M:

Sucrose: 38.508 g

Imidazole: 85 mg

Buffer A 1.65 M sucrose:

Sucrose $1.65 \text{ mol/l} \times 342.3 \text{ g/mol} = 564.79 \text{ g/l} \times 0.1 \text{ ml} = 56.479 \text{ g/l}$

Sucrose: 28.23 g

Imidazole: 85 mg

50 ml distilled water

Buffer 1.1 M:

Sucrose: 18.85

Imidazole: 85 mg/l

50 ml distilled water

Sucrose buffer 0.4 M:

Sucrose:

Sucrose: 6.8 g per 50ml of Milli-Q water

Imidazole: 85mg

50 ml Milli-Q water

Both buffers (A and B) were adjusted to pH 7.0 and stored at 4° C.

Buffer B:

25 mM imidazole-HCl (85 mg)

50% (v/v) glycerol containing protease inhibitors

100 ml Milli-Q water

Sodium carbonate:

10 ml of 0.2 mol/l sodium carbonate

Sodium Carbonate 0.21198 g/l

Urea:

10 ml of 6 M urea

Molecular weight: 60.06

$60.06 \text{ g/mol} \times 6 \text{ mol/l} = 360.36 \text{ g/l}$

$360.36 \text{ g} \times 0.01 \text{ l} = 3.60 \text{ g}$

Staining solution

80 ml Milli Q water

100 ml methanol

20 ml acid acetic

Destaining solution:

600 ml methanol for 1 liter of solution

300 ml Milli-Q water

100 ml of acid acetic

2.6.2 Running buffer 5X:

Tris 15 g for 1 liter

Glycine 72 g

SDS 10 g

1000 ml of Milli-Q water (Motojima, 2018).

NA Niloofer Ahmadi
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Mon 6/9/2025 12:30 PM

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Dear Madam Sa Coresia,

I hope this message finds you well.

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Dear Niloo,
Thank you for your kind message. Of course, you are welcome to use the figure.
All the best for you MSc thesis,
Isabel



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