ANA RITA LOPES NOGAL LEMOS DE SOUSA

TOOL GENERATION TO CHARACTERIZE *DTR1*, A MEMBER OF THE POORLY CHARACTERIZED DHA1 TRANSPORTER FAMILY OF PROTEINS IN YEAST

UNIVERSIDADE DO ALGARVE
Faculdade de Ciências e Tecnologia
2021

ANA RITA LOPES NOGAL LEMOS DE SOUSA

TOOL GENERATION TO CHARACTERIZE *DTR1*, A MEMBER OF THE POORLY CHARACTERIZED DHA1 TRANSPORTER FAMILY OF PROTEINS IN YEAST

Mestrado Integrado em Engenharia Biológica
Trabalho efetuado sob a orientação de Drª Isabelle Georis
e coorientação de Profª. Drª. Leonor Faleiro

UNIVERSIDADE DO ALGARVE
Faculdade de Ciências e Tecnologia
2021

Tool Generation to characterize *DTR1*, a member of the poorly characterized DHA1 transporter family of proteins in yeast.

Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito.
Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

(Ana Rita Nogal Sousa)

“Nothing is impossible, the word itself says 'I'm possible'!”

— *Audrey Hepburn*

Acknowledgements

First, I would like to thank the University of Algarve and the Université Libre de Bruxelles for providing me with the amazing opportunity of doing my dissertation project abroad. The execution of this work was only possible thank to the support, availability and encourage of a handful of people that somehow contribute to the execution of this dissertation.

I wish to particularly express my deepest gratitude to my supervisor Prof. Dra Maria Leonor Faleiro, without her guidance, support, attention, sympathy, motivation and availability for my every doubt this work would not be possible and Prof. Isabelle Georis for her guidance and support throughout my work in Brussels. Their useful insight and feedback was thoroughly appreciated throughout this project.

I am also very grateful to my ERASMUS friends that I have met along this journey for their assistance, advice, teaching and patience. Their friendships was of tremendous help to me during this period of time away from home. Thank you, I had an incredible time, which I'll remember for the rest of my life.

Finally, I would like to thank to all my teachers from the Biological Engineering course at the University of Algarve, for their support all throughout my course.

Thank you all.

The following personal acknowledgements will be addressed in Portuguese.

E como os últimos são sempre os primeiros, e agora na língua materna que me é muito querida, quero agradecer à minha querida mãe, Alexandra, à minha irmã Inês e a todos os meus tios e tias, por todo o apoio que me deram e por terem tornado possível esta experiência.

Em especial ao meu namorado Jarred, pelo seu apoio e amor incondicional ao longo destes 10 anos.

À minha amiga Carolina, agradeço por todo o apoio e pela sua amizade ao longo de mais de 20 anos e ao meu afilhado Tomás que tornou a vida mais risonha, aos meus amigos Diogo e Teresa e às minhas colegas de curso e amigas, agradeço a ajuda, motivação e amizade dados ao longo desde percurso académico e que nunca serão esquecidos.

E, por fim, a todos aqueles que, de uma forma mais ou menos direta, me apoiaram ao longo desta caminhada.

Muito Obrigada.

Abstract

Nitrogen is necessary for the synthesis of a large number of compounds, such as amino acids, which are very important for several yeast dynamics, and for industrial purposes.

This project aims at the identification and characterization of new transporters involved in the excretion of amino acids through the study of a specific gene of the MFS superfamily of transporters in eukaryotes, the *DTR1* gene.

In the MDR-MFS (MultiDrug Resistance-Major Facilitator Superfamily) family of proteins, up to 100 are unknown, 24 proteins are Multidrug Resistance, and may be involved in the excretion of amino acids.

DTR1 is a multi-drug resistance protein with a physiological role assigned to the yeast cell where the layers of chitosan and dityrosine on the external spore wall provide greater resistance to environmental stresses.

In the present study, techniques such as *E. coli a*nd *S. cerevisiae* transformation, PCR, RT-PCR, qPCR analysis were applied.

The characterization of the *DTR1* gene was carried out through its location and expression using various tools, such as the Green Fluorescent Protein by observing when it is expressed in the control of its own promoter, and a fusion with the GAL1 promoter, verifying overexpression of the protein or whether the expression is normally done. The construction of the P_{GAL1}-DTR1-GFP cassette did not occur as expected, therefore was not possible to observe the overexpression of the *DTR1* gene and its location.

As for characterization on a plasmidic level, plasmids containing the GAP1 promoter were used and the promoter induced, this was possible to observe the expression analysis of the GAP1-*DTR1* construction with a change of medium.

Additionally, a study of the influence of glycerol and temperature, on S. cerevisiae culture was carried out where it was observed that high temperature and presence of glycerol might be stress conditions enough to *DTR1* to be expressed.

Keywords: MFS-MDR; *DTR1*; *Saccharomyces cerevisiae*; GFP; P_{GAL1}.

Resumo

O azoto é um nutriente mineral crítico em todos os organismos vivos, pois é necessário para a síntese de um grande número de compostos, incluindo hormonas, nucleotídeos e aminoácidos. Os aminoácidos são muito importantes para várias dinâmicas da levedura, como síntese de proteínas, metabolismo de hormonas, transmissão nervosa, crescimento celular, geração de energia, metabolismo do azoto e síntese de bases azotadas. Os aminoácidos também são importantes para fins industriais, como aplicações em alimentos como o aminoácidos glutamato (intensificador de sabor) ou aspartato, fenilalanina (adoçantes); para alimentação, tais como os aminoácidos lisina, metionina, treonina; e para aplicações farmacêuticas, como soluções de infusão e blocos de construção, triptofano (indutor do sono) e fenilalanina (antidepressivo).

Este projeto visa a identificação e caracterização de novos transportadores envolvidos na excreção de aminoácidos por meio do estudo de um gene específico da superfamília MFS (*Major Facilitator Superfamily*) de compostos azotados em eucariontes, o gene *DTR1*.

De acordo com, Sá-Correia et al., (2009) vários transportadores MDR foram identificados e estudados em diferentes organismos, particularmente aquele pertencentes à superfamília *ATP-binding cassette* (ABC). Um grupo de transportadores envolvidos na resistência a multidrogas menos caracterizado pertence à família MFS-MDR. Proteínas deste grupo têm vindo a receber mais atenção, maioritariamente em bactéria. Em *Saccharomyces cerevisiae*, a maioria dos membros desta família foi apenas descobertos aquando revelada a sequencia genómica desta levedura e caracterizados em alguns aspetos nos últimos 12 anos.

A família MDR-MFS (*Multidrug Resistance-Major Facilitator Superfamily*) de proteínas tem aproximadamente 300 proteínas transportadoras de membrana, com até 100 sendo

desconhecidas. 24 proteínas são MDR da superfamília MFS, algumas delas podem estar envolvidas na excreção de aminoácidos, como *DTR1*.

Dentro da DHA1 (Drug: H+ antiporter family 1) que é composta por 46 proteínas, sete genes são individualmente necessários para conferir resistência à quinidina, um fármaco antiarrítmico e anti malária, entre esses genes encontram-se os genes *AQR1*, *TPO1* e *DTR1*. Estes sete transportadores podem, também, proteger a célula contra outros compostos que estão normalmente ausentes no ambiente natural de células de levedura e poderão ter substratos fisiológicos específicos, onde fármacos são transportados fortuitamente ou oportunisticamente.

Ainda na família DHA1, 9 proteínas mostraram ser bombas de multirresistência, 15 são provavelmente bombas de efluxo específicas para drogas e 22 são proteínas hipotéticas ou não caracterizadas. Os genes de resistência a múltiplas drogas, isto é, a aquisição simultânea de resistência a uma variedade de químicos citotóxicos, é encontrada numa grande variedade de organismos, desde bactérias a mamíferos, e isto poderá causa um severo problema clínico, principalmente no tratamento de cancros humanos e infeções de origem bacteriana e fúngica, tendo atingido proporções alarmantes nos últimos anos.

DTR1 é a primeira proteína de resistência a múltiplas drogas da superfamília de facilitadores principais com um papel fisiológico atribuído na célula de levedura, mas o transporte por *DTR1*p pode não ser restrito ao seu substrato natural, bisformil ditirosina. As camadas de quitosana e ditirosina da parede externa dos esporos conferem maior resistência a estresses ambientais no esporo, incluindo a capacidade de passar pelo trato digestivo dos insetos, permitindo a dispersão para o meio ambiente.

No presente estudo é realizada a caraterização do gene *DTR1* através da sua localização e expressão com recurso a variadas ferramentas, tais como a proteína Green Fluorescent Protein, produzida pelo cnidário *Aequorea victoria* que emite fluorescência na zona verde do espectro visível, observando quando esta está a ser expressa no controle de seu próprio promoter. Uma fusão com o promotor Gal1, um operão procariótico que codifica as enzimas necessárias para o metabolismo da galactose, para verificar a sobrexpressão da proteína ou se a expressão é feita normalmente como no seu estado natural.

Com essas fusões podemos analisar se a proteína está bem expressa, superexpressa e onde ela ocorre na célula e, usando plasmídeos identificados como GAP1 e UGA4 ambos contendo o promotor *GAP1*, ativado pela falta de azoto, para observação do comportamento do gene em estudo. Essas ferramentas contribuem para a caracterização

a nível genómico e plasmídico da proteína de interesse, *DTR1*, para compreender sua função e localização no genoma da levedura.

O objetivo principal deste trabalho foi construir uma cassete combinando primers para o plasmídeo, primeiro e para localização do *DTR1* foi utilizado o plasmídeo PKT140, que contém a proteína GFP e resistência à canamicina. Os primers construídos são homólogos à proteína GFP, no caso do F5, e à resistência à canamicina, no caso do primer R3.

A estratégia adotada foi a utilização de um fragmento do plasmídeo contendo os primers, GFP e a resistência à canamicina, que foi amplificado com um tamanho esperado de 2515 pares de bases e transformado em *S. cerevisiae*.

A tag GFP foi aplicada a jusante do gene de interesse, *DTR1*. A canamicina foi usada como marca de seleção no momento da transformação e a integração no genoma da levedura foi concluída por recombinação homóloga.

Para compreender se a proteína de interesse, *DTR1*, está a ser superexpressa, um fragmento do plasmídeo P_{GAL1} foi usado, novamente os primers foram construídos contendo parte de *DTR1* e o promoter Gal1 no caso do iniciador R2 e parte de *DTR1* e resistência à nourseotricina em o caso do primer F4.

Para análise deste estudo recorreu-se a técnicas como transformação em *E. coli* e em *S. cerevisiae*, digestão com recurso a enzimas de restrição, PCR, RT-PCR (Reverse Transcriptase PCR), análise de expressão por qPCR (Quantitative Real Time PCR), Cross-feeding e ainda Western Blotting.

O fragmento do plasmídeo mais primers foi amplificado por PCR com 1850 pares de bases e a integração no genoma da levedura é feita por recombinação homóloga.

A nível funcional concluímos que não foi possível a construção da cassete P_{GAL1}-*DTR1*-GFP tendo sido efetuadas várias tentativas da mesma não tendo sido possível a observação da sobrexpressão do gene *DTR1* e a sua localização.

A nível plasmídico foi possível uma observação de analise de expressão de diferentes estudos de comportamento da construção GAP1-*DTR1* com alteração de meio.

Foi ainda feito um estudo de influência do glicerol e temperatura em cultura de *S. cerevisiae* em meio mínimo. Tendo em conta que o gene *DTR1* faz parte da reprodução de *S. cerevisiae* através esporulação em condições de stress, verificou-se que uma temperatura de 30ºC e glicerol 10% poderão representar condições de stress suficientes para que este gene seja expresso.

Palavras chave: MFS-MDR; *DTR1*; *Saccharomyces cerevisiae*; GFP; P_{GAL1}.

Contents

List of Figures

List of Tables

List of Acronyms

A. gossypii	*Ashbya gossypii*
ABC	ATP Binding Cassette
Amp	Ampicillin
Bp	Base pairs
DHA	Drug: H (+) antiporter
DHA1	Drug:H;+ antiporter1
DNA	Desoxirribonucleic acid
DTR1	Gene coding for Dityrosine transporter 1
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
GAP1	Gene coding for the Geral Amino acid Permease
GFP	Green Fluorescent Protein
KAN	Kanamycin
MDR	Multi Drug Resistance
MFS	Major Facilitator Superfamily
NAT	Nourseothricin
OD	Optical density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
P_{GAL1}	Galactose promoter
qPCR	Real-time polymerase chain reaction
rpm	Rotations per minute
RT-PCR	Reverse transcription polymerase chain reaction

S. cerevisiae	*Saccharomyces cerevisiae*
SDS-PAGE	Sodium Sodecyl Sulphate-Polyacrylamide Gel Electrophoresis
Wt	Wildtype

1 Introduction

1.1 *SACCHAROMYCES CEREVISAE*, AN IMPORTANT TOOL!

Yeasts are of major economic, social and health significance in human culture and have been often described as mankind's oldest 'domesticated' organisms, in particular, *Saccharomyces cerevisiae* has been widely used by humans for thousands of years and is arguably one of the most important microbial species in human history and it owes this distinction to its ability to produce alcohol from sugar. In fact, it was the demand for alcoholic beverages that motivated the scientific study of yeast by Pasteur in 1897 and the Carlsberg Research Laboratories in 1896, since they are present in a wide range of applications, such as producing alcoholic beverages and leaven bread dough that are aid by yeasts for millennia, and in biomass production such as single-cell protein (SCP) (Chambers & Pretorius, 2010; Cherry et al., 2012; Goddard & Greig, 2015). There is still some ignorance within known species relating to cell physiological biodiversity, for example around 50% of the 6000 genes identified by the *S. cerevisiae* genome project are of unknown function, yet *S. cerevisiae* is the best understood genetic model organism, since it was the first eukaryote to have its genome completely sequenced, and its genome is still the best annotated and most tractable to genetic manipulations and analysis, and has been key to numerous major breakthroughs in genetics, biochemistry and cell biology (Walker, 1998).

It is important to notice that there is high homology between yeast genes and mammalian ones, this is because the basic cellular mechanics of replication, recombination, cell division, and metabolism are generally conserved between yeast and larger eukaryotes and they constitute a good model for studying different processes such as aging, regulation of gene expression, signal transduction, cell cycle, metabolism, apoptosis, neurodegenerative disorders, and others (Żymańczyk-Duda et al., 2017).

Most recently, yeasts have also been used in the biofuel industry and for the production of heterologous compounds, since their metabolic capacity allows them to carry out the transformation of sugars into ethyl alcohol and carbon dioxide under anaerobic conditions. The main factors influencing yeast metabolism are the oxygen availability and the type of carbon source, although many yeast strains can function under both anaerobic as well as aerobic conditions of environment, switching their metabolism types easily, showcasing that the main metabolic pathways courses are conserved but some regulative mechanisms denote unusual metabolism flexibility. A highly unique and unusual, as for eukaryotes, feature of *S. cerevisiae* genome is the presence of DNA plasmids, this forms of genetic material and protein expression systems shows great potential in the field of molecular biology and biomedicine and *S. cerevisiae* is one of very few eukaryotic organisms that contain circular DNA in the form of plasmids. Almost every strain of this yeast has the 2-µm plasmid, which can constitute the outstanding basis for cloning vectors, that enables a variety of genetic manipulations and are of great importance for modern and future molecular biology (Walker, 1998; Żymańczyk-Duda et al., 2017).

In summary, culturing yeast is simple, economical, and rapid which is their big advantage over the insect or mammalian cells and can be conducted under aerobic and anaerobic conditions. As a non-pathogenic and nontoxic organism, they are safe for laboratory work, without any special precautions. Large accessibility as well as easy culturing on both liquid and solid medium makes yeast a cheap and handy organism with significant biotechnological capabilities. They easily undergo genetical manipulation and adapt to fermentation processes, therefore, using yeasts as a cell factory is the best, most convenient and also enables obtaining a fair amount of the target protein (Żymańczyk-Duda et al., 2017).

1.1.1 **Reproduction Cycle under stress conditions**

S. cerevisiae typically grows asexually by budding, the most common vegetative reproduction in yeasts and in this species, its life cycle include haploid and diploid phase. Individual bud growth is known to depend critically on the dynamism of the cytosketal actin fibres and microtubules. Bud site selection in this yeast is governed by several genes which predispose cells in a non-random budding pattern. The cell cycle consists of four distinct phases(G1, S, G2, and M), and is regulated in a similar way to that of the cell cycle in larger eukaryotes, as is illustrated in Figure 1.1.

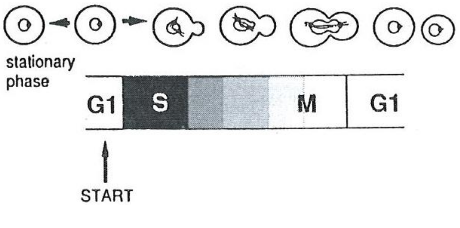

Figure 1.1 Schematic representation of cell cycle phases in *S. cerevisiae*. The status of the haploid cell relative to phases of the cell cycle are characterized and it is important to note that in *S. cerevisiae* the mitotic spindle forms very early, and the S and M phases overlap (resulting in an indistinct G2 phase). Cells can bud only after they have passed Start and can divide only after they have inactivated the cyclin-dependent kinase-cyclin complex known as MPF, making it easy to monitor these cell cycle transitions in living cells. (Walker, 1998)

Haploid yeast cells can be either mating type a or α and under normal condition can mate together to generate a/α diploids. The diploid cells cannot mate but can reproduce asexually by budding like haploids. However, under specific circumstances, like unfavourable environment conditions (lack of nutrients), diploid cell can undergo meiosis to produce haploid spores. Each single haploidic spore from tetrad arising after meiosis to environmental stress can be isolated and analysed by various micromanipulation methods. Haploid states cell can be also used for recessive mutation studies, while diploid strains can be exploited for complementation tests (Walker, 1998; Żymańczyk-Duda et al., 2017). In this thesis, the aimed gene to go under study is *DTR1*, a protein revealed under specific stress conditions, as the one mentioned above, of *Saccharomyces cerevisae* reproduction cycle and spores formation.

As stated, sporulation involves meiosis and ascopore development in cells and in *S. cerevisiae* can be initiated by depriving diploid cells of nitrogen and providing acetate as a respiratory carbon source. Meiotic division in *S. cerevisiae* produces four-lobed nuclei from which four spores are formed. The spindle pole body (SPB) in each nuclear lobe polarizes the meiotic spindles and forms thickened plaques in the outer nuclear membrane which serve as beginnings of spore wall formation. After meiosis is complete yeast spore walls develop within the ascus to form a structure known as the forespore membrane. Several genes of both the mating type control pathway and the nutritional control pathway are involved in regulating meiosis and sporulation in *S. cerevisiae*.

Yeast cells generally arrest in the G1 phase and rest in the G0 state of their cells division cycle when deprived of essential nutrients such as carbon, nitrogen or phosphorus sources. In sexual yeasts, depletion of carbon or nitrogen source may lead to sporulation as a survival mechanism. In *S. cerevisiae* the glucose transport system is inactivated during sporulation (Walker, 1998).

The yeast cell physiologist can manipulate the growth behaviour of cells in both solid and liquid media. For instance, by controlling the supply of nutrients and the physical growth environment, yeast can be induced to grow randomly or non-randomly with respect to the cell cycle, and continuously or non-continuously with respect to growth rate.

Sexual reproduction in *S. cerevisiae* mating involves the conjugation of two haploid cells of the opposite mating types, designated a and α. These cells synchronize each other’s cell cycles at Start in response to peptide mating pheromones known as a **a factor** and **α factor**. Once starved of nutrients or if grown on a non-fermentable carbon source such as ethanol or acetate, the *S. cerevisiae* cells are induced to undergo meiosis and sporulation results in the formation of four haploid spores (2**a** and 2α). These spores can in turn germinate in rich media and mate once again to form diploids. After germination, haploid cells of *S. cerevisiae* have the capability of undergoing mating type switching, which maximizes the chances of diploidy, the formation of spores is illustrated in Figure 1.2.

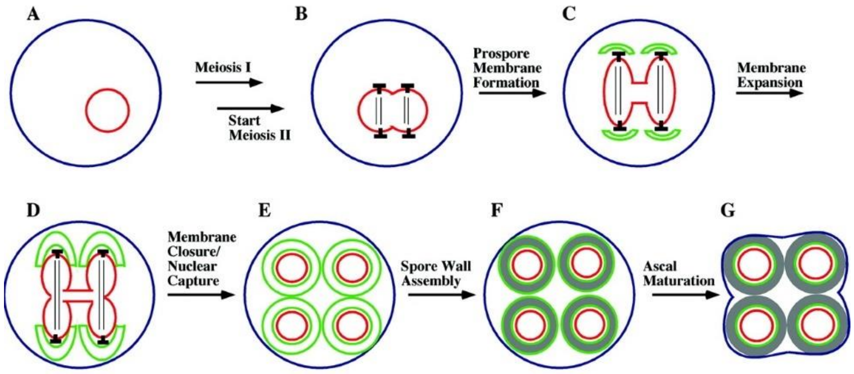

Figure 1.2 (A to G) Overview of the stages of spore and ascus formation. In the presence of a nonfermentable carbon source, diploid cells starved for nitrogen will undergo meiosis. During the second meiotic division, the SPBs (Spindle pole body, indicated as ⫟), which are embedded in the nuclear envelope (displayed in red), become sites for formation of prospore membranes (displayed in green). As meiosis II proceeds, the prospore membranes expand and engulf the forming haploid nuclei. After nuclear division, each prospore membrane closes on itself to capture a haploid nucleus within two distinct membranes. Spore wall synthesis then begins in the lumen between the two prospore membrane-derived membranes. After spore wall synthesis is complete, the mother cell collapses to form the ascus. (Neiman, 2005)

Spores are protected from adverse environmental conditions by the spore wall, especially the surface layers that contribute both to the spores' mechanical rigidity and their resistance against chemical and enzymatic attack. Spore wall synthesis begins with the formation of the prospore membrane, a bi-layered electron-dense structure that starts to form during the second meiotic division on the cytoplasmic side of each of the four spindle pole bodies by fusion of secretory vesicles. As meiosis progresses, the prospore membrane extends along the outer surface of the nuclear envelope. The major component of the surface layer is the amino acid, dityrosine, a highly fluorescent dimer of tyrosine. Dtr1p, a sporulation-specific member of the major facilitator superfamily involved in multidrug resistance (MFS-MDR), is shown to be the membrane transporter responsible for the translocation of bisformyl dityrosine from the prospores cytoplasm to the maturing spore wall during spore formation, and, as stated, this thesis is based on the study of this particular protein (Felder et al., 2002; Walker, 1998).

1.1.2 Membrane transport in *Saccharomyces cerevisiae*

There are four basic mechanisms whereby nutrients are taken up, and metabolites exported, across yeast membranes: free diffusion, facilitated diffusion, diffusion channels (pores), and active transport.

The free diffusion is the simplest and slowest mode of nutrient transport in yeast which involves passive penetration of lipid-soluble solutes through the plasma membrane. Solutes move by the law of mass action from high extracellular concentration to a lower intracellular concentration. Passive diffusion may account for the uptake of undissociated organic acids, short-chain alkanes and long-chain fatty acids into yeast cell and the export of ethanol and gaseous compounds from the yeast cell (André, 1995).

Facilitated diffusion is faster that simple diffusion since solutes are translocated down a transmembrane concentration gradient in an enzyme-mediated fashion. The enzyme is referred to as a permease, or carrier, or facilitator, which traverses the membrane and exhibits stereospecificity for the transported solute. Certain sugars are transported in this matter, notably glucose in *S. cerevisiae*. Many facilitators/transporters have been identified in yeast and can be divided into several families on the basis of sequence similarities. Some transporters, called uniporters, catalyse the transport of (charge) salute down its (electro)chemical gradient, i.e. mediate *facilitated diffusion* of this compound. Many others are coupled to a symport or antiport of protons, which enables them to operate against (electro)chemical gradients, these symporters and antiporters are usually referred to a *secondary active co-transport systems* (André, 1995).

Diffusion channels for some ions exist in yeast as voltage-dependent 'gates' to move transiently ions down concentration gradients. Such proteinaceous channels are normally closed at the negative membrane potential of resting yeast cells and open when the membrane potential becomes positive. Plasma membrane diffusion pores in the yeast cell membrane may also exist to allow the passage of water and sugar alcohols. In addition, under conditions of hypoosmotic stress, *S. cerevisiae* facilitates glycerol permeation through a membrane channel, the FPS1 facilitator protein.

Active transport is a concentrative, energy dependent mechanism based on chemiosmotic principles which is responsible for the uptake of the majority of nutrients into yeast cells. Represented by Figure 1.3 are the three types of integral membrane proteins, which participate in moving molecules across the cell membrane. Specifically, they take part in active transport, but uniporters are involved in facilitated diffusion, an example of an uniport carrier is the GLUT1 glucose carrier, found within the plasma membrane of various cells. An example of a symport carrier protein is the lactose permease. As for the antiport protein which is a transport protein where an exchange is made through the membrane, one solute for another an example can be the adenine nucleotide translocated (also referred as the ADP/ATP exchanger).

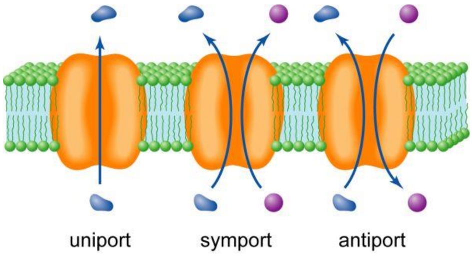

Figure 1.3 Schematic representation of three types of proteins that are used in the transport of substances across a cell membrane in yeast. (Kodíček, 2007)

For many yeasts concentrative sugar uptake occurs by proton symport. It should also be noted that metabolites can be expelled from yeast by such means (Cherak et al., 2016).
Specific permeases, which may either be constitutive or inducible, facilitate the translocation of solutes with simultaneous movement of protons across the yeast cell membrane.
Most species can grow perfectly well in the presence of simple carbon and nitrogen backbone compounds together with inorganic ions and a few growth factors. Yeasts absorb these nutrients through the cell membrane as low molecular weight compounds dissolved in water. Sugars are translocated into yeasts cells by active transport or facilitated diffusion mechanisms which are controlled at the plasma membrane and which depend on sugar, yeast species and growth conditions. For example, *S. cerevisiae* translocates glucose by facilitated diffusion and maltose by active transport. Active transport means that plasms membrane ATPases act as directional proton pumps in accordance with Peter Mitchell's chemiosmotic hypothesis. pH gradients thus drive nutrient transport either via proton antiporters (as is the case with certain sugars and amino acids), or via proton antiporters (as is the case with certain inorganic ions, such as potassium).
Yeast cells may also employ two different transport systems for the same sugar, depending on its availability. There is still a great deal to learn about precise mechanisms of sugar transport, not only in *S. cerevisiae*, but also in other exploitable yeasts. Understanding the regulation of how yeasts acquire sugar and other nutrient solutes is important in

biotechnology, for the reason that efficient transport links to efficient yeast growth and metabolism (Mitchell, 1963; Walker, 1998).

1.1.3 The GAP1 permease in *Saccharomyces cerevisiae*

The founding genetic studies on transport proteins in *Saccharomyces cerevisiae* concerned amino acid permease and led to the genetic notification of a general amino acid permease (*GAP1),* sensitive to the nitrogen suppression and to several more specific transport systems (André, 1995). *S. cerevisae,* among other yeasts, have remarkably proven to be trustworthy system of expression for both endogenous and heterologous eukaryotic proteins and, as any other eukaryotic organism, possess a typical internal organization with membrane delimited organelles. This yeast has been used and studied for many years and a large number of expression vectors are available for protein production and transformation- associated *in vivo* recombination in these vectors allows to easily test various plasmid constructs (Debailleul et al., 2013).

Over 20 specific transport systems mediate active transport of amino acids across the plasma membrane in *S. cerevisae,* also, a number of these permeases have been identified genetically and each is specific for just one of a few related L-amino acids. *GAP1*, the general amino acid permease, can mediate uptake of all protein amino acids, several non-protein amino and toxic analogues. This permease is a member of the amino acid-polyamine-organocation (APC) superfamily and shows very high affinity for most of its natural substrates. These proprieties are well suited to the physiological role of Gap1, which is synthesized and most active under conditions of poor nitrogen supply (e.g. proline, urea, low ammonium, etc.). The role of Gap1 under these conditions is to scavage external amino acids to be used as nitrogen sources or directly as building blocks for protein synthesis. The regulation of the GAP1 permease is complex, a remarkable feature of this regulatory pattern is its pleiotropic character: it is shared by the specific proline permease (PUT4) and the ureidosuccinate-allantoate permease, and these depend on the nature of the nitrogen source(s) in the medium. The presence of ammonia, glutamine or asparagine prevents the development of permease activity and according to Jauniaux & Grenson (1990) it appears that two distinct control mechanisms are involved: repression of permease synthesis and reversible permease inactivation. (Jauniaux & Grenson, 1990).

The transcription of the *GAP1*gene is promoted by two GATA-family factors, Gln3 and Gat1, which are mostly active when the nitrogen supply conditions are cell-growth limiting. If cells shift to more favourable nitrogen supply conditions, the Gln3 and Gat1 factors are inhibited by the mechanism of Nitrogen Catabolite Repression (NCR), leading to a string decrease in *GAP1*'s expression (Debailleul et al., 2013).

According to the study of Debailleul *et al.* (2013), genes under the regulation of P_{GAL1} or P_{GAP1} are only produced when minimal medium is used, in order to impose a specific carbon source for P_{GAL1} (galactose) or a specific nitrogen source for P_{GAP1} (proline), and as *GAP1* is a non essential gene, the result presented for that study has shown that direct genome integration can be used to avoid plasmid instability which is a recurring problem in yeast protein expression, without affecting the number of copys of the gene. The NCR in general and *GAP1* in particular can be used to create a robust, cost-effective and high-throughput expression system in *S. cerevisiae* that can be used when specific requirements are needed such as eukaryotic system, membrane integration or difficult protein secretion.

1.2 THE IMPORTANCE OF AMINO ACIDS TRANSPORTATION

In addition to being the building blocks of proteins, amino acids have a central role in general metabolism. A major achievement in yeast research has been the determination of the complete metabolic pathways for amino acid utilization as carbon and nitrogen sources, amino acid biosynthesis, and the conversion of amino acids to other metabolites including nucleotides.

The function of catabolic nitrogen source utilization, anabolic amino acid and nucleotide biosynthetic pathways are in parallel so these processes must be well coordinated to enable cells to manifest a proper response to nutrient availability. To fulfil these requirements it's important to monitor concentrations of nutrients in extracellular environment and within the cells. Furthermore, catabolic and anabolic pathways generate multiple metabolic intermediates that provide information about the metabolic cell status and exerting regulatory effects (Ljungdahl & Daignan-Fornier, 2012).

Amino acids are used as nitrogen source and yeast cells react to the nitrogen content of the growth environment by controlling nitrogen source uptake and by regulating catabolic and anabolic processes. Yeast can use a variety of nitrogenous compounds as sole sources of nitrogen growth, although some strain variability exists, all L-amino acids, with the exception of lysine, histidine and cysteine, can support growth as the sole nitrogen source.

Amino acids transported across the yeast cell membrane can either be incorporated intact into proteins or they can be intracellularly catabolized to serve as nitrogen or carbon sources, while the growth of yeasts is often better with ammonium salts than when any single amino acid is employed, growth of some yeasts is more rapid in mixtures of amino acids. This is the case with brewer's yeast, where in fact the presence of ammonium ions may inhibit amino acid uptake.

A general succession of events is involved in yeast amino acid transport*: Binding:* this is accomplished by recognition of the amino acid at a specific receptor. *Translocation:* this is mediated by permeases in the plasma membrane and results in concentrative accumulation of amino acids. *Coupling:* uptake is driven by spontaneous influx of protons coupled to ion efflux. *Release:* amino acids are released inside the cell and may be compartmentalized into vacuoles which accounts for the uptake irreversibly (Walker, 1998).

Two classes of amino acids uptake system are synthesized in yeast. One is broadly specific and effects the uptake of all naturally occurring amino acids including citrulline. This is referred to as the general amino acid permease, or Gap1. The other system displays specificity for one or a small number of related amino acids. Amino acid transport (both general and specific) in *S. cerevisiae* and *C. albicans* has been shown to be active and dependent on proton-symport mechanisms.

Considering regulatory aspects of yeast amino acid uptake, Gap1 may be viewed as a scavenger whose expression strongly depends on the nitrogen source availability, as it’s reflected when we try to induce the Gap1 promoter in this study. The Gap1 is not synthesized when yeasts are grown in nitrogen-rich media, for example, containing ammonium ions. While Gap1 is subject to ammonium catabolic repression, consequently, nitrogen catabolite repression in yeasts enables cells to adapt to the changing availability of amino acids (Walker, 1998).

1.3 NITROGEN SOURCE TYPE DEPENDENCY TO GROWTH

Yeasts are capable of employing a range of different inorganic and organic sources of nitrogen for integration into the structural and functional nitrogenous components of the cell, by regulating catabolic and anabolic processes. In industrial fermentation media, available nitrogen is usually in the form of complex mixtures of amino acids, instead of ammonium salts, however, media are often supplemented with inexpensive inorganic nitrogen forms such as ammonium sulphate.

The ability to use amino acids and other nitrogenous compounds requires their internalization, and yeast cells possess multiple permeases to facilitate their transport across the plasma membrane, as mentioned before. Additionally, the presence of external amino acids induces the expression of various broad-specificity permeases; hence amino acids induce their own uptake (Walker, 1998).

Once internalized, nitrogenous compounds can be used directly in biosynthetic processes, be deaminated to generate ammonium, or serve as substrates of transaminases that transfer amino groups to α-ketoglutarate to form glutamate.

In the Ljungdahl & Daignan-Fornier (2012) article it's confirmed that in cells grown on glucose, ammonium can be a simulated by two anabolic reactions, *i,e.*, the synthesis of glutamate from ammonium and α-ketoglutarate catalysed by NADP-dependant glutamate dehydrogenase (*DH1*), and the synthesis of glutamine from ammonium and glutamate by glutamine synthetase (*GLN1*). The fundamental importance of glutamate and glutamine in biosynthesis of nitrogenous compounds is associated to incorporation of nitrogen in the cell, as 85% of the total cellular nitrogen is incorporated via the amino nitrogen from glutamate and the remaining 15% is derived from the amine nitrogen of glutamine, consequently the central core reaction in nitrogen metabolism, becomes apparent by highlighting their involvement in transamination reactions essential in the synthesis of each amino acid.

Ammonium sulfate is a commonly used nitrogen source in yeast growth media since it also provides a source of assimilable sulphur. A variety of organic nitrogen compounds: amino acids, peptides, purines, pyrimidines and amines can also provide the nitrogenous requirements of the yeasts cell. Glutamine and aspartic acids are readily deaminated by yeasts and therefore act as good nitrogen sources. It is clear that cells need to adapt to the quality of the nitrogen source to achieve a balanced state of growth. Nitrogen regulation of transcription is general suprapathway response that is commonly referred to as nitrogen catabolite repression (NCR). NCR primarily functions to guarantee that cells selectively use preferred nitrogen sources when they are available, and in the absence of a preferred nitrogen source, the general derepression of NCR-regulated genes enables cells to indiscriminately scavenge alternative, nonpeferred nitrogen sources (Ljungdahl & Daignan-Fornier, 2012; Walker, 1998).

1.4 AMINO ACID EXCRETION

Yeast cells can excrete metabolites such as amino acids, although this occurs under particular conditions, *e.g* when the amino acid is produced at atypically high levels as a result of deregulated anabolism or when amino acid metabolism rapidly shifts to an imbalanced situation. Since amino acid excretion exists in other eukaryotes as well, it is very interesting to investigate the molecular details of this cell process in yeast.

As already referred, yeast cells hold suprapathway mechanism that, in response to metabolic changes, can reprogram large-scale patterns of gene expression. Suprapathway control is exerted at both the transcriptional and the translational levels. In contrast to this general modes of control, cells can also respond very precisely by regulating the activity of specialised transcription factors that bind the particular metabolite and in response activate or repressed expression of specific sets of genes. These mechanisms are complemented by post-translational modes of regulation, which provides cells with the means to rapidly adjust the catalytic properties of enzymes, modulating the degradation rates of enzymes and permeances and regulating the flow of metabolites in and out of intracellular organelles. With regard to amino acid biosynthesis in yeasts, nitrogen from simple nitrogenous compounds such as ammonium may be assimilated into amino acid *families* (Ljungdahl & Daignan-Fornier, 2012; Velasco et al., 2004; Walker, 1998). The carbon skeletons of these amino acids originate from common precursors of intermediary carbon metabolism as depicted in Figure 1.4.

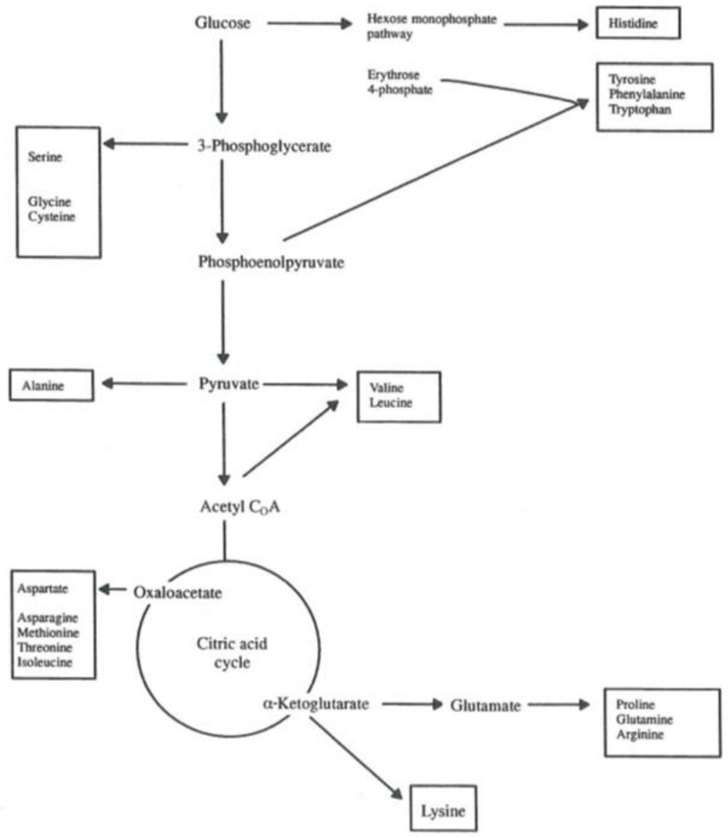

Figure 1.4 Summary of amino acid biosynthetic pathways in yeast (Walker, 1998).

Yeast cells provided with an appropriate source of carbon and ammonium can synthesise all L-amino acids used in protein synthesis. The families of amino acids derived from a common molecule are readily identifiable and include the glutamate family (glutamate, glutamine, arginine, proline, and lysine); the aromatic family (phenylalanine, tyrosine, and tryptophan); the serine family (serine, glycine, cysteine, and methionine); the aspartate family (aspartate, asparagine, threonine, and the sulphur containing amino acids cysteine and methionine); and finally the pyruvate family (alanine and the branched amino acids valine, leucine, and isoleucine). The histidine and nucleotide biosynthetic pathways are connected and the importance of glutamate and glutamine, and consequently the central core reactions in nitrogen metabolism, becomes apparent by highlighting their involvement in transamination reactions required in the synthesis of each amino acid (Ljungdahl & Daignan-Fornier, 2012). Potassium limitation is intimately intertwined with ammonium toxicity.

- Growth of *Saccharomyces cerevisiae* is limited at low concentrations of potassium ions by ammonium toxicity.
- Ammonium toxicity also occurs, even in the presence of high concentrations of potassium ions, if ammonia transporters are constitutively expressed.

o the physiological response of yeast to ammonium toxicity involves excretion of large quantities of amino acids.

According to Hess et al., it was found, via metabolite profiling that, when experiencing ammonium toxicity, yeast respond by excreting amino acids, such amino acids may constitute a rudimentary ammonia detoxification mechanism in yeast.

The potassium limitation affects not only yeast growth but also gene expression, since a large number of genes affected are known to be involved in nitrogen metabolism (Hess et al., 2006). Studies on bacteria, also have shown that the excretion of amino acids can occur when central metabolism is imbalanced: e.g. when carbon source and energy sources are present in excess and massively used by cells but growth is limited by lack of an essential nutrient or other compound, the excretion of amino acids is also observed under conditions of limited peptide catabolism , for instance, L-lysine excretion is observed in bacteria using L-lysine-containing peptides as a carbon source but lacking L-lysine-degrading enzymes. Deregulated anabolism is yet another situation favoring excretion of amino acids by bacteria. As for studies using yeast as a model have shown that this simple eukaryote can also release detectable amounts of amino acids into the medium, this was typically observed with cells producing unusually large amounts of an amino acid as a result of deregulated anabolism and impaired catabolism, it was also reported that mutants also traffic for specific amino acid or for pyramiding excrete a number of different and unrelated amino acids once the cells are transferred to a medium lacking the required compound, such conditions causing growth arrest. These studies have also shown that excretion is usually more pronounced if cells are flawed in the permeases that mediate high-affinity uptake of the excreted compound, this has led to the notion that high-affinity permeases may play an essential role in retention of intracellular compounds. In pluricellular organisms the transfer of amino acids between tissues is done through transport in and out of the cell, in the case of plants, root cells are known to release amino acids into the xylem sap for subsequent distribution to different organs, but the mechanism of this excretion is yet to be recognized. Continuing on the study of Velasco et al. (2004) it was shown that the overexpression of the protein Aqr1, a member of the multidrug resistance transporter family characterized, resulted in the excretion of several amino acids such as glutamate and alanine and is involved in the excretion of Homoserine. It was speculated that close homologues of Aqr1 might be involved in excretion of amino acids or derivatives (Velasco et al., 2004).

1.5 THE MAJOR FACILITATOR SUPER FAMILY

The major facilitator superfamily (MFS) is the largest known superfamily of secondary carriers found in the biosphere. According to Pao *et al.* in 1998, MFS transporters are single-polypeptide secondary carriers capable only of transporting small solutes in response to chemiosmotic ion gradients. Although well over 100 families of transporters have now been recognized and classified, the ABC superfamily and MFS account for nearly half of the solute transporters encoded within the genomes of microorganisms.

The MFS was originally believed to function primarily in the uptake of sugars but, subsequent studies revealed that drug efflux systems and Krebs cycle metabolites belong to this family (Pao et al., 1998; Reddy et al., 2012).

Members of the MFS family are divided into 17 families. The members of each family all proved to be more closely related in sequence to each other than they were to any of the other MFS proteins. This fact presumably reflects the evolutionary histories of these proteins and, remarkably, we find that phylogenetic family correlates with function, thus each of the families recognizes and transports a distinct class of structurally related compounds.

The largest family is the sugar porter (SP) family, with 133 identified members. Families DHA1 and DHA2 consist of drug efflux systems which possess 14 and 12 TMSs (transmembrane spanner), respectively, since these permeases uniformly catalyze drug:H+ antiport. Respectively, a total of 30 and 46 sequenced members are currently recognized in these two families. Members of both families are found in bacteria and eukaryotes, and DHA1 family members have also been identified in archaea (Saier Lab Bioinformatics Group, 2020), the phylogenetic tree as viewed below, in Figure 1.5.

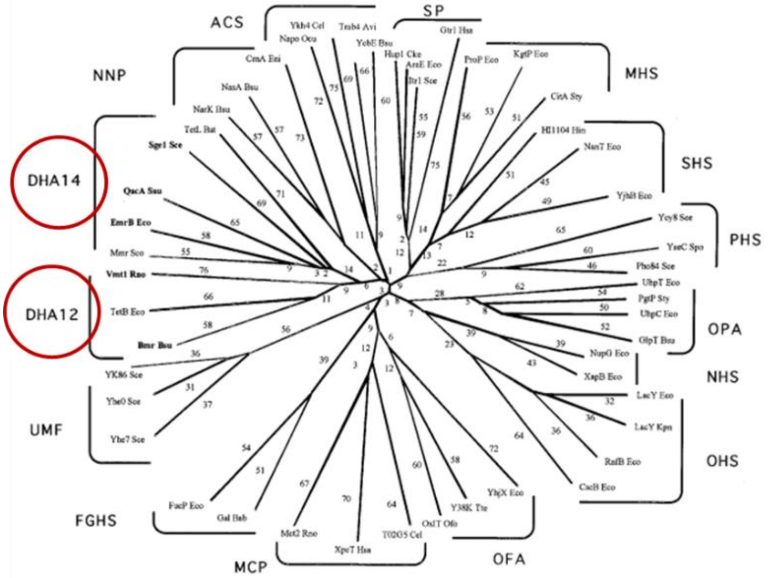

Figure 1.5 Phylogenetic tree for the MFS including representative members of most of the currently recognized constituent families (Pao et al., 1998).

The DHA1 and DHA2 families branch off from each other after the initial divergence from the center of the tree, suggesting that they are more closely related to each other than to other MFS families.

1.5.1 Multidrug resistance transporter genes

The simultaneous acquisition of resistance to a wide range of structurally and functionally unrelated cytotoxic chemicals, is found in a wide variety of organisms, from bacteria to mammals. Multidrug resistance (MDR), is an universal biological phenomenon that causes serious problems in the treatment of human cancers and infections of bacterial and fungal origin, since cells can develop resistance to a variety of drugs that can lead to treatment failure. It is usually associated with specific transport systems that catalyze efflux of structurally and functionally unrelated transports out of the cell. The most important MDR transporters belong either to the ABC family (ATP-bidding cassette) or to the MFS (major facilitator family) which differ by the way they are energized (ABC transporters bind ATP and require ATP hydrolysis for transport activity; MFS-mediated transport is driven by the proton-motive force.). Although the phylogenetic trees of ABC and MFS transporters reveal striking differences in the evolution of the gene families and subfamilies, the individual gene families already exited in the common ancestor of all these fungal species and were remarkably conserved in evolution.

In the *S. cerevisiae* genome the products of 22 genes are categorized as primary active transporters of the ABC family Among those, 16 proteins are about twice as long as the other six, since the larger proteins have 12 transmembrane-spanning domains (TMS) and probably result from by duplication of the half- size ABC transporters with six TMS. (Gbelska et al., 2006).

1.5.2 **MFS Drug: H+ Antiporters, the DHA1 family**

The MFS-MDR transporters are classified into two families according to the number of predicted transmembrane spans: The Drug:H+ Antiporter-1 (12-Spanner; DHA1) Family, and the Drug:H+ Antiporter-2 (14-Spanner; DHA2) Family. On average the DHA2 family is more variable between genomes than the DHA1 family and both are more variable than the ABC transporters. In the DHA1 family 51 members were found in the five genomes. The DHA1 drug efflux family, consists of 46 proteins, of these, 9 have been shown to be multidrug resistance pumps, 15 are probably drug-specific efflux pumps, and 22 are hypothetical or uncharacterized proteins. Like the DHA2 family, functionally characterized members of the DHA1 family exhibit specificities only for drugs. As described in Figure 1.6 cluster 1 is represented by the S. cerevisiae genes *AQR1*, *QDR1/2*, *DTR1*, *QDR*3, and Cluster 2 by *TPO1*, *TPO2/3*, *TPO4*, *FLR1* (and *HOL1*) (Gbelska et al., 2006).

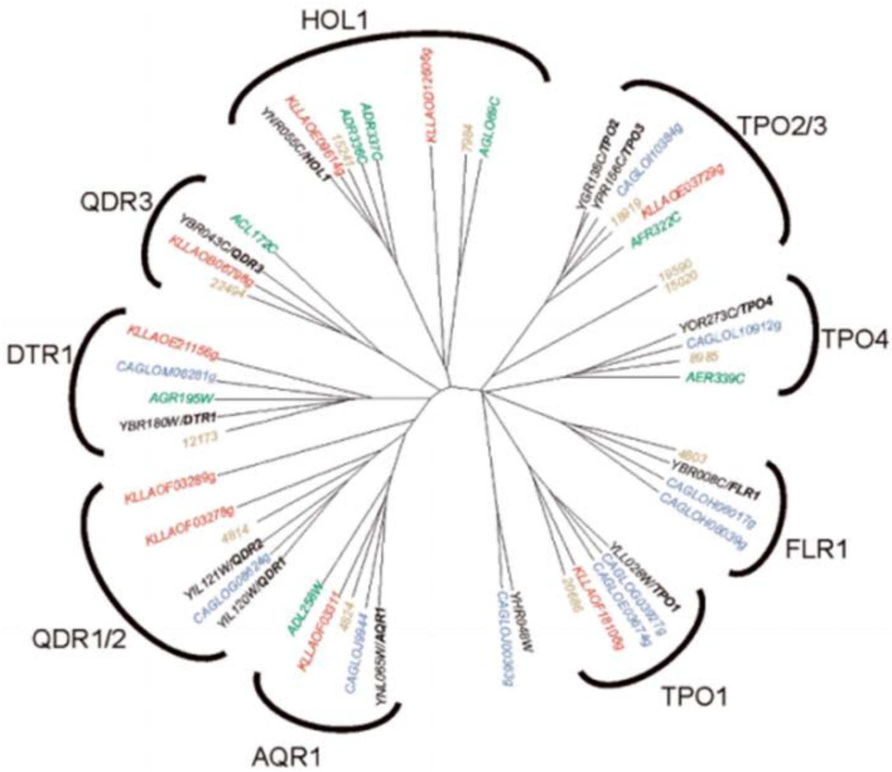

Figure 1.6 Phylogenetic relationship of the DHA1 family of Drug:H+ antiporters (major facilitator superfamily). ORF names for the different species are colour-coded: *Saccharomyces cerevisiae* black, *Candida glabrata* blue, *Kluyveromyces lactis* red, *Kluyveromyces waltii* brown, and *Ashbya gossypii* green. (Gbelska et al., 2006).

Most gene families of MDR-related MFS transporters have also diverged early in evolution and orthologues of the *S. cerevisiae* genes are found in other species. The DHA2 family shows the highest degree of variation and in several cases true orthologues cannot be assigned simply based on sequence comparison. Nevertheless, the phylogenetic trees are a good indication for potential functional equivalence.

In DHA1 family the function of the 12 *S. cerevisiae* family members as MDR determinants has been confirmed following their systematic individual disruption (Gbelska et al., 2006).

The subclusters represented by the *DTR1* and the *AQR1* genes have the five-ended branches typical for the ABC transporters. The strict conservation of gene number indicating selection pressure against gene duplication suggests that the drug resistance phenotypes associated with mutations in these genes may be only indirectly related to the physiological function of the encoded transporters. Its known that, the *AQR1* gene product was shown to be involved in excretion of excess amino acids whereas *DTR1*, which is localized in the prospore membrane, plays an important role in spore wall since it facilitates the translocation of bisformyl dityrosine, the major building block of the spore surface, through the prospore membrane synthesis (Felder et al., 2002; Velasco et al., 2004). The products of the genes *QDR1*, *QDR2* and *QDR3* were characterized as drug efflux pumps and they are located in the plasma membrane and confer resistance to quinidine and to the anticancer agents cisplatin and bleomycin, also the genes *QDR1/2*, *QDR3*, *AQR1*, *DTR1*, *TPO1* and, *TPO4* are able to protect the cell against other compounds that usually absent from natural environment of yeast cells, this, according to Sá-Correia et al. (2009) provides the hypothesis that their natural physiological role might have nothing to do with large chemoprotection as proposed for bacterial multidrug transporters but that they might have specific physiological substrates, whereas drugs would be transported fortuitously or opportunistically. The expression of *DTR1* in vegetative cells renders the cells slightly more resistant against antimalarial drugs and food grade organic acid preservatives. (Gbelska et al., 2006; Sá-Correia et al., 2009).

1.6 *DTR1* GENE

*DTR1*p is the first multidrug resistance protein of the major facilitator superfamily with an assigned physiological role in the yeast cell. As already mentioned, *DTR1*p facilitates the translocation of bisformyl dityrosine trough the prospore membrane during the spore wall maturation. *DTR1*p is also a MDR determinant in yeast cells, conferring resistance to several unrelated drugs, such as quinine or benzoic acid (Felder et al., 2002; Sá-Correia et al., 2009). *DTR1* might transport other amino acids, and the aim of study is to know if that happens and how does it happen by study this protein on a genomic and plasmidic level through overexpressing it.

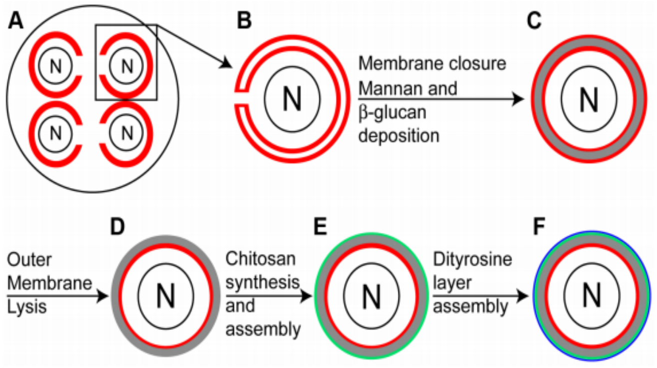

Figure 1.7 Overview of spore wall formation. (A) Each of the four nuclei (N) in a sporulating cell are engulfed by a prospore membrane (red). B) A single prospore membrane prior to closure. C) After closure of the prospore membrane, mannans and β-glucans (gray) are deposited in the lumen between the spore plasma membrane and outer membrane derived from the prospore membrane (both in red). D) The outer membrane disappears, exposing spore wall material to the ascal cytoplasm. E) Chitosan is synthesized and assembled as a discrete layer (green) on the outside of the β-glucan. F) The dityrosine layer (blue) is formed on the outside of the chitosan layer (Lin et al., 2013).

In Figure 1.7 is represented that the spores are formed by an unusual cell division in which daughter cells are generated within the cytoplasm of the mother cell. Intracellular membranes named prospore membranes absorb and eventually enclose each of the 4 nuclei generated by meiosis, bringing out the daughter cells. Closure of a prospore membrane envelops a nucleus within two membranes; the plasma membrane of the spore and an outer membrane that separates the spore plasma membrane from the mother cell cytoplasm. After prospore membrane closure a spore wall is formed around each spore.

In Figure 1.7, the letter C illustrates the completed spore wall consists of four distinct layers. The first two layers are composed of mannan and b-glucan and are similar in composition to the vegetative cell wall, these are formed between the spore plasma membrane and the outer membrane. Represented by letter D, is the b-glucan layer is completed, after, the outer membrane is lost exposing the spore wall directly to the cytoplasm of the surrounding ascus. The two outer layers of the spore wall are made from components unique to the spore, next, represented by letter E is the layer of chitosan, which is formed on top of the b-glucan layer after outer membrane lysis. Once assembled, the chitosan layer forms a surface on which the outermost layer of the spore wall assembles. Finally, the letter F represents the major component of this outer layer, the cross-linked amino acid dityrosine. Dityrosine is formed in the spore cytoplasm by the Dit1 and Dit2 enzymes, these dityrosine molecules are then moved to the spore wall by the action of transporters localized in the spore plasma membrane. The primary transporter is encoded by *DTR1*, together, the outer spore wall chitosan and dityrosine layers confer enhanced resistance to environmental stresses on the spore, including the ability to pass through the digestive tracts of insects, permitting dispersal in the environment.(Felder et al., 2002; Lin et al., 2013).

On the study of Felder (2002) it was confirmed that the expression of *DTR1* increased during sporulation and that it is a sporulation-specific gene. It is referred that the properties of *DTR1*p make it an excellent marker to study the fate of the prospore membrane in mutants with impaired spore wall assembly and morphogenesis and for the isolation and purification of the still poorly characterized prospore membrane. It was confirmed that dityrosine transport by *DTR1*p is unidirectional from the inside to the outside of the cell and observed a slight but consistent increase of resistance in the presence of *DTR1*p against the important food-grade organic acid preservatives propionic acid, benzoic acid, and butyric acid, as well as against the antimalarial drugs quinine and quinidine (Felder et al., 2002).

1.7 OBJECTIVE

This thesis is integrated in the project of the Institut de Recherches microbiologiques Wiame (IRMW) in the CERIA campus in Brussels (now renamed Labiris).
Was integrated in the study of various genes belonging to the poorly characterized DHA1 transporter family of proteins in yeast.
In this thesis it was proposed the characterization of the *DTR1* gene through its overexpression knowing that *DTR1* as already a role in yeast physiology. The specific aims of this study are to study DTR1 on a genomic level with GFP protein fusion downstream in order to observe its location on the cellular membrane under its own promotor, and with the *gal1* promotor upstream DTR1 we can verify its expression and if this expression occurs the same way as in its natural state, also the overexpression of *DTR1* on a plasmidic level by transforming the plasmids GAP1 and UGA4, by removing the coding part of UGA4 and GAP and replacing it with our gene of interest, the *DTR1*gene and analysis under the *GAP* promoter, that is influenced by nitrogen source, so its possible to observe its behavior when overexpressed.

2 Materials & Methods

2.1 MATERIALS

Media compositions for cultures as well as stock solutions for media preparation and antibiotics were prepared in the laboratory.

The media composition presented in Table 6-2 in the appendices section are specific media prepared in the laboratory therefore the names they have are specific to the laboratory in question. The media YNB and 164 are yeast specific minimum media, 165 is a media based on 164 not containing ammonium. To this media it was added (as it was necessary) a carbon source, a nitrogen source and amino acids to cover auxotrophic requirements necessary for yeast growth.

2.1.1 **Strains**

All work was performed using the yeast *Saccharomyces cerevisiae* strain 23344c, MG408, MG401, MG360, 27061, JA188 and 32501d and the strain of *E. coli* strain *MC1061 and XL1Blue.*

2.1.2 Primers

The primers used throughout this study are in Table 2-1. Note that, P is for a presence of a promoter, U for the region upstream the promoter, O for the ORF (***O**pen **R**eading **F**rame*) and D to indicate the primers found in the zone located directly after the stop codon of the ORF. Every primer represented in the 5’ →3’ sense.

Table 2-1. Primers classification according to the study’s aim.

Diagnosing deletion of genes	KanV	GTATGGGCTAAATGTACG
	KanR	CCTCGACATCATCTGCCC
GFP Fusion	R3 *DTR1*	TTCTAAAAGTTAAATTTTATAGCTAAACATAAACTATTTCTCGATGAATTCGAGCTCG
	F5 *DTR1*	TCTTCTCATTTTTCAAAGAAAATATACTGCCAAAGAATTTGGTGACGGTGCTGGTTTA
P_{GAL1} Insertion	R2 *DTR1*	TTTGCAGACCCAAATTCTTTTTCTGAAACGGTTCGCTTCCCATTTTGAGATCCGGGTTTT
	F4 *DTR1*	TCAAAAAAGAGTTTCAAGAGCTGGGAAAAGAATTAAGGCAGAATTCGAGCTCGTTTAAAC
PCR verification	A1 *DTR1*	GGGCTACTGTAACCTGCGTTAT
	A2 *DTR1*	TTTTGGGCCCAATGTCTGGA
	A3 *DTR1*	ACAGCTCTGTAACGCAATGG
	A4 *DTR1*	GTCAGAAACGCTCAAGCTCA
plasmid construction	R6 *DTR1*	CTCATTTTTCAAAGAAAATATACTGCCAAAGAATTTCTAGAAGTTCTATTTCAAGGTCCA
	F6 *DTR1*	ACAGCAACATTTATAAGAAACAAAAAAAAGAAATAAAAAATGGGAAGCGAACCGTTTCAG
qPCR	TBP1 O1	TATAACCCCAAGCGTTTTGC
	TBP1 O2	GCCAGCTTTGAGTCATCCTC
	DTR1 O1	TGCTGGAACTGTTGCTGT
	DTR1 O2	AGACCGAGCCCTGTAAAA

It is important to refer that the cassettes used for the deletion of genes confer resistance to an antibiotic. Although the resistance marker may sometimes differ, it possesses common parts. This allows us to limit the number of oligonucleotides required to verify the deletion/insertion into the transformed strains.

2.2 METHODS

2.2.1 *E. coli* -specific procedures.

2.2.1.1 Preparation of E. coli competent cells

The capability to introduce plasmid DNA molecules into the cells has been of key importance to the advancement of molecular biology. There are various techniques on how to introduce plasmid DNA into the cell, *e.g.* chemical treatment, electroporation, use of biolistic gun, polyethylene glycol (Bergmans et al., 1981; Dower, 1990; Drury, 1994; Kurien & Scofield, 1995; Smith et al., 1992). The physiological state of cells that enables them to bind and take up high molecular weight exogenous DNA is called “competence”. Uptake of free DNA by *E. coli* cells which have become competent by treatment of chemicals providing Ca^{2+} ions followed by a heat shock pulse was first reported by Mandel and Higa (1970) Among many cations tested (Ca^{2+}, Mn^{2+}, Sr^{2+}, Ba^{2+}, Mg^{2+}, Na^{+} and Rb^{+}), Ca^{2+} (100-200 mM range) provided comparatively better transformation of *E. coli*. Transformation frequencies obtained using these methods range approximately 105 -107 transformants/µg of DNA (Eynard & Teissié, 2000; Tsai et al., 1989).
Successfully transformed cells are usually identified by selection or screening markers, such as drug resistance or fluorescence. This technique is commonly used to transform cells with plasmids for various purposes like recombinant protein expression, cloning, and long term storage of plasmids (Chang et al., 2017).

2.2.1.1.1 Preparation of $CaCl_2$ competent cells

A preculture of *E. coli* strain *XL1Blue* in 853 medium was prepared by growing them overnight under agitation (150 rpm) at 37°C.

The day after, the culture was diluted to an optical density at 660 nm of 0.1 in prewarmed 853 medium (10 mL of culture for each transformation) and was incubated until the optical density reached about 0.6 (exponential phase). A 500 µl volume of the culture was transferred to a Falcon tube and centrifuged at 16 000 xg for 5 minutes at 4°C. The cells were washed with 1/2 volume of 50 mM $CaCl_2$ cold and sterile. Afterwards the culture was centrifuged at 4000 xg for 5 min at 4°C and the supernatant was eliminated. The pellet was washed with 1/10 volume of cold and sterile 50mM $CaCl_2$ and centrifuged at

4000 xg for 5 min at 4°C following the removal of supernatant. The pellet was washed with 1/50 volume of cold and sterile 50mM $CaCl_2$ following new centrifugation at 4000 xg for 5 min. at 4°C for removing the supernatant. The pellet was maintained on ice for 30 min. The pellet was resuspended in 1/50 volume of cold and sterile 50mM $CaCl_2$ supplemented with 15% glycerol and then distributed (150 µL) in Eppendorfs and flash freezed in liquid nitrogen. The treated cells were stored at -80°C.

2.2.1.1.1.1 Transformation

For *E. coli* transformation all work was performed on ice and with cold solutions.

$CaCl_2$-competent cells aliquots were placed on ice to thaw. To 100 µL competent cells 1µL (1-100ng) of the DNA was added and the incubation was done for 30 minutes on ice. Then, the cells were subjected to a heat shock at 42°C for 2 minutes in a water bath. And finally 500 µL of 853 medium was added to each transformation tube.
Afterwards the cells were recovered for 30 minutes at 37°C. Samples were centrifuged at room temperature at 13 000 rpm for 2-3 minutes and grossly the supernatant was removed.
The pellet was resuspended in the remaining supernatant and spread on a Petri dish containing the selective medium and incubated overnight at 37°C.

2.2.1.2 Small-scale isolation of plasmidic DNA (Mini Preps)

This protocol was performed using the DNA & RNA Purification Plasmidic Miniprep Kit. *(Sigma Aldrich, Belgium).*

In the preparation of the column it was added 500 µL of column Preparation Buffer added to the column and centrifuged for 1 minute at 13000 rpm at room temperature.
The supernatant was transferred to the column and centrifuged for 1 minute at 13000 rpm at room temperature
Then, 500 µL of Wash 1 Buffer was added and centrifuged for 1 minute at 13000 rpm into the same conditions at room temperature.
A quantity of 750 µL of Wash 2 Buffer was added and centrifuge for 1 minute at 13000 rpm at room temperature. Another centrifugation was done, under the same conditions to

dry the column, for 3 minutes, and then the column was transferred to a new Eppendorf with a capacity for 1.5 mL and eluted with 50µL of water.
Finally, the Eppendorf tube containing the purified plasmid was centrifuge for 1 minute at 13000 rpm at room temperature and the plasmid concentration measured.

2.2.1.3 Plasmid Isolation

This protocol was performed using a kit from Qiagen©, QIAfilter Plasmid Midi Kit *GenElute HP Plasmid DNA Midiprep Kit - 25 Purifications | Gen Elute | (Sigma-Aldrich, Belgium*).

The plasmid DNA isolation began harvesting the *E. coli* culture and proceeded to centrifuge in Falcon tubes at 6000 xg in a Sorvall BioFuge Primo model centrifuge (Thermo Scientific) for 15 minutes at 4 °C. Then, the supernatant was pooled, and the pellet resuspended with 4 mL of Buffer P1. A quantity of 4 mL of Buffer P2 (Lysis Buffer) was added, mixed by inverting the tube 4-6 times, and incubated at room temperature for up to 5 minutes. During incubation, the cap onto the outlet nozzle of QIAfilter Cartrige was screwed and placed on a rack. Then, 4 mL of prechilled Buffer P3 (neutralization Buffer) was added to the lysate and mixed immediately and thoroughly by inverting 4-6 times. The lysate was poured into the barrel of the QIAfilter Cartridge and incubated at room temperature for up to 10 minutes.
Equilibrating the QIAGEN-tip by applying 4 mL Buffer QBT, allowed the column to empty by gravity flow. Next, the cap from the QIAfilter Cartridge outlet nozzle was removed and the plunger was gently inserted into the QIAfilter Cartridge, and the cell lysate filtered into the equilibrated QIAGEN-tip. The lysate was allowed to enter the resin by gravity flow. Subsequently, the QIAGEN-tip was washed with 2x10 mL Buffer QC and the DNA eluted with 5 mL of Buffer QF. Finally, the DNA precipitated by adding 3,5 mL room temperature isopropanol, mixed and centrifuged at 6000 xg for 1 hour and then carefully the supernatant was decanted.

2.2.1.4 Colony PCR

For the preparation of the DNA template, one *E. coli* colony was resuspended in the PCR reaction mixture (see 2.2.3.1) and 1 µL 20% Triton X-100 was added to 20 µL PCR reaction (see 2.2.3.1).

2.2.2 **Yeast-specific procedures**

2.2.2.1 Transformation

The day before 20 mL of culture with the yeast strain was inoculated and cells were grown overnight under agitation (200 rpm) at 30°C. Then the yeast culture was diluted to an OD_{600nm} of 0.1 in prewarmed 863 medium (10 mL of culture for each transformation) and grown overnight until the optical density at OD_{600nm} reached about 0.6 (exponential phase).

A volume of 10 mL of the culture was transferred to Falcon tubes with a capacity for 50 mL per transformation and centrifuged at 6000 xg for 5 minutes and the supernatant was removed.
The pellet was washed with 20 mL of sterile water, and after centrifuged again at 7000 rpm for 5 minutes and the supernatant removed.
The flasks with 5 mL of 100 mM Lithium Acetate were washed and centrifuged again at 7000 rpm for 5 minutes and the supernatant. removed . The pellet was resuspended in 50 µL of 100 mM Lithium Acetate per sample and finally incubated for 20 minutes at 30°C. During incubation, the DNA carrier (DNA from fish sperm 10 mg/mL) was boiled during 10 minutes and then maintained on ice.
For transformation each Eppendorf (with a capacity of 1.5 mL) was prepared containing 5 µL or 10µL of DNA, 50 µL of cells to be transformed, 5 µL of DNA vector.
These Eppendorf tubes were incubated at 30°C for 20 minutes and gently 300µL of Lithium Acetate /PEG 40% were added and homogenized by Vortex for 1 minute. Following, the tubes were incubated for 20 minutes at 30°C and then were transferred to a thermic bath at 42°C for 20 minutes Afterwards, the tubes were centrifuge for 5 minutes at 7000 rpm and the supernatant removed and resuspended in 1 mL of 863 medium.

The transformant cells were transferred to a Falcon tube of 15 mL containing 3 mL of rich media and incubated for 4 hours at 30°C under agitation (200 rpm). Then, the culture was centrifuged for 5 minutes at 7000 rpm and the supernatant removed. Finally, the yeast pellet was resuspended in 100 µL of water and spread on a Petri dish containing selective media and incubated overnight at 30 ºC.

2.2.2.2 Transformation by electroporation

Electroporation refers to the process of subjecting living cells to a rapidly changing, high-strength electric field, producing transient pores in their outer membranes, if pores occur in adjacent cells, this can facilitate fusion of the cells (Zimmerman, U., 1982). The electrical apparatus used to operate cells for transformation and gene expression experiments has relied upon the discharge of a capacitor through the cellular suspension to generate the required electric field. (Calvin & Hanawalt, 1988). ‘‘Electropermeabilization’’ or ‘‘electrotransfection’’ or ‘‘electroinjection’’, as it is also called, has become an increasingly popular technique for introducing foreign DNA or plasmid DNA into various cell types as it offers several advantages over conventional techniques of gene transfer as technical simplicity, ease of operation, rapidity and reproducibility as well as greater transformation efficiencies (expressed as the number of transformants per µg of input plasmid DNA) compared to the best chemical methods like CaCl2-mediated and PEG mediated transformation and also avoids the deleterious toxic side effects of chemicals like PEG and though the technique is a useful and important mean for introducing exogenous DNA it can also be used to translocate other substances like proteins, antibodies, drugs, enzymes hormones, nucleoside triphosphates and nucleoside analogues, etc (Prasanna & Panda, 1997).

2.2.2.2.1.1 Preparation of electrocompetent cells

A pre-culture of 863 medium with the *S. cerevisiae* 23344c to be transformed was prepared in order to obtain a culture of 200 mL with an optical density at OD_{600nm} of about 0.8. Afterwards, 100 mL of culture was transferred into a Falcon tube and centrifuged at 4°C at 4000 gx for 5 minutes. The supernatant was eliminated, and the pellet resuspended in 100 mL of sterile water following a centrifugation at 4°C at 4000 g for 5 minutes and the supernatant was removed. The pellet was resuspended with 8 mL of 1M Sorbitol and centrifuged at 4000 g at 4°C for 5 minutes and the supernatant eliminated. Finally, the pellet was resuspended in 100 µL of 1M Sorbitol.

2.2.2.2.1.2 Transformation

A volume of 100 µL of the competent cells were transferred into an Eppendorf tube and 10 µL of the DNA fragment was added . The electroporation cuvettes were maintained on ice for 5 minutes before electroporation. After that the competent cells were submitted to an electro shock under the conditions: 1.5 KV, 25µF and 200 Ω. Then the cells were resuspended in 1 mL ice cold 1M Sorbitol. All the content was then transferred to an Eppendorf tube and centrifuged for 5 minutes at 4°C at 4000 xg. About 800µL of the supernatant was removed and the cells were resuspended in the remaining liquid.

In Petri dishes containing the selective medium, 100 µL of cells were inoculated and were incubated overnight at 30°C.

2.2.2.3 DNA isolation

A volume of 10 mL of culture with the transformed *S. cerevisiae* 23344c cultures with the plasmids/DNA of interest, was prepared by growing the cells overnight under agitation (200 rpm) at 30°C. The day after 6 mL of culture was centrifuged in Eppendorf tubes, with a capacity of 2 mL each, for 2 minutes (3 x 2mL each time with removal of supernatant in between) at 13 000 rpm. A quantity of 0.2 mL of lysis solution, 0.2 mL of phenol-chloroform-isoamyl alcohol (25:24:1) and 0.3 g of glass beads was added and then proceed to vortex for about 2 minutes continuously. The Eppendorfs were centrifuged for 5 minutes at 13000 rpm. Supernatant was transferred to an Eppendorf and proceed to DNA precipitation by adding 1/10 volumes of Sodium Acetate 50mM (NaAc) and 2 to 2.5 volumes of pure ethanol, the tubes were placed in -20°C for 15 to 20 minutes. Then, the tubes were centrifuged for 20 minutes at 4°C at maximum speed in a Heraeus Fresco 17 model centrifuge (Thermo Scientific). The supernatant was grossly removed and 300 µL of 70 % ethanol was added. After that the sample was centrifuged for 5 minutes at 4°C at maximum speed. The supernatant was grossly removed and the tubes were left to dry at 37°C for 30 minutes. Finally, the pellets were resuspended in 100 µL of water. Genomic DNA concentration was determined using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific) absorbance reports at 230, 260 and 280nm allow to quantify the purity of the nucleic acid, the 260/280 ratio of 1,8 is generally accepted as pure for DNA, as for the 260/230 ratio values for pure nucleic acid should be higher than the respective 260/280 values as expected 260/230 values are commonly in the range of 2.0-2.2.

2.2.2.4 Colony PCR

For the preparation of the DNA template, 20 µL of SDS 0.25% was transferred to PCR tubes and a sample of each colony was resuspended in SDS. Then, the tubes were treated at 95°C for 10 minutes. Afterwards were centrifuged for 3 minutes at 7000 rpm. Subsequently 1µL of supernatant and 1µL 20% Triton X-100 were transferred to a 20 µL PCR reaction (see 2.2.3.1).

2.2.3 **DNA Procedures**

2.2.3.1 DNA amplification via PCR

The PCR reaction was prepared containing 25 µL/10 µL (for 50 µL tubes and for 20 µL tubes, respectively) DreamTaq Green PCR Master Mix (2X); 1µM of each primer; 1 µL target DNA and water to complement the volume. It is important to note that Thermo Scientific DreamTaq Green PCR Master Mix (2X) is a ready-to-use solution containing DreamTaq DNA Polymerase, optimized DreamTaq Green buffer, MgCl2, and dNTPs. (Catalog number K1081, Thermo Scientific™).

For analytical PCR amplifications, the PCR reaction volume was 20 µL and for preparative PCR amplifications, the PCR reaction volume was 50 µL. The PCR amplification was performed onto a model by MJ Research, the Bio-Rad PTC 200, and the amplification programme was as follows:

1. Denaturation at 95°C for 10 minutes
2. Denaturation at 95°C for 1 minute
3. Annealing at 50°C for 1 minute
4. Elongation at 72°C for 2:30 minutes
5. Repeat 34 times steps 2-4
6. Final elongation at 72°C for 10 minutes

The whole content of the PCR tubes was loaded into an agarose gel (see 2.2.3.3). The run was performed at 100 V during approximately 30 minutes on an Advance Mupid-exu Submarine Electrophoresis system. To the gel, was added 5 µl of the intercalant agent Sybr Green I 10,000x concentrated, in order to be able to see the gel under UV light.

2.2.3.2 Purification of fragments of DNA from agarose gel

This protocol was performed using the High Pure PCR Product Purification Kit from Roche Company.

Part of the gel containing the desired fragment was cut and transferred into an Eppendorf tube and the gel fragment was weighted. Then 300 µL of Bidding Buffer was added for each 100 mg of gel. The Eppendorf was placed in the thermomixer without agitation at

56°C for 10 minutes and gently mixed every 2 or 3 minutes to melt the gel, the machine used is a model Thermomixer Comfort by Eppendorf.

The column was centrifuged for 1 minute at 13000 rpm and the supernatant removed, a volume of 500 µL of washing buffer was added and centrifuged for 1 minute at 13000 rpm and finally the supernatant removed. Afterwards 200 µL of washing buffer was added, centrifuge for 1 minute at 13000 rpm and supernatant removed. Once again, it was centrifuged under the same conditions for 3 minutes in order to dry the column. Ultimately the column and the tube were separated and placed in a new Eppendorf tube where 40 µL of water was added and the tube was centrifuged for 1 minute at 13000 rpm.

2.2.3.3 Agarose gel for electrophoresis

The Gel running was performed in a Mupid® -exU New generation Submarine Electrophoresis System model by Advance Co.

For small 1% agarose gel (13 slots), 50 mL of Buffer TBE 10x was added, with 0.3 g of Agarose and 3 µL SybrSafe 10,000x concentrated. As for preparation of a large 1% agarose gel (26 slots), 100 mL of Buffer TBE 10x was added, plus 0.6 g of Agarose and 5 µL SybrSafe 10,000x concentrated.

The agarose was dissolved into the buffer onto an Erlenmeyer flask laying it in to the microwave for 2 minutes and then cool down under tap water. Then, SybrSafe was introduced left to cool down for 30 minutes. The running Buffer was TBE 10x.

2.2.3.4 Quantitative Real Time PCR – qPCR

Quantitative PCR follows the same principle as the classic PCR but allows to quantify the DNA present in samples through the cycles of amplification, particularly at the end of each step elongation, allowing to deduce the initial DNA concentration before amplification. This measurement is made using a fluorescent marker (SYBR Green I) that intercalates in the DNA, the intensity of the signal emitted being proportional to the quantity of double stranded DNA present in the well. The excitation and emission maxima of SYBR Green I are at 494 nm and 521 nm.

The qPCR was performed using the LightCycler® 96 System model (Roche Life Science), results obtained from its own software, LightCycler® 96 version 1.1, and then transferred to an Excel sheet, under the following parameters:

Table 2-2. qPCR parameters.

Parameters	Cycles	Description
Pre-incubation	1	95°C for 600 seconds
2 step amplification	35	95°C for 10 seconds
		60 °C for 30 seconds
Melting	1	95°C for 10 seconds
		65 °C for 60 seconds
		90 °C for 1 second
Cooling	1	37 °C for 30 seconds

The fluorescence measures taken into account are performed at the end of each exponential phase of the PCR amplification. For each sample, when the reaction reaches a fluorescent intensity above the background, the cycle at which the sample reaches this level (named Cycle Threshold, Ct), is recorded.

Also, for each primer pair, a standard curve is performed to test the effectiveness of the PCR reaction and calculate DNA concentrations based on arbitrary DNA quantities in the standard DNA sample. To produce this, four dilutions from 10 to 10000 are prepared from a purified DNA extract solution from PCR product.

The results of qPCR were calculated by comparison of the target DNA with a reference gene *TBP1* since this gene does not present changes in the different culture conditions that were used. The results are presented based on a comparison of target DNA concentrations (*DTR1*) divided by *TBP1* concentrations.

The mixture reaction for 1 sample (to be multiplied by the number of samples) was 5.1µL qPCR Mix Green (Contains FastStart Taq DNA Polymerase, reaction buffer, dNTP mix, SYBR Green I dye, and MgCl2); 1.6µL water; 0.4µLof each primer (5 µM).

The mix reaction was distributed in Eppendorf tubes with a capacity for 1,5 mL, homogenized by vortexing following a quick spin and placed on ice while preparing the standard dilutions.

Meanwhile the standard dilutions were prepared by adding 45 µL of water in 4 Eppendorf tubes.

Starting from the 10000 standard dilution (the most highly concentrated standard, corresponding of a genomic DNA sample of known concentration) 5 µL were transferred to the Eppendorf tube marked as 1000 dilution, then of then 5 µl and place it into the

Eppendorf marked as 100 and from that on until the Eppendorf marked as 1 (10000, 1000, 100, 10 or 1) The Eppendorf tube marked with 0 only has water inside.

Then, 2.5 µL of cDNA was loaded and the plate slightly tapped in order to make the drops of sample go to the bottom of the plate. Afterwards, the mixtures (7.5 µL) were loaded into the appropriate wells. The plate was closed with the plastic cover and centrifuged for 30 seconds at maximum speed in a SIGMA 2-16PK centrifuge model by SIGMA Laborzentrifugen GmbH.

2.2.4 **RNA Procedures**

2.2.4.1 RNA isolation from yeast.

For RNA isolation, all work was performed on ice, with gloves to avoid RNase contamination.

When a culture of yeast achieved an optical density of 0.5-0.6 at OD_{660nm}, 2 times 2 mL of culture was taken, transferred to an Eppendorf of 2 mL capacity and placed on ice for 1 minute. The Eppendorf containing the 2 mL of culture was then centrifuged for 3 minutes at 4°C at the maximum speed and the supernatant was removed and each cell pellet resuspended in 1 mL of ice cold H_2O. This was then centrifuged for 3 minutes at the maximum speed and at 4°C. Supernatant was once again removed and flash freeze in liquid nitrogen. The cell pellets were stored at -80°C.

For the RNA isolation procedure the aliquots were laid to unfreeze on ice before resuspended in 750 µL AE Buffer (50mM NaAc pH 5.2; 10mM EDTA) the, 40µL of SDS solution at 25% was added as well as 750µL of Acid Phenol and finally well homogenized.

Afterwards the mixture was incubated at 65°C under shaking in a thermo-cycler at maximum speed for 15 minutes before laid on ice for 5 minutes. This was then centrifuged at 4°C for 15 minutes at maximum speed and the upper phase was recovered and transferred to another Eppendorf of 2 mL capacity. A volume of 1 mL of chloroform was added and homogenized. Once again, this was centrifuged at 4°C for 10 minutes at maximum speed and the upper phase recovered and transferred to another Eppendorf to which 70 µL of 3M Sodium Acetate (pH5.2-5.6) and 700 µL of Isopropyl alcohol were added and gently mixed.

Another centrifuge step at 4°C for 40 minutes at maximum was done and also the supernatant eliminated before the addition of 1 mL of 70% ethanol. A final centrifuge step for 20 minutes in the same conditions as before was done and the ethanol excess removed with absorbent paper. The isolated RNA was left to dry completely overnight.

The day after the isolated RNA was resuspended with 100 µL of nuclease-free H_2O and the concentration of RNA determined using a NanoDrop ND-1000 spectrophotometer.

2.2.4.2 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The reverse transcriptase (RT) protocol was performed using a kit RevertAid™ H Minus First Strand cDNA Synthesis Kit *(Thermo Scientific™, ref 1632, Belgium)*. The following quantities for preparation of the mixture for the RT reaction were based in one single sample and composed by: 0.5µL Primer Oligo(dT) (100µM, 0.5µg/L); 5x 2µL Reaction Buffer (250mM Tris HCl (pH 8.3), 250 mM KCl, 20mM $MgCl_2$, 50 mM DTT; 4.5µL H_2O; 0.5µL RT enzyme (200 U*/µL); 0.5µL Ribonuclease Inhibitor (20 U**/µL); 1µL of dNTPs (10mM).

(*According to the kit, one unit of RevertAid H minus M-MuL V RT incorporates 1nmol of dTMP into a polynucleotide fraction (adsorbed on DE-81) in 10 min at 37ºC; ** One unit of Ribonuclease Inhibitor inhibits the activity of 5ng RNase A by 50%)

In an Eppendorf tube it was placed the quantities mentioned above, in the following order: H_2O, Reaction Buffer, dNTPs, primers and finally the enzyme RT. Then, 9 µL of the mixture was transferred to a PCR tube to which 1µL of RNA extract was added.The tube was placed in the thermocycler (Bio-Rad PTC 200) and the appropriate program was selected, 42°C for 1 hour and the Reverse Transcriptase Inactivation at 70°C for 10 minutes.

For application with qPCR, the specific details are mentioned in section 2.2.3.4.

2.2.4.3 Purification of the PCR products

This protocol was performed using a kit from Promega Company® Wizard SV Gel and PCR clean-up system.

In an Eppendorf tube with a capacity for 1.5 mL, 40µL of water was placed and 10 µL of RT product added. Then, 250 µL of Binding Buffer was added and transferred to a column. Following a centrifuge step for 1 minute at 13000 rpm at room temperature was performed and the supernatant eliminated, then, 250 µL of Washing Buffer was added and centrifuged for 1 minute at 13000 rpm at room temperature and the supernatant eliminated. A volume of 250 µL of Washing Buffer was added and centrifuged for 1 minute at 13000 rpm at room temperature and the supernatant eliminated. Afterwards it was centrifuged under the same conditions for 3 minutes to dry the column. In a new Eppendorf tube with a capacity for 1.5 mL, it was placed the column and 100 µL of water was added, then remain for 1 minute and finally centrifuged for 1 minute at 13000 rpm at room temperature.

2.2.5 **Protein Procedures**

The techniques described below were used to verify protein extracts with the aid of antibiotics and as has a validation for expression analysis of *DTR1*, via western blotting, the antibodies used are in section 6.1.3.

2.2.5.1 Protein Extraction- membrane proteins

Twenty five mL of culture in a Falcon tube (with a capacity of 50 mL) was washed with 10 mL 50 mM Tris pH8 and then centrifuged at 4°C for 5 minutes at a speed of 13000 rpm and the supernatant removed. This was then flash freeze in liquid nitrogen and store at -80°C.

The Eppendorf tubes were then laid to unfreeze on ice and resuspended with 2 mL of lysis solution (100mM Tris pH8, 150mM NaCl, NP40 1%, 1mM EDTA, 15 µL PMSF) and 400 µL were transferred into a 2 mL Eppendorf tube where it was added 400 µL of glass beads and 120 µL of protein inhibitor. This was subjected to a Vortex in a VXR basic Vibrax® model by IKA for 30 minutes at 4°C at maximum speed.

The supernatant was recovered in a new Eppendorf with a capacity of 2 mL and 200 µL of lysis solution (100mM Tris pH8, 150mM NaCl, NP40 1%, 1mM EDTA PMSF 0,2M, Protease Inhibitor Cocktail from Roche) was added before transferred to a Vortex to homogenize. Afterwards it was centrifuged at 4°C for 3 minutes at low speed (3000 rpm), and the supernatant recovered. Then it was centrifuged at 4°C for 45 minutes at high speed (12000 rpm), the supernatant was pooled, and the pellet resuspended with 500 µl Lysis Buffer with and addition of Urea 5M. This was centrifuged once again under the same conditions and for 45 minutes and resuspended with 500 µl Lysis Buffer with 120 µl of TCA 10%. Final centrifugation for 5 minutes at maximum speed was performed, and the supernatant recovered to which was added the Blue loading buffer 5X: 70µL extract + 25µL blue loading buffer + 10 µL β-mercaptoethanol and aliquoted. The aliquots were stored at -80°C flash frozen by liquid nitrogen.

Before loading the SDS-PAGE gel, aliquots were incubated for 15 minutes at 37ºC.

2.2.5.2 SDS-PAGE

The step of SDS-PAGE and Western Blotting was performed with The NuPAGE Bis-Tris Electrophoresis System, *"a revolutionary neutral pH, pre-cast, discontinuous SDS-PAGE mini-gel system providing maximum stability of both proteins and gel matrix during electrophoresis, and better band resolution than other gel systems"* (NuPAGE ® Technical Guide General information and protocols for using the NuPAGE ® electrophoresis system, 2010).

The NuPAGE Novex Pre-Cast Gel cassette is 10 cm × 10 cm in size, the gel formulation was Bis-Tris-HCl buffer (pH 6.4), Acrylamide, Bis-acrylamide, APS, Ultrapure water its stacking gel was4% and its separating gel 12% with a pH of 7.0

The first step was assembling the XCell SureLock MiniCell (Thermo Scientific) by cutting open the gel cassette pouch with scissors and remove cassette. Then, the gel packaging buffer was drained away. The gel cassette was removed from the bag rinsed with deionized water. Then we peeled off the tape covering the slot on the back of the gel cassette and pulled the comb out of the cassette in one fluid motion to expose the gel loading wells. Afterwards we gently washed the cassette wells with sterile water. The next part was to invert the gel and shake it to remove the buffer. This procedure was repeated twice and the gel cassette was inserted into the XCell SureLock and the Gel Tension Lever locked. The aliquots previously unfrozen were then loaded into the wells of the gel, along with molecular weight markers and the XCell SureLock MOPS SDS 1x Buffer filled up to cover the gel cassette. The samples in the gel were run for 1 hour at 100-150 V and used immediately in Western Blotting. For this the blotting pads were soaked in NuPAGE Transfer Buffer until they were saturated with buffer and by squeezing the pads while they were submerged in buffer the air bubbles were removed.

2.2.5.3 Western Blotting

The preparation of the transfer membrane and filter paper (Whatman cellulose filter paper) was cut to the dimensions of the gel and the membrane was placed directly into a shallow dish containing about 50 mL of NuPAGE Transfer Buffer for several minutes. The filter paper was soaked briefly in NuPAGE Transfer Buffer immediately prior to use.

The step of transferring the protein from the gel to the membrane was done by removing the lid and unlock the Gel Tension Lever. The gel cassettes were removed from the mini-cell and the gel cassettes (well side up) laid on a flat surface, such as the benchtop. Then, carefully inserted the Gel Knife's bevelled edge into the narrow gap between the two plates of the cassette and pushed up and down gently on the knife's handle to separate the plates. Afterwards it was removed and discard the plate without the gel, allowing the gel to remain on the other plate.

Subsequently it was placed a piece of pre-soaked filter paper on top of the gel. Where its kept saturated with the transfer buffer and all trapped air bubbles were removed by gently rolling over the surface using a glass pipette as a roller. Then, the plate was turned over so the gel and filter paper were facing downwards in order to separate the gel from the plate. Two soaked blotting pads were placed into the cathode (–) core of the blot module and, carefully the gel/membrane assembly was picked up and placed on blotting pad.

The assembled gel/membrane/filter paper (Whatman cellulose filter paper) sandwich on top of the anode plate (+) is illustrated in Figure 2.1.

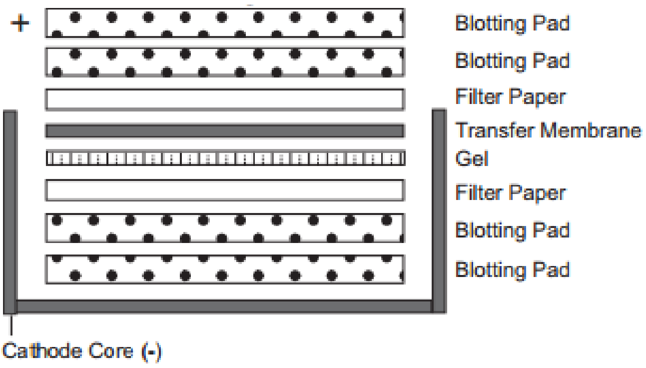

Figure 2.1 Western Blotting assembling example (Invitrogen, 2009)

After the assembly of positioning the gel/membrane and blotting pads into the XCell II Blot Module it was locked it and the blot module filled with 1X NuPAGE Transfer Buffer until the gel/membrane assembly is covered in Transfer Buffer and the Lower Buffer Chamber with deionized water. The lid is placed and run for about 1 hour under the following conditions: 200V; 120 mA; 250 W. (NuPAGE ® Technical Guide General information and protocols for using the NuPAGE ® electrophoresis system, 2010)

As for the Immunodetection- Antibody Incubation, the blocking solution of about 30 mL per gel revelation was prepared by placing the membrane in a plate and start by rinsing it with sterile water. Then, the water was removed and poured about 5 mL of Ponceau Solution so the total protein bands on the membrane can be revealed. The Ponceau solution was rinsed off with three washes with 10mL TBST, 10 minutes each wash and 10 mL of blocking solution was poured and placed it under agitation for 1 hour.

The solution was removed, and the primary antibody solution was placed along with 10 mL of blocking solution under agitation for 30 minutes and incubated at 4°C.

The antibody solution was then recovered to a Falcon tube and the membrane rinsed 3 times 10 minutes with 10 mL TBST. For the second antibody solution, this was placed along with Blocking Solution and was let it under agitation for 1 hour. The membrane was finally rinsed 3 times 10 minutes with 10 mL TBST. Antibodies used can be found in section 6.1.3.

Ultimately for the Imaging and Data Analysis, the membrane was placed on a plate and the Revelation Solution* transferred to cover all the membrane. This was left for 5 min for revelation. A plastic cover was placed on top of the membrane avoiding the formation of bubbles and proceed to the CCD camera-based imager and use image analysis software to read the band intensity of target proteins (BioRad, 2016).

*Revelation Solution is composed by 2 solutions, 400 µL of each. The two solution are added to an Eppendorf and vortexed before covering the membrane (SuperSignal West Femto Maximum Sensitivity Substrate Kit, from Thermo Scientific).

3 Results & Discussion

3.1 OVER EXPRESSION FROM GENOMIC *DTR1* LOCUS

The aim for the functional analysis of *DTR1* protein was to study its function and what happens when it is overexpressed. A functional analysis allows the verification of the overexpression of *DTR1* protein on genomic level. In order to induce the overexpression of the *DTR1* gene, it was decided to build a cassette containing the GAL1 promoter and the GFP tag.

Promoters are employed in yeast cloning vectors to enhance transcription of the gene of interest and its sequences may be yeast derived (homologous) or heterologous. The former are generally more efficient and can either be constitutive or regulated.

The regulated promoters facilitate yeast cell growth in the absence of heterologous gene expression, i. e., biomass increase can be temporarily separated from gene expression by controlling the availability of certain nutrients (*e.g.* galactose in the case of GAL1, phosphate in the case of PHO5, methionine in the case of MET25).

Several physiological factors are important in governing the expression of foreign genes and the subsequent modification of foreign proteins in yeast cells. With regard to nutrient and temperature inducible transcription, there are several examples of physiologically regulated promoters operate in yeast, such as the GAL1 promoter, that is repressed by glucose and induced by galactose (Walker, 1998).

Genes of interest may be fused with the GFP gene and the subcellular destiny of the expresses fusion proteins followed by fluorescence microscopy (blue light at 395 nm). Such techniques employing GFP are likely to assist in the functional analysis of yeast genomes. For example, immunofluorescence has enable tubulin and actin to be localized during the *S. cerevisiae* cell division cycle.(Kilmartin & Adams, 1984; Walker, 1998)

3.1.1 Construction strategy of P_{GAL1} -GFP

This requires the transformation with P_{GAL1} promoter upstream to our protein and GFP tag downstream (to our protein) through PCR-directed homologous recombination, this technique allows the homology necessary to promote efficient recombination-mediated gene disruption in yeast to be quite small, around 3050bp, small enough to be synthesized as part of a PCR primer. The general procedure of the PCR-directed homologous recombination begins with a linearized target plasmid containing a selectable marker is co—transformed with a PCR fragment and by homologous recombination a subset of the cut plasmids are re-circularized and simultaneously acquire a DNA segment of interest (Oldenburg et al., 1997).

For this purpose, plasmids containing these proteins were digested, purified, transformed and results analyzed.

From PKT140 plasmid the GFP tag was isolated and from P_{GAL1} plasmid, the Gal1 promoter was isolated and homologous primers were prepared for cassette building, these primers were constructed with 40 base pairs corresponding to *DTR1* gene and 20 base pairs corresponding to the sequences of GFP and Kanamycin resistance that is found on PKT140 plasmid. For the Gal1 promoter fusion another pair of homologous primers containing 40 base pairs corresponding to *DTR1* and 20 base pairs corresponding to the sequence of Gal1 promoter plus Nourseothricin resistance were prepared from the P_{GAL1} plasmid (Table 2-1).

3.1.1.1 Insertion of restriction zones

A successful *E. coli* transformation from *XL1Blue* strain in medium 853 with ampicillin antibiotic- as selector marker allowed the selection of competent cells in the exponential phase of growth and the introduction of restriction zones with specific primers to our proteins, GFP and P_{GAL1} promoter via digestion with restriction enzymes.

The insertion of restriction zones in the PKT140 and P_{GAL1} plasmids are for cassette construction with the finality of builing one single cassette, the P_{GAL1} promoter upstream to *DTR1* protein and the GFP tag downstream, as mentioned before.

Figure 3.1 represents the PKT140 plasmid containing GFP tag protein and the specific primers, F5 and R3, that were introduced after linearization with *Sal*I restriction enzyme. It also represents the Ampicillin and Kanamycin resistance by AmpR and KanR respectively as well as the size of the plasmid. The linearization facilitated the introduction of restriction zones with the primers selected, F5 and R3.

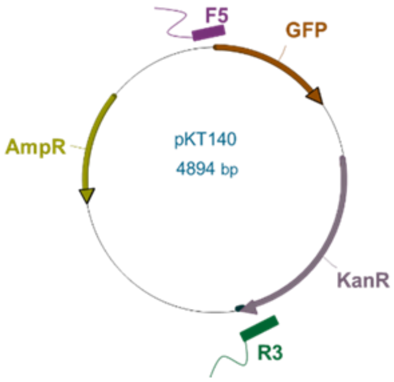

Figure 3.1 **PKT140 plasmid representation containing F5 and R3 primers specific to GFP tag fusion**. Represented in yellow is the ampicillin resistance, AmpR, and the Kanamycin resistance, in light purple, KanR. In purple and green are the primers F5 and R3 respectively and, in brown is represented the GFP tag. The size of this plasmid is 4894 bp.

The cassette fusion with GFP tag is built with homologous primers, F5/R3 and these are constructed with 40 base pairs corresponding to *DTR1* gene and 20 base pairs corresponding to the sequence of GFP and Kanamycin resistance that is found on PKT140 plasmid as it is illustrated in Figure 3.2.

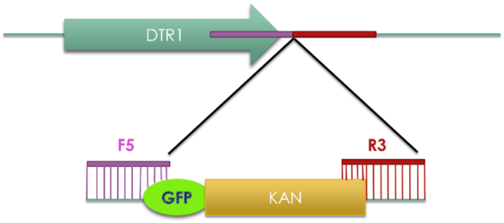

Figure 3.2 Schematic representation of cassette fusion with GFP tag and homologous primers F5/R3.

As for the cassette fusion of the Gal1 promoter and *DTR1* gene, the building with homologous primers, F4/R2, is constructed with 40 base pairs corresponding to *DTR1* and 20 base pairs corresponding to the sequence of Gal1 promoter as it is illustrated in Figure 3.2 and in more detail, a representation of the P_{GAL1} plasmid is represented in Figure 3.4. The restriction enzyme used is the same in both plasmids.

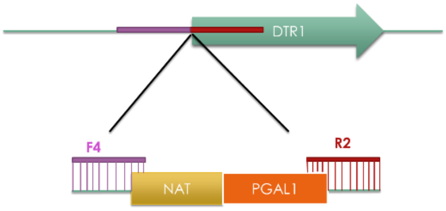

Figure 3.3 Schematic representation of cassette fusion with Gal1 promoter and homologous primers F4/R2.

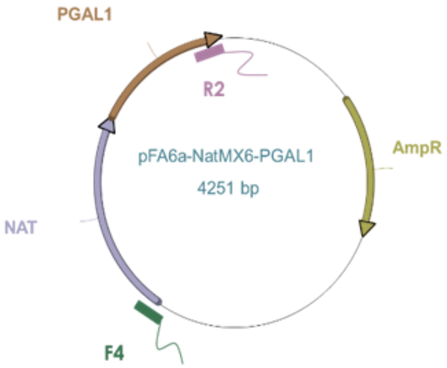

Figure 3.4 ***P**_{GAL1}* **plasmid representation containing F4 and R2 primers specific *P*_{*GAL1*} insertion** and the site of its introduction. Represented in yellow is the ampicillin resistance, AmpR, and the Nourseothricin resistance in light purple, NAT. . In purple and green are the primers R2 and F4 respectively and, in brown is represented the Gal1 promoter. The size of this plasmid is 4251 bp.

The amplified fragments have an expected length of 2515 bp for the GFP tag plus primers fragment as illustrated Figure 3.5 in lane 1 and the expected length of 1850 bp for the P_{GAL1} plus primers is illustrated on lane 2. Therefore, the bands visualized on agarose gel electrophoresis which migrates close to 2500 bp and just above 1500 indicate a correct digestion of the plasmid.

In Figure 3.5 are only represented the marks of 5000, 1500 and 500 bp since these are the ones that will be used as reference throughout the work.

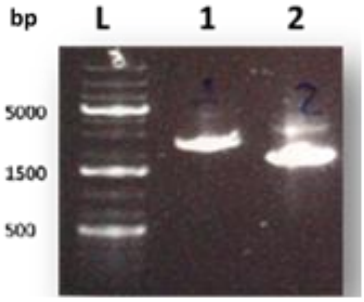

Figure 3.5 .**Plasmid fragments PCR amplification after digestion with *Sal*I restriction enzyme**. Gel was stained with Sybr green I to allow UV visualization. Lane L corresponds to the ladder GeneRuler 1kb Plus DNA Ladder (Thermo Scientific). Lane 1 shows the PKT140 digestion product. Lane 2 shows the P_{GAL1} digested product.

3.1.1.2 DNA fragments purification

In Figure 3.6 is illustrated the fragments digested with 3 and 4 replications respectively, in a gel with ethidium bromide prepared for purification. These were cut and weighted to proceed to chromatographic columns for purification.

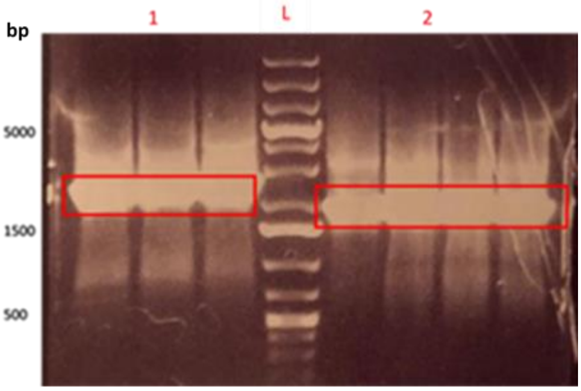

Figure 3.6 **DNA fragments replications for purification procedure**. Lane 1 GFP tag plus F5/R3 primers and Lane 2 corresponds to P_{GAL1} plus F4/R2 primers. Lane L corresponds to the ladder GeneRuler 1kb Plus DNA Ladder (Thermo Scientific).

Ilustrated in Figure 3.7 is the gel result from purified fragments of GFP tag plus F5/R3 primers characterized by number 1 and P_{GAL1} plus F4/R2 primers by number 2, respectively.

These purified fragments were obtained from plasmid linearizartion for yeast tranformation as they contain not only GFP tag (from PKT140 plasmid) and P_{GAL1} promoter (from P_{GAL1} plasmid) but also part of *DTR1* gene sequence (from the specific primers) as mentioned before.

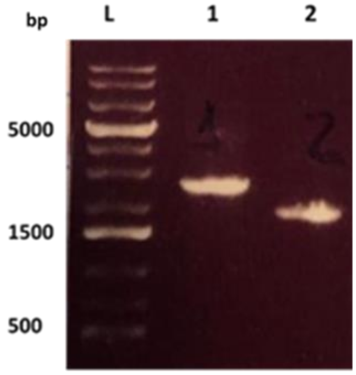

Figure 3.7 **Purified DNA fragments R3/F5 and R2/F4**. Lane 1 R3/F5 fragment. Lane 2 R2/F4 fragment Lane L corresponds to the ladder GeneRuler 1kb Plus DNA Ladder (Thermo Scientific).

3.1.1.3 Transformation and fusion of the fragments P_{GAL1} and GFP

Yeast transformation began first with GFP tag and secondly with P_{GAL1} promoter plus the respective primers R3/F5 and R2/F4 by homologous recombination to compose the aimed cassette P_{GAL1}-*DTR1*-GFP (Table 2-1). The yeast selected was the 23344c strain, cultured in medium 863 with Ura3$^-$, meaning Ura3 deficient. It is expected that a culture well transformed would contain in its genome a resistance to antibiotic that in this case is Gentamicin (Kan) and Nourseothricin (Nat), containing Ura3 thanks to pGal and PKT140 plasmids. Cultures grew over-night at 27ºC until they achieved exponential phase related to a DO$_{660nm}$ between 0.5 and 0.6.

This transformation did not occur. The colonies did grow between four or five days but neither the fragments of P_{GAL1} nor GFP tag had integrated the yeast genome.

Repeated yeast transformations were tried and carried on via Electroporation with Sorbitol since the normal transformation procedure did not provide valid results.

The transformation approach only provided results for the *DTR1*-PKT140. It was then decided to repeat the plasmid digestion process of P_{GAL1} plasmid with the *Nde*I enzyme, and the PKT140 plasmid with the *Bgl*II enzyme. This last plasmid digestion was done just to be prepared if further work was needed, though it was not used since there were still results to be shown, as concentration.

Illustrated in Figure 3.8 is the PCR analysis for the construction of the fragment F2/R4 from PGAL1 plasmid. This fragment was amplified. On lane 1 it is possible to verify the size of 1850 bp which means a good amplification after digestion. After purification of the fragment, a yeast transformation was carried out.

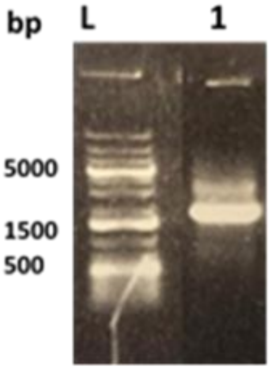

Figure 3.8 **PCR amplification of fragment F2/R4 from *P*$_{GAL1}$ digested plasmid-** Lane 1 corresponds where the size of fragment is around 1850 bp fragment Lane L corresponds to the ladder GeneRuler 1kb Plus DNA Ladder (Thermo Scientific).

As mentioned before, transformation with the PKT140 linearized plasmid did result in colonies in the medium containing KAN antibiotic as selection marker, so some of the colonies were transferred and transformation for fusion of the *P*$_{GAL1}$ promoter was used.

The transformation and fusion were verified via PCR using the primers A1A3Kv and A2A4Kr (Table 2-1), in order to fuse the gal1 promoter upstream to *DTR1* gene and then the GFP tag downstream.

15 samples in Kan medium were prepared with selected colonies from GFP tag transformed culture for diagnostic PCR. The expected results for this diagnostic PCR are described bellow for *P*$_{GAL1}$-*DTR1* and *DTR1*-GFP transformation, respectively:

For GFP tag:

Wildtype (wt)*: A3A4 *DTR1	508 bp
***DTR1*+GFP A4Kr**	524bp
***DTR1*+GFP A3Kv**	1238bp

For *P*$_{GAL1}$:

Wildtype (wt.)*: A1A2*DTR1	923 bp
***P*$_{GAL1}$*-DTR1* A1Kr**	680 bp
***P*$_{GAL1}$*-DTR1* A2Kv**	1066 bp

Fifteen samples from PKT140 plasmid were prepared containing two controls, and for the *P*$_{GAL1}$ 7 samples were prepared, using 1 as control as exemplified in Figure 3.9 and

Figure 3.10, respectively, the control strain is the *23344c wildtype* and it contains only the primers. For PCR mix for GFP fusion we placed the primers A3, A4 and Kv and for the PCR mix of P_{GAL1} we introduced the primers A1, A2 and Kv, with the obtained results shown in the figure below.

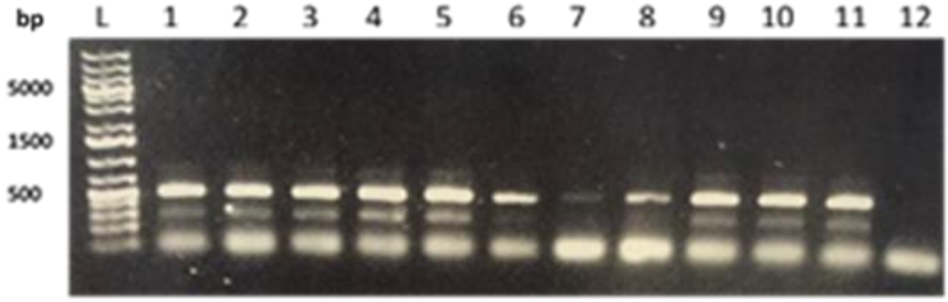

Figure 3.9 **Diagnostic PCR for *DTR1*-GFP construction with A3A4Kr primers**. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 correspond to 11 samples out of 15; Lane 12 corresponds to the control for GFP fragment (the control contains the primers A3A4Kv); Lane L corresponds to the ladder GeneRuler 1kb Plus DNA Ladder (Thermo Scientific).

By observation of Figure 3.9 from Lane 1 to 11 and Figure 3.10, Lane 1 and 2, it is notorious that this diagnostic PCR resulted in no amplification from the fragment F5/R3 (*DTR1*-GFP) from PKT140 plasmid since only the *wildtype* with 508 bp appears on the agarose gel, which denotes that GFP fusion did not occur as it was not integrated in the gene sequence.

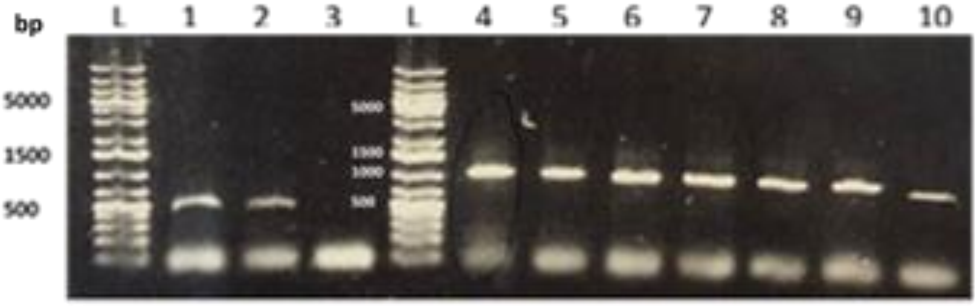

Figure 3.10 **Diagnostic PCR for *DTR1*-GFP construction with A3A4Kr primers (samples 1 and 2) and Diagnostic PCR for *PGAL1-DTR1* with A1A2Kv primers** Lane 1 and 2 represent the last 2 samples of GFP tag and Lane 3 represents the second control containing only the A3A4Kr primers. Then L is, again, the molecular ladder as is for separation of samples; Lane 4, 5, 6, 7, 8 and 9 represent the samples for P_{GAL1} construction and on Lane 10 is the control for the primers A1A2Kv fragment; Lane L corresponds to the ladder GeneRuler 1kb Plus DNA Ladder (Thermo Scientific). Sample from lane 4 was the one selected throughout the study.

As for the for the F4/R2 fragment (*P_{GAL1}-DTR1*) from *P_{GAL1}* plasmid, illustrated on Figure 3.10 from Lane 4 to 9, there was however a successful amplification with all the candidates having the expected size of 1066 bp. Lane 4 (Figure 3.10) represents a good candidate and it was the one used to proceed for the GFP fusion as proposed for this study. As for the GFP tag fusion, a new amplification of fragment F5/R3 from *DTR1*-GFP was evaluated. The result is represented on Figure 3.11 and it confirms that this fragment had the expected size of 2515 bp, so it was purified and the concentration verified with a value of 68 ng/µl after precipitation.

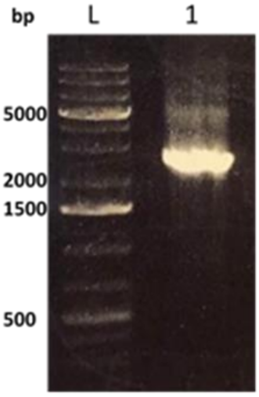

Figure 3.11 **F5/R3 fragment amplification for purification and final yeast transformation**. Lane L corresponds to the ladder GeneRuler 1kb Plus DNA Ladder (Thermo Scientific).

Preparation for the GFP tag integration was made by using pre-cultures containing the *P_{GAL1}-DTR1* and 23344c yeast strains. They were prepared to be planted on Petri dish containing KAN antibiotic as selection marker since F5/R3 is part of the *DTR1*-GFP construction and contains the antibiotic resistance itself. This procedure resulted in one single colony with the complete fragment *P_{GAL1}-DTR1*-GFP. To this colony we did use it in another diagnostic PCR that resulted in a non verification of the GFP tag.

3.1.2 Expression levels of *DTR1* gene analysis via RT-qPCR

A pre-culture in different conditions described in Table 3-1 was prepared, knowing that when Gal1 promoter is present in glucose, expression and transcription are repressed since it connects to DNA regulation sites and to the transcriptional activator Gap4. With galactose, the Gal1 promoter is activated and there is expression.

Table 3-1 Pre-culture for RNA extraction with *P_{GAL1}-DTR1* fusion in different conditions and RNA concentration after extraction. To samples 1 and 4 was added 100 µl of H_2O since the concentrations were to high to proceed to Reverse Transcriptase.

	Strains	**Medium 863 with:**	**RNA concentration (ng/µl)**
1	23344c	Glucose 3%	1250.0
2	23344c	Galactose 2%	655.4
3	23344c	Glycerol 3%	453.0
4	23344c	Glycerol 3% + Galactose 2%	451.9
5	*P_{GAL1}-DTR1*	Glucose 3%	1103.1
6	*P_{GAL1}-DTR1*	Galactose 2%	431.3
7	*P_{GAL1}-DTR1*	Glycerol 3%	542.1
8	*P_{GAL1}-DTR1*	Glycerol 3% + Galactose 2%	554.4

Readings for optical density were made until the OD_{660nm} was around 0.6 for glucose and galactose containing medium and around 0.4 for Glycerol containing medium. Afterwards the products were purified according to the Promega Company protocol, referred in 2.2.4.3. This process allowed us to measure gene expression, in this case the expression of genes in the control of Gal1 promoter.

To confirm over-expression, it is important that we verify the cDNA since the cell only expresses genes when it is multiplying, *i.e.* after transcription.

Once reverse transcriptase was done and we obtained the cDNA for each of the 8 samples (

Table 3-1), we proceeded to qPCR, where parameters are referred in Table 2-2, using *TBP1* gene as a reference since this gene is not affected by imposed conditions as glucose, galactose and glycerol.

As mentioned above, the Gal1 promoter is only activated when galactose is present, it was expected that our samples provided us with some results, the samples with *P_{GAL1}-DTR1* sequence under the Galactose 2% medium under the and Glycerol 3% + Galactose 2% had some expression, this means that our gene *DTR1* is well regulated by the carbon source and confirms the correct insertion of the promoter upstream of the gene. The results shown, in Figure 3.12 are based on a comparison of target DNA concentrations (*DTR1*) divided by *TBP1* concentrations.

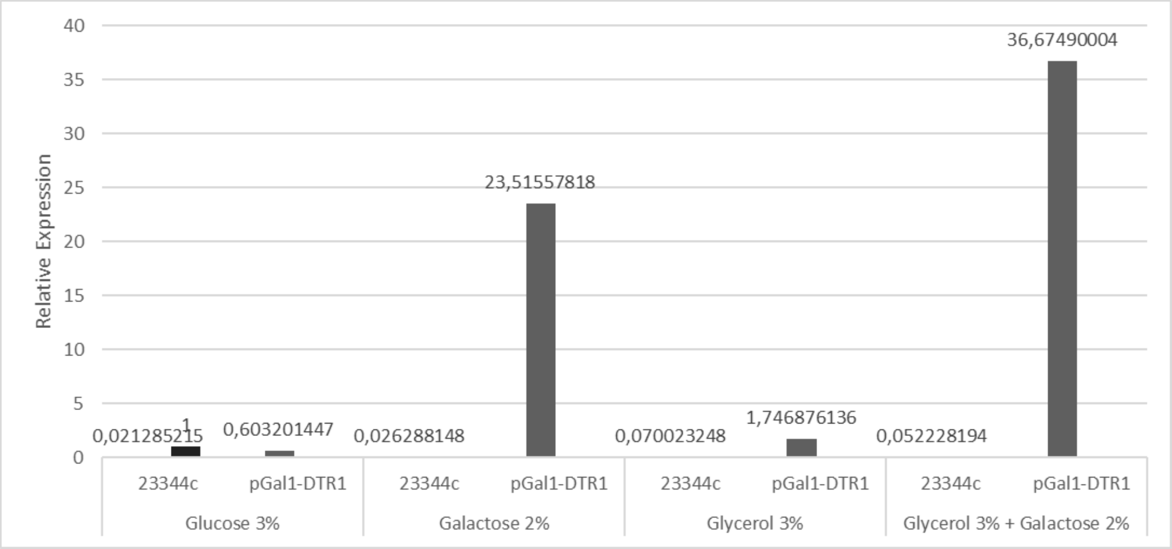

Figure 3.12 **Expression levels of DTR1 gene via qPCR.** Represented, are the samples 23344c and the P_{GAL1}-*DTR1* fusion under different conditions: in medium 863 containing glucose 3%, containing galactose 2%, containing glycerol 3% and containing glycerol 3% + galactose 2%. Normalization was made using *TBP1* as reference gene.

3.1.3 Physiological consequences of *DRT1* over expression-Cross-Feeding analysis.

With the intention to validate the *P_{GAL1}-DTR1* construction functionality and verify if amino-acids are excreted a cross-feeding study was prepared with Petri dishes containing minimum media 164 plus Uracil. For such, 8 strains were incubated, from which 5 were missing a specific amino acid, and added a drop of the strain that excrete amino acid. The five auxotrophic strains were MG408, MG401, MG360, 27061, JA188 and the excrete strains were the P_{GAL1}-*DTR1* construction, the 23344c wildtype and the positive control, the 32501d strain. Table 3-2. shows the missing amino acid in each auxotrophic strain.

Table 3-2 Auxotrophic strains and their missing amino acids.

Strain	**MG408**	**MG401**	**MG360**	**27061**	**JA188**
Amino acid	Arg	His	Lys	Trp	Tyr

The expected results for this experience were: Positive control, strain 32501d, will excrete the missing amino acid in plate, which is Trp (tryptophane) so around it there will always be growth of the auxotrophic strain.

Around the wildtype strain, 23344c, there will be no growth since there is no amino acid excretion. Around P_{GAL1}-*DTR1* there will be growth if the missing amino acid in plate is the one excreted by this strain. This test was performed in order to verify if and what amino acids are excreted by our construction strain, P_{GAL1}-*DTR1*.

This strain does not grow in plates containing glucose since in has the gal1 promoter which is repressed in the presence of glucose. These strains were first grown on an 863 medium over-night and diluted until OD_{660nm} was around 0.1-0.2, and then planted on Petri dishes.

This procedure did not result in any excretion from our construction, therefore we do not have representative results display. In order to validate our results the following step would be to try the western blotting and verify our protein expression but unfortunately this revelation did show negative results, suggesting that the protein was not being expressed through this system.

3.2 OVEREXPRESSION OF *DTR1* GENE FROM PLASMID

The aim of this construction allows us to verify the overexpression of *DTR1* gene on a plasmidic level, this study required the transformation of UGA4 and GAP1 plasmids by removing the coding part of UGA4 and GAP and replacing it with our gene of interest, the *DTR1* gene.

3.2.1 **Construction strategy of PGAP-GFP**

Both GAP1 and Uga4 proteins are located in the plasma membrane and have different functions for yeast metabolism, GAP1 codes for the general amino acid transport permease and Uga4 is a permease GABA-specific, *i.e.* specific transporter of ϒ-aminobutyric acid.

By placing a culture growing in a medium Ura3$^-$, meaning uracil deficient, we could verify transformed colonies since the integration of *DTR1* in the plasmids receives the auxotrophic mark of the gene Ura3 providing resistance.

For this purpose, the plasmids were digested, purified and transformed into yeast. Protein expression was analyzed by fluorescence analyses of different approaches of our construction.

3.2.1.1 Plasmids UGA4 and GAP1 linearization

This construction was made with the plasmids GAP1 and UGA4, by switching the coding part of UGA4 for the gene of interest, *DTR1*, and by placing them in a growing culture with a medium containing Ura3$^-$, *i.e.* Ura3 deficient. This procedure allows for the

verification that transformed colonies have in their construction the Ura3 gene that offers resistance and allows for the growing of the culture. This is an auxotrophic marker.
The first step was to linearize the plasmids and introduce restriction zones. So, we can begin transformation with pUGA4, taking UGA4 gene and switching for *DTR1* gene.

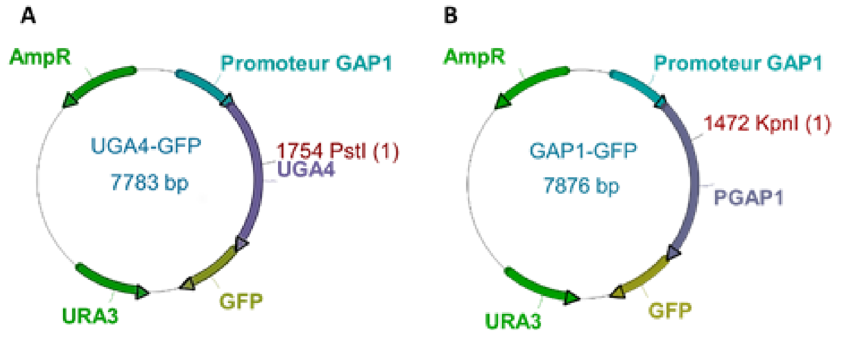

Figure 3.13 **pUGA4 (A) and pGAP1 (B) Plasmid representation**. In both is illustrated, in green, the URA3 for uracil resistance and AmpR as antibiotic resistance, GAP1 promoter in blue and GFP tag in yellow. UGA4 and GAP1 sequence originally from plasmid to be substituted for our gene of interest, *DTR1*, in purple. It is also represented its size and the excision site for restriction as well as the enzyme used and the restriction zone, in red.

It is known that UGA4 sequence is between 1079 bp and 2792 bp and that the GAP1 sequence is between 1079 bp and 2885 bp so with the help of Nebcutter platform we have chosen *Pst*I and *Kpn*I enzymes to cut UGA4 and GAP1 plasmids. *Pst*I enzyme was cut in the position 1758/1754 and *Knp*I in the position 1476/1472. The expected size after linearization is 7783 bp for UGA4 plasmid and 7876 bp for the GAP1 plasmid. Therefore, these restriction enzymes are good option for linearization.

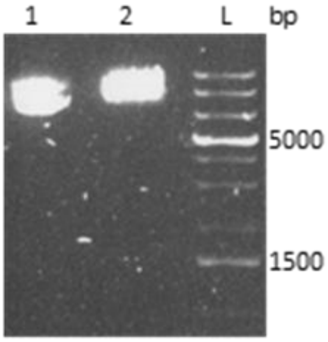

Figure 3.14 **Plasmid linearization.** Lane 1 corresponds to the UGA4 plasmids digested with PstI restriction enzyme. Lane 2 corresponds to GAP1 plasmids digested with KpnI restriction enzyme. Lane L corresponds to the ladder GeneRuler 1kb Plus DNA Ladder (Thermo Scientific).

For yeast transformation with UGA4 plasmid, the lineage used does not grow in medium containing Ura3 since it is deactivated because the plasmid is not circular, so its sequence containing our gene of interest was amplified and yeast transformation done with the linearized plasmid and expect homologous recombination was completed in the process. The plasmids sizes after the cut with restriction enzymes was, for UGA4, 7783 bp and, for pGAP1, 7876 bp.

3.2.1.2 Extraction of genomic DNA.

The extraction was done for UGA plasmid in the first place with the objective to proceed to PCR amplification of *DTR1*-UGA4. Examining the concentration of genomic DNA of pUGA4 we validated the value of 2243.7 ng/ µL. This extraction is from the 23344c strain and its digestion via ECOR1 enzyme.

The agarose gel represented in Figure 3.15 for verification of genomic DNA provide us the information of this genomic DNA having a large size since it represents a chromosome, this, is symbolized by Lane 1. Represented by Lane 2 well represents the smear of the digested genomic DNA since EcoRI enzyme cuts in two different sites and the representation of the lanes are from 3 replications of each.

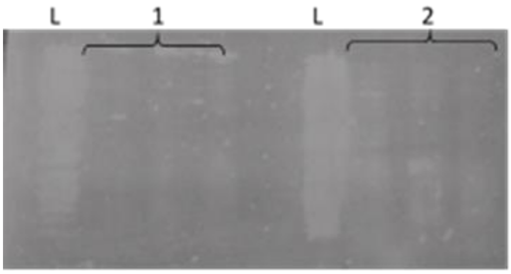

Figure 3.15 **Genomic DNA and its digestion from UGA4 plasmid** Lane 1 represents Genomic DNA from pUGA4 with the concentration of 2243,7 ng/ µL; Lane 2 corresponds to the digestion of genomic DNA - Smear well represented as expected since EcoRI cuts in two different sites. Lane L corresponds to the ladder GeneRuler 1kb Plus DNA Ladder (Thermo Scientific).

At the same time, a pre-culture was prepared in order for yeast transformation with the vectors R6 and F6 (Table 2-1) and our gene of interest, *DTR1,* that has 1842bp in the pUGA4 plasmid.

3.2.1.3 Insertion of restriction zones F6/R6 primers in UGA4 plasmid

Different essays for PCR with genomic DNA were made and new conditions were imposed on PCR: the annealing temperature was adapted for 50ºC and the genomic DNA was diluted by 1:20. The result for this PCR is represented on Figure 3.16.

Represented in Figure 3.16 by Lane 1 is the genomic DNA extracted plus F6/R6 primers with a size expected above 1800 bp. Lane 2 illustrates the genomic DNA extracted with L1 and L4 primers from Aqr1 gene, which is a gene that encodes a plasma membrane transporter of the major facilitator superfamily that confers resistance to short-chain monocarboxylic acids and quinidine in saccharomyces cerevisiae (Tenreiro et al., 2002) and overexpressed under the gal1 promoter in the presence of galactose (our unpublished observations).

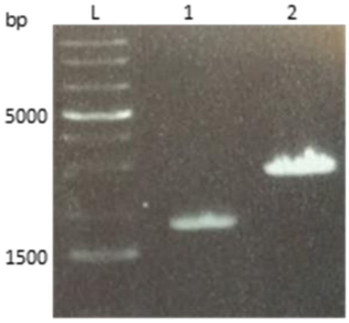

Figure 3.16 **Agarose gel of PCR under new conditions** Lane 1 corresponds to Genomic DNA diluted 1:20 plus primers R6/F6; Lane 2 represents the Control (with different primers L1 Aqr1 and L4 Aqr1). Lane L corresponds to the ladder GeneRuler 1kb Plus DNA Ladder (Thermo Scientific).

After we have verified the PCR amplification, we proceeded to purification of the genomic DNA plus primers F6/R6. This amplification is illustrated in Figure 3.17 where is verified the primers, described by number 1 which is the lightest band, and the fragment of interest, the genomic DNA, represented by number 2 on the heaviest band, with a size of around 1800 bp. The concentration of the UGA4 genomic DNA fragment equated to 78.6 ng/µl before purification.

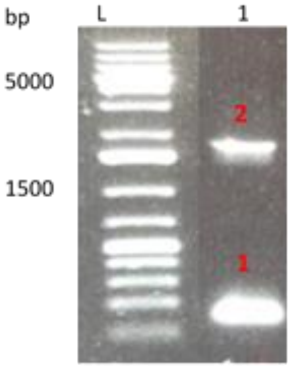

Figure 3.17 **Agarose gel from PCR amplification of genomic DNA**. Lane 1 represents the primers R6/F6 (1); (2) fragment of interest. Lane L corresponds to the ladder GeneRuler 1kb Plus DNA Ladder (Thermo Scientific).

Table 3-3 Concentration of F6/R6 fragments and digested UGA4 plasmid after purification.

	Concentration (ng/ µl)
F6/R6 fragments	26.6
pUGA4 digested	96.1

Transformation occurred in Petri dishes containing 164 mineral media with $+NH_4^+$ as nitrogen source and with h different ratios between the plasmid and the fragments of R6/F6, 1:1 and 1:3. Unfortunately, this preparation resulted in no colonies to be analysed.

A different approach was arranged for the UGA4 plasmid. We found two different enzymes that cut a big part in the UGA4 genome to optimize transformation so yeast cannot re-connect to restriction zones or complete them with its own genome. This verification was made using Nebcutter platform as an assistant, this platform predicts and indicates a graphical representation of restriction sites and their location based on nucleotide sequence.

Knowing that the gene from UGA4 is between 1079 and 2791 bp, we opted for *Pst*I enzyme (cuts in 1758/1754bp) for the begging cut and *Sph*I enzyme (cuts between 2634/2630 bp) for the end cut. These enzymes use the same buffer and have an activity between 100% with it.

This new digestion of the plasmids was used to transform yeast and it was planted in media uracil deficient, $Ura3^-$, and with this procedure resulting in colonies where we took samples and did a diagnostic PCR to verify insertion of our gene, *DTR1,* in UGA4 and GAP1 plasmids.

The PCR to verify construction with GAP1 plasmid was made using the primers described below as for the expected sizes for each combo.

- P7 – Gap1 promoter
- O4 – from GAP1 plasmid
- A2 – from the interest gene (*DTR1*) (Table 2-1)

After PCR amplification we get the described products: P7-A2 primers have the size of 663 bp, P7-O4 corresponding to wt, *wildtype,* in GAP1 is 540 bp and in UGA4 is 568 bp.

For UGA4 were prepared 8 samples plus 1 control with the primers P7, O4 and A2.

From 9 transformants in total, from individualized colonies, 4 were resulted in positive candidates meaning that it was expected to have the insertion of *DTR1* gene, a streak of these positive candidates is illustrated in Figure 3.18, the numbers 3, 4, 5 and 9 are representative from each of the originated colony.

c

Figure 3.18 Streak of selected colonies with good insertion of our gene, *DTR1,* in Petri dish containing Ura3$^-$. The numbers 3, 4, 5, and 9 correspond to previous colonies from where we did the replicate to this Petri dish.

A diagnostic PCR of those colonies was made, but the result was not expected since some might have the circular plasmid but not the fragment inserted in their genome, candidates corresponding to lanes 2, 3, 4 and 6 were selected since they seamed to have a good insertion size on the agarose gel around 663 bp. This is illustrated in Figure 3.19 which is the PCR analysis from our transformed colonies.

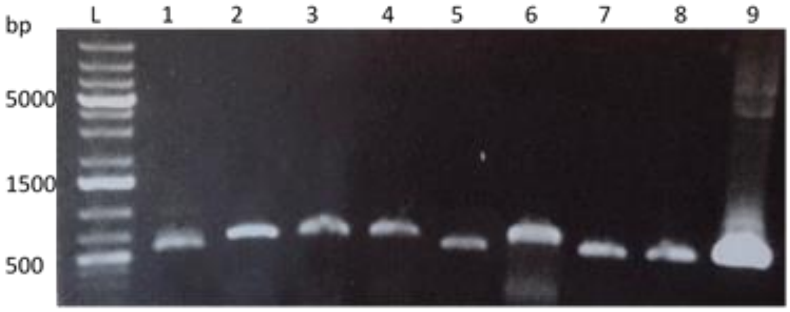

Figure 3.19 **pUGA4 diagnostic PCR from plasmidic construction with the 3 primers**. Lanes 2, 3, 4 and 6 were selected for further analysis since they have the pGAP1-*DTR1* insert. Lane 9 represents the control having only the *wt* in the sample. Lane L corresponds to the ladder GeneRuler 1kb Plus DNA Ladder (Thermo Scientific).

For GAP1 were prepared 11 samples plus 1 control with the primers P7 and A2, and did a diagnostic PCR of the colonies, again, some might have the circular plasmid but not the fragment inserted in their genome. So, the result is below, on Figure 3.20, and it shows that only Lanes 1, 5, 6, 7, 8, 10 and 11 seamed to have a good insertion, corresponding to 663 bp.

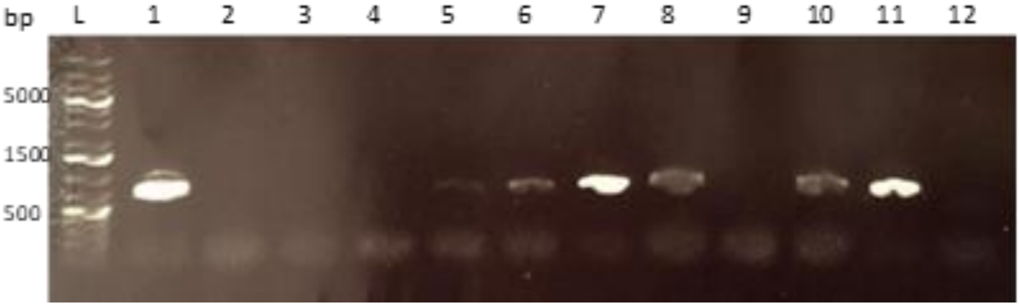

Figure 3.20 **pGAP1 diagnostic PCR from plasmidic construction with the 3 primers** Lanes 1, 5, 6, 7, 8 10 and 11 represent good candidates for insertion with 663bp. Lane 12 represents the control having only the *wt* in the sample. Lane L corresponds to the ladder GeneRuler 1kb Plus DNA Ladder (Thermo Scientific).

3.2.1.4 GAP1-DTR1 plasmidic extraction

From the UGA4 plasmid we did a GAP1-*DTR1* insertion meaning that our gene was inserted in the place where once was the UGA4 gene. For preparation for the plasmidic extraction an *E. coli* transformation was made, the genomic DNA extracted and then the plasmid isolation done in yeast.

E. coli strains *MC1061, XL1Blue* competent cells and the selected candidates from transformation 3, 4, 5 and 9 (corresponding to lanes 2, 3, 4 and 6 in Figure 3.19) were used and this transformation was made in dishes containing ampicillin antibiotic as selection marker.

A Petri dish (we have called it M4) containing the transformed strain *MC1061* with the candidate 5 from GAP1-*DTR1* construction was well transformed so a streak from the selected colonies was made. Figure 3.21 illustrates those streaks.

Figure 3.21 **Petri dish containing streaks from selected colonies from MC1061 strain**, these colonies were well transformed with the GAP1-*DTR1* insertion.

This plasmid was purified and the concentration for candidate 1 was 794.7 ng/µl, for candidate 2 was 673.1 ng/µl and for candidate 3 was 524.5 ng/µl.

The plasmid was digested with *Hind*III restriction enzyme where it resulted one only candidate belonging to candidate 4 from GAP1-*DTR1* construction. This candidate was sent for sequencing and the results showed it having silent mutations, and also a mutation where the amino acid aspartate was replaced by glycine. The combination affects the structure since aspartate is responsible for helix α and glycine offers flexibility to the structure.

So, the Petri dish (M2) was selected containing the transformed strain *MC1061* with the candidate 3 (lane 2 in Figure 3.19) from GAP1-*DTR1* construction was well transformed, having resulted in one colony (Figure 3.22, middle-bottom).

Figure 3.22 Petri dish with ampicillin containing medium containing the single candidate from *MC1061* strain, this colony was well transformed with the GAP1-*DTR1* insertion.

With this colony, a new *E. coli* transformation was made containing 100µl of cells and three new approaches for plasmidic DNA, *i.e.* different quantities, none, 2 µl and 10 µl. this samples were inoculated and planted on Petri dishes in medium with ampicillin.

Afterwards the plasmid was purified from an yeast colony and digested with *Hind*III enzyme, and concentration of each plasmid purified was measured. The result of this measurement resulted in sending the highest concertation for sequencing, which was the sample containing 10 µl of plasmidic DNA with a concentration of 39.4 ng/µl although the ideal for sequencing would be 50 ng/µl. This candidate was the one used in the subsequent analysis.

3.2.2 GAP promoter induction with proline 0.3% as a nitrogen source

As mentioned in the introduction of this study, *GAP1*, is synthesized and most active under conditions of poor nitrogen supply (*e.g*. proline, urea, low ammonium, etc.) and its role under these conditions is to scavage external amino acids to be used as nitrogen sources or directly as building blocks for protein synthesis. The regulation of the GAP1 permease is complex, and depends on the nature of the nitrogen source(s) in the medium. *GAP1* gene transcription is promoted by two GATA-family factors, Gln3 and Gat1, which are mostly active when the nitrogen supply conditions are cell-growth limiting. If there is a shift to a more favourable nitrogen supply conditions, these factors are inhibited by the mechanism of Nitrogen Catabolite Repression (NCR), leading to a string decrease in *GAP1*'s expression.

The expression analysis of the GAP1 promoter was made via its induction with proline as nitrogen source, this was prepared using the *wildtype* strain 23344c as negative control, pGAP1-UGA4 as a positive control and GAP1-*DTR1* from the first sample from M2 (candidate 3) that we have send to sequencing. For the *wildtype* strain it was always added of Ura 0.25% to medium.

A pre-culture, prepared with medium 165 containing Proline 10%, Glucose 3%, Traces (metal based) and vitamins (Table 6-2), was incubated for 24 hours with an addition, afterwards of part pre-culture and part of 863 rich medium. The OD_{660nm} and relative expression was measured through time.

The graph presented below and illustrated by Figure 3.23, indicates the fluorescence measurements emitted during qPCR that provide us with the quantity of amplified product, cDNA, present, in our samples as well as the OD_{660nm} at the following times:

- Before medium shift to proline 0.3%;
- Two hours after;
- 24 hours after.

This analysis revealed that it took about 22 hours for OD_{660} to double which may determine that the number of cells had lowered or that they were in stress.

RNA concentration was measured (Table 3-4) after extraction, cDNA obtained by reverse transcriptase and purified by the Promega Company protocol, referred in 2.2.4.3.

On the first analysis, it was added zymolyase to half of the samples in order to verify protein extraction.

Table 3-4 **RNA concentration after extraction for qPCR expression levels analysis.** These concentrations were diluted accordingly to be around 500 ng/µl.

		RNA Concentration (ng/µl)
Before shift	**23344c**	1059,5
	PGAP1-UGA4	3071,3
	PGAP1-*DTR1*	3211,3
2 hours after shift	**23344c**	1497,6
	PGAP1-UGA4	3055,5
	PGAP1-*DTR1*	4665,8
24 hours after	**23344c**	1075,6
	PGAP1-UGA4	2761,3
	PGAP1-*DTR1*	3087,5

Note that all the values of expression levels quantified by relative fluorescence units are means of raw fluorescence data measured by qPCR with TRIS mean value subtracted.

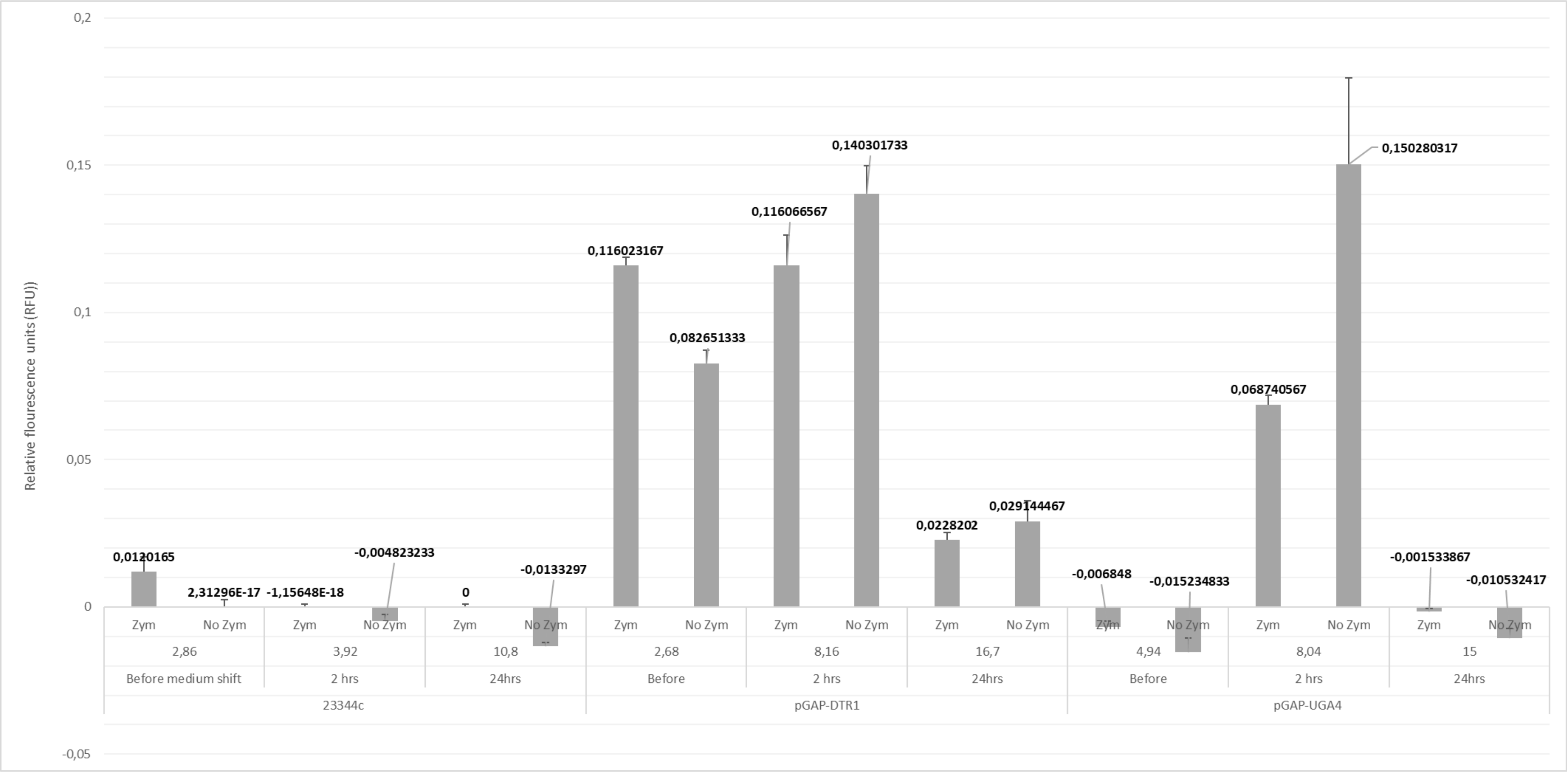

Figure 3.23 **DNA quantification measured by fluorescence in qPCR, between wildtype, pGAP-*DTR1* and pGAP-UGA4 samples with and without the presence of zymolase** trough a period of 24 hours before, 2 hours after and 24 hours medium shift of proline 0.3%. Represented between the bars and sample-name is the respective $OD_{660\ nm}$ measurements. Values are average ± standard deviation. TRIS 7.5 was used as a calibrator sample.

Examining the graph represented by Figure 3.23 is verified that GAP promoter relative to the *pGAP-UGA4* construction with no zymolase present and 2 hours after medium shift, has the higher fluorescence-mean value, which is 0,150280317 that might indicate a high expression level of *DTR1*.

The fluorescence detection of our gene, *DTR1*, under the same condition as the UGA4 gene, meaning with no zymolase present and with 2 hours after the medium shift does not have a higher fluorescence value but, after 24 hours either with zymolase or not, *DTR1* protein has a higher fluorescence value, meaning a higher quantification of target DNA amplified. Our best result can be found without the presence of zymolase and after the 2 hour medium shift (0.140301733).

In order to have a clearer and careful analysis of DNA quantification via fluorescence-qPCR, the samples, *wt*, pGAP-*DTR1* and pGAP-UGA4, were washed with solution TRIS with pH 7.5 this analysis can be found of the graph indicated below by Figure 3.24.

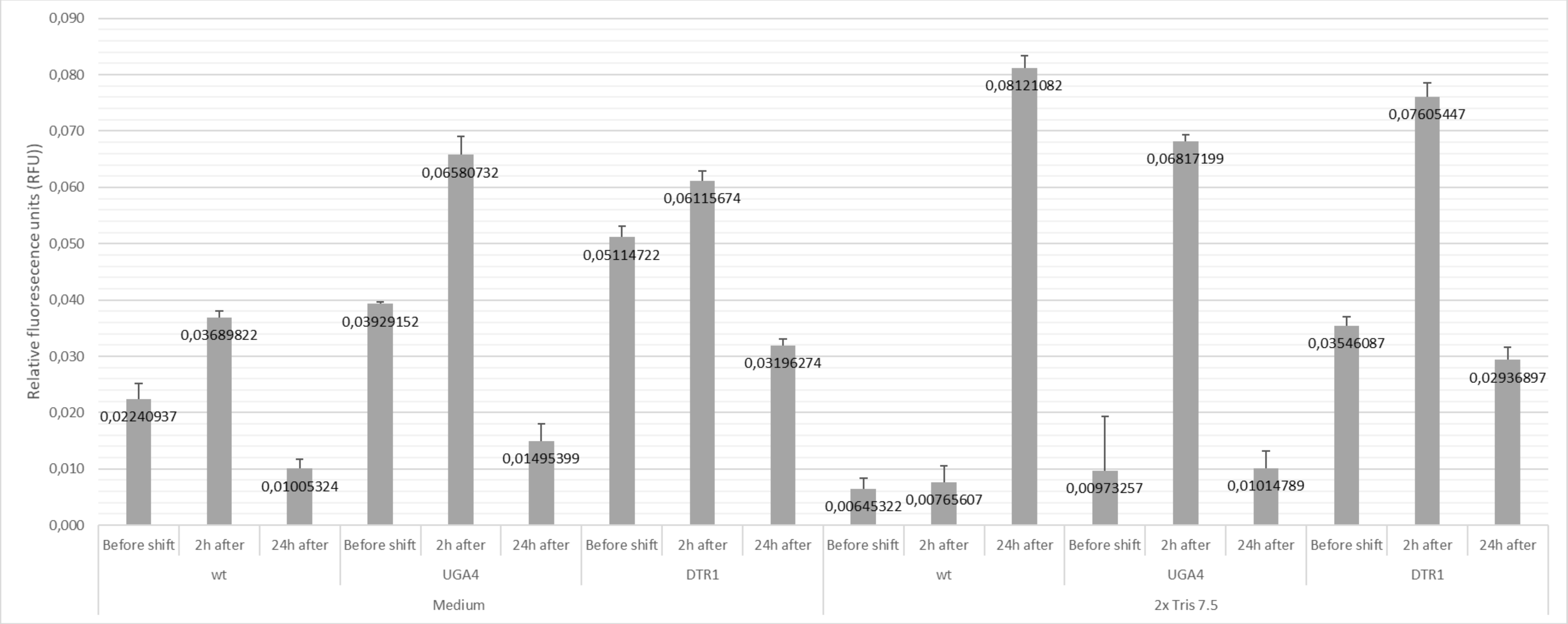

Figure 3.24 **DNA quantification measured by fluorescence in qPCR, between wildtype, pGAP-*DTR1* and pGAP-UGA4 samples of the GAP promoter with the medium shift and refined with two times TRIS 7.5 washing**. Values are average ± standard deviation. TRIS 7.5 was used as a calibrator sample.

A carefully analysis of the graph indicates that washing with TRIS 7.5 solution provide a clearer cDNA product amplification quantification, the 2 hours after the medium shift was the best value of fluorescence emitted for quantifying our protein of interest, *DTR1*, (0.07605447).

Since qPCR resulted in quantified levels that provide us with interesting values from our protein it was decided to validate those via western blotting.

For the western blotting 3 antibodies were used, details for these antibodies can be found in section 6.1.3.

1º Antibody BSA-free Tetra-His

2º α- Mouse

3º PGK after revelation

It was expected a protein size of about 92.6 kb for our GAP-*DTR1*-GFP construction, unfortunately these revelations did not have the expected results, one possible explanation might be that probably an antibody used did not aggregated well to the membrane.

A final qPCR analysis was preprepared by comparison of 4 different experiments, Figure 3.25 illustrates the DNA quantification means measured by fluorescence marker (SYBR) and respective relative expression levels. In the experiences referred as number 3 and 4 an extra condition was applied, it added 1 ml of 863 medium and 4 ml of 863 medium to the cultures respectively.

This quantification of expression levels of *DTR1* gene made via qPCR used *TBP1* gene as a reference, since this gene does not pose changes in different culture conditions. The results shown are the quantified levels of expression from the construction GAP1-*DTR1* and the respective quantified DNA amplification product measured by fluorescence-qPCR.

Our best value of would be 0,285046783 corresponding to experiment 3, 24 hours after the medium shift to proline with addition of 1 ml of 863 medium, since it shows a high value of fluorescence/quantification of amplified product representing our gene of interest and a value of relative expression corresponding to 302,02 and OD_{660} being the highest (25,7) meaning good culture growth.

Although there is a high level of relative expression in the 1st experiment 24 hours after medium shift the quantification of DNA is too low so we did not consider it for good result, and in the same experiment, is noted that we considered a high value of quantified DNA in Figure 3.24 but as far as relative expression goes, this was very low compared to experiment 3.

All fluorescence quantification levels represented values are means, subtracted with TRIS mean values, this results are illustrated in Figure 3.25.

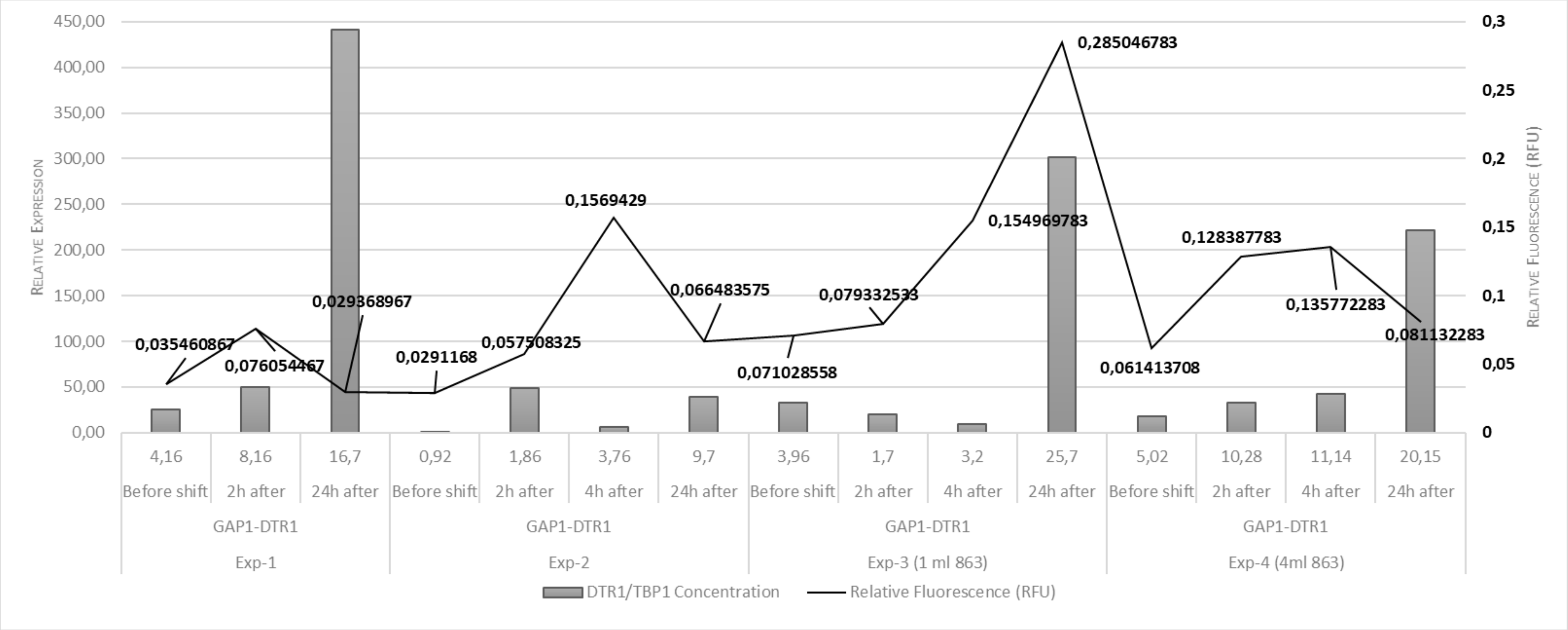

Figure 3.25 **-Expression levels of GAP1-*DTR1* and DNA quantified by fluorescence measurements in qPCR from in 4 different experiences comparison**. Experience 3 and 4 differ in medium quantities added 1ml vs 4ml respectively. Represented in grey is the expression levels of *DTR1* and represented in black is the DNA quantified by fluorescence-qPCR of the GAP-*DTR1* construction measured trough 4 experiments. Normalization was made using *TBP1* as reference gene. TRIS 7.5 was used as a calibrator sample. Represented between the bars and sample-name is the respective $OD_{660\ nm}$ measurements.

3.1 INFLUENCE OF GLYCEROL AND TEMPERATURE IN YEAST CULTURE

The final experience for GAP promoter induction proposed was an analysis of OD_{660nm} to measure the growth of three different cultures under two different conditions of medium and two different temperatures. These cultures were prepared in 165 minimum medium with glucose 3% and proline 10% where we added glycerol 10% to 6 cultures and left 6 without. Also we place three cultures under 25ºC and another three at 30 ºC.

The OD_{660nm} was measured every 2 hours, making a total of four readings (OD1, OD2, OD 3 and OD 4) and we verified that samples that were in medium that did not contain glycerol and were at 30ºC grew quicker than culture in medium without glycerol and at 25ºC, this is depicted in Figure 3.26.

In relation to culture with *DTR1* construction we verified that the best result can be found in medium containing glycerol 10% with a temperature of 30ºC, based on previous works and observation we know that under stress conditions *DTR1* is expressed meaning that maybe a high temperature and glycerol represents a stress condition to our protein.

The analysis of our protein was tested with western blotting verification, and the antibodies used were: 1º α-His RGS overnight and α- mouse; 2º αGFP overnight and α- mouse (6.1.1.3).

These westerns were repeated a few times but no good representations were revealed in our process.

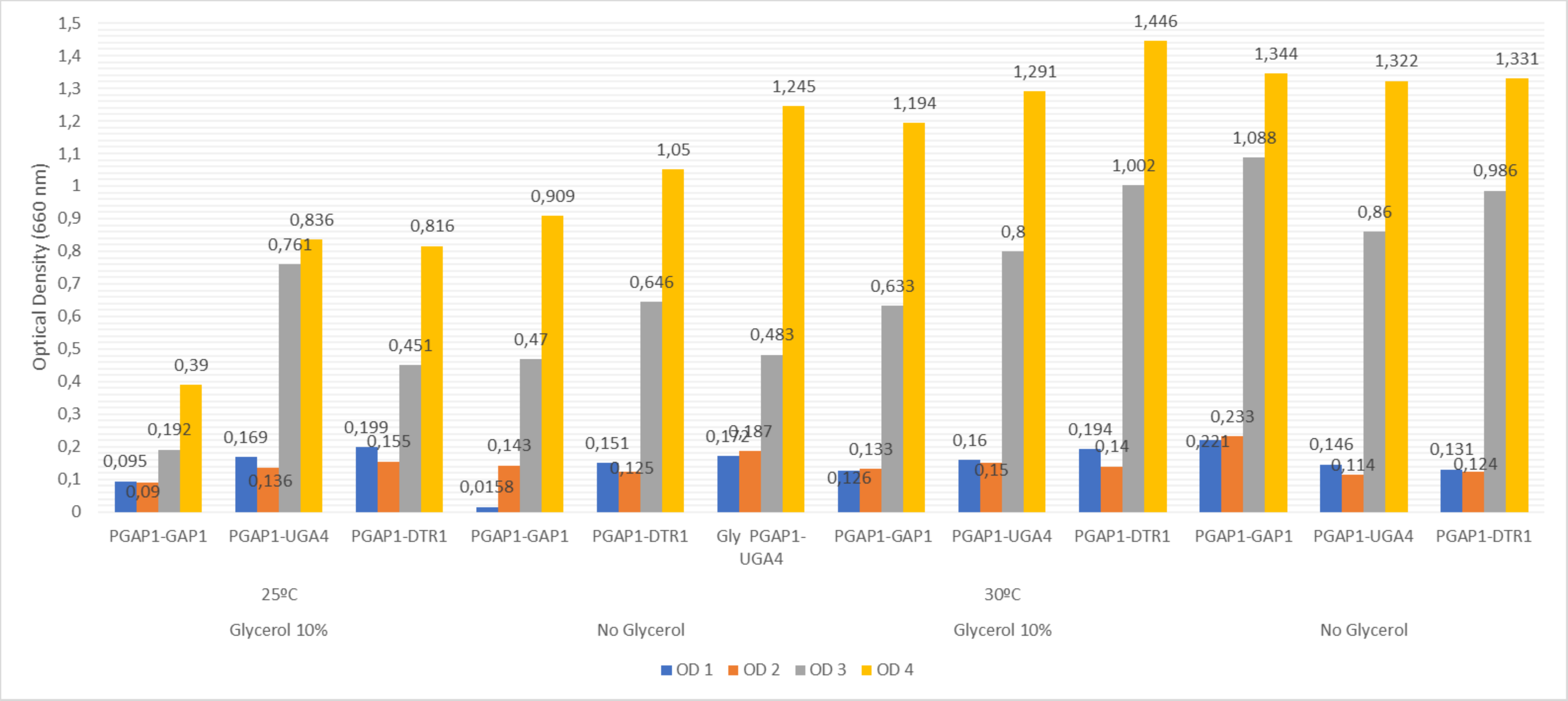

Figure 3.26 **Influence of Glycerol and temperature in cultures.** Represented are the samples: *PGAP1-GAP1*, *PGAP1-UGA*4 and *PGAP1-DTR1* in medium containing glycerol 10% and not containing glycerol 10% , under 25ºC and 30ºC and respective OD_{660nm}.

4 Conclusions

As far as functional analysis work was done, by overexpression from genomic DTR1 locus, the conclusion appeared to be that tagging *DTR1* gene with the GFP tag is very difficult and different approaches were made but were unsuccessful which prevented western blotting to work. Maybe if the digestion in the beginning was made with a different enzyme, perhaps the RNA concentration was not sufficient or the transformation in yeast was not well made.

In the medium 863 containing Glycerol 3% + Galactose 10% there was some relative expression measured (36,67490004) meaning a good insertion of the gal1 promoter and that our gene, *DTR1*, was well regulated by the carbon source. Cross-feeding analysis as an intention to validate the *P_{GAL1}-DTR1* construction functionality and verify if amino-acids were excreted did not produced the expected outcome since GFP tag never connected to the construction, so this experience did not continue.

As for the overexpression from plasmid analysis, we can conclude that removing a gene from a plasmid and placing it with our gene of interest was of somewhat successful since transformation did occur but did not provides us with many candidates, so perhaps its needed a different approach for this transformation process.

RNA concentration increased with the time when medium shift to proline 0.3% being that our best result was at the time of two hours after medium shift after a wash with TRIS solution. Also, and by examining the DNA amplified quantified by fluorescence, emitted by Sybr, in qPCR is noted that GAP promoter relative to the *pGAP-DTR1* construction with no zymolase present and 2 hours after medium shift, has the higher value, of 0,140301733 and that might indicate a higher quantification of target DNA product amplified.

After washing twice with TRIS with pH 7.5 the quantification of amplified cDNA via fluorescence-qPCR, provided us with a result of, again 2 hours after the shift being the best result, a value of 0.07605447. As for expression quantification levels of the construction GAP1-*DTR1*, using *TBP1* as a refence gene, the results shown a values of 302,02 of relative expression and a value of 0,285046783 corresponding to quantified DNA amplification measured by fluorescence-qPCR.

This experience also did not provides us with the best results since we were not able to go to western blotting analysis, maybe because of the antibodies used or possibly because our gene, *DTR1*, was not being expressed like it was expected.

Finally we have tested the influence of glycerol and temperature in different cultures of *S cerevisiae* and it was verified that the best result found was in medium containing glycerol

10% with a temperature of 30ºC, and this was expected since under stress conditions *DTR1* is expressed, therefore high temperature and glycerol represents a stress condition to our protein. Again, western blotting was a step to analyse the expression of the gene but no good revelations were obtained.

For further work, maybe it is possible that the antibodies used on the westerns tested might have to be different.

An alternative option is that, maybe *DTR1* is responsible for one the outer layers of the spore wall produced during sexual reproduction under stress conditions and does not excrete amino acids.

.

5 References

André, B. (1995). An overview of membrane transport proteins in Saccharomyces cerevisiae. *Yeast*, *11*(16), 1575–1611. https://doi.org/10.1002/yea.320111605

Bergmans, H. E. N., Van Die, I. M., & Hoekstra, W. P. M. (1981). Transformation in Escherichia coli: stages in the process. In *Journal of Bacteriology* (Vol. 146, Issue 2, pp. 564–570). https://doi.org/10.1128/jb.146.2.564-570.1981

BioRad. (2016). General Protocol for Western Blotting. In *Boletín 6376* (Vol. 20, Issue 20).

Calvin, N. M., & Hanawalt, P. C. (1988). High-Efficiency Transformation of Bacterial Cells by Electroporation. In *JOURNAL OF BACTERIOLOGY* (Vol. 170, Issue 6).

Chambers, P. J., & Pretorius, I. S. (2010). Fermenting knowledge: the history of winemaking, science and yeast research. *EMBO Reports*, *11*(12), 914–920. https://doi.org/10.1038/embor.2010.179

Chang, A. Y., Chau, V. W., Landas, J. A., & Pang, Y. (2017). Preparation of calcium competent Escherichia coli and heat-shock transformation. *JEMI Methods*, *1*(June), 22–25.

Cherak, S. J., Gugala, N., & Turner, R. J. (2016). *Membrane Transport*.

Cherry, J. M., Hong, E. L., Amundsen, C., Balakrishnan, R., Binkley, G., Chan, E. T., Christie, K. R., Costanzo, M. C., Dwight, S. S., Engel, S. R., Fisk, D. G., Hirschman, J. E., Hitz, B. C., Karra, K., Krieger, C. J., Miyasato, S. R., Nash, R. S., Park, J., Skrzypek, M. S., … Wong, E. D. (2012). Saccharomyces Genome Database: The genomics resource of budding yeast. *Nucleic Acids Research*, *40*(D1), D700–D705. https://doi.org/10.1093/nar/gkr1029

Debailleul, F., Trubbia, C., Frederickx, N., Lauwers, E., Merhi, A., Ruysschaert, J. M., André, B., & Govaerts, C. (2013). Nitrogen catabolite repressible GAP1 promoter, a new tool for efficient recombinant protein production in S. cerevisiae. *Microbial Cell Factories*, *12*(1), 129. https://doi.org/10.1186/1475-2859-12-129

Dower, W. J. (1990). Electroporation of bacteria: a general approach to genetic transformation. In *Genetic engineering* (Vol. 12). https://doi.org/10.1007/978-1-4613-0641-2_14

Drury, L. (1994). Transformation of bacteria by electroporation. In *Methods in molecular biology (Clifton, N.J.)* (Vol. 31, pp. 1–8). https://doi.org/10.1385/0-89603-258-2:1

Eynard, N., & Teissié, J. (2000). General Principles of Bacteria Electrotransformation: Key Steps. In *Electrotransformation of Bacteria* (pp. 3–22). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-662-04305-9_1

Felder, T., Bogengruber, E., Tenreiro, S., Ellinger, A., Sá-Correia, I., & Briza, P. (2002). Dtr1p, a multidrug resistance transporter of the major facilitator superfamily, plays an essential role in spore wall maturation in Saccharomyces cerevisiae. *Eukaryotic Cell*, *1*(5), 799–810. https://doi.org/10.1128/EC.1.5.799-810.2002

Gbelska, Y., Krijger, J. J., & Breunig, K. D. (2006). Evolution of gene families: The multidrug resistance transporter genes in five related yeast species. *FEMS Yeast Research*, *6*(3), 345–355. https://doi.org/10.1111/j.1567-1364.2006.00058.x

GenElute HP Plasmid DNA Midiprep Kit - 25 Purifications | Gen Elute | Sigma-Aldrich. (n.d.). Retrieved September 16, 2020, from https://www.sigmaaldrich.com/catalog/product/sigma/na0200?lang=pt&region=PT&gclid=CjwKCAjw74b7BRA_EiwAF8yHFO5jVywUxkk1TkP6aD6PkXWOgfIu7eJBuww-qRO9r8sePtWXZzly_hoCY6wQAvD_BwE

Goddard, M. R., & Greig, D. (2015). Saccharomyces cerevisiae: a nomadic yeast with no niche? ADAPTATION AND THE ECOLOGICAL NICHE. *FEMS Yeast Research*, *15*, 9. https://doi.org/10.1093/femsyr/fov009

Hess, D. C., Lu, W., Rabinowitz, J. D., & Botstein, D. (2006). Ammonium Toxicity and Potassium Limitation in Yeast. *PLoS Biology*, *4*(11), e351. https://doi.org/10.1371/journal.pbio.0040351

Invitrogen. (2009). *XCell II ™ Blot Module*. http://tools.thermofisher.com/content/sfs/manuals/blotmod_pro.pdf

Jauniaux, J.-C., & Grenson, M. (1990). GAP1, the general amino acid permease gene of Saccharomyces cerevisiae. Nucleotide sequence, protein similarity with the other bakers yeast amino acid permeases, and nitrogen catabolite repression. *European Journal of Biochemistry*, *190*(1), 39–44. https://doi.org/10.1111/j.1432-1033.1990.tb15542.x

Johnson, E. A. (2013). Biotechnology of non-Saccharomyces yeasts-the basidiomycetes. In *Applied Microbiology and Biotechnology* (Vol. 97, Issue 17, pp. 7563–7577). Springer. https://doi.org/10.1007/s00253-013-5046-z

Kilmartin, J. V., & Adams, A. E. M. (1984). Structural rearrangements of tubulin and actin during the cell cycle of the yeast Saccharomyces. *Journal of Cell Biology*, *98*(3), 922–933. https://doi.org/10.1083/jcb.98.3.922

Kodíček, M. (2007). *Biochemické pojmy (výkladový slovník)*. Http://Vydavatelstvi.Vscht.Cz/Knihy/Uid_es-002/Ebook.Html?P=uniport. http://147.33.74.135/knihy/uid_es-002/ebook.html?p=uniport

Kurien, B. T., & Scofield, R. H. (1995). Polyethylene glycol-mediated bacterial colony transformation. *BioTechniques*, *18*(6), 1023-1024+1026.

Lin, C. P. C., Kim, C., Smith, S. O., & Neiman, A. M. (2013). A Highly Redundant Gene Network Controls Assembly of the Outer Spore Wall in S. cerevisiae. *PLoS Genetics*, *9*(8), e1003700. https://doi.org/10.1371/journal.pgen.1003700

Ljungdahl, P. O., & Daignan-Fornier, B. (2012). Regulation of amino acid, nucleotide, and phosphate metabolism in Saccharomyces cerevisiae. *Genetics*, *190*(3), 885–929. https://doi.org/10.1534/genetics.111.133306

Mandel, H. (1970). Calcium-dependent Bacteriophage DNA. *J. Mol. Biol.*, *53*, 159–162.

Mitchell, P. (1963). The Structure and Function of the Membranes and Surfaces of Cells. In D. J. B. J. K. Grant (Ed.), *Biochemical society symposia*.

Neiman, A. M. (2005). Ascospore Formation in the Yeast Saccharomyces cerevisiae. *Microbiology and Molecular Biology Reviews*, *69*(4), 565–584. https://doi.org/10.1128/mmbr.69.4.565-584.2005

NuPAGE ® Technical Guide General information and protocols for using the NuPAGE ® electrophoresis system. (2010).

Oldenburg, K. R., Vo, K. T., Michaelis, S., & Paddon, C. (1997). Recombination-mediated PCR-directed plasmid construction in vivo in yeast. *Nucleic Acids Research*, *25*(2), 451–452. https://doi.org/10.1093/nar/25.2.451

Pao, S. S., Paulsen, I. T., & Saier, M. H. (1998). Major Facilitator Superfamily. *Microbiology and Molecular Biology Reviews*, *62*(1), 1–34. https://doi.org/10.1128/MMBR.62.1.1-34.1998

Prasanna, G. L., & Panda, T. (1997). Electroporation: Basic principles, practical considerations and applications in molecular biology. *Bioprocess Engineering*, *16*(5), 261–264. https://doi.org/10.1007/s004490050319

Reddy, V. S., Shlykov, M. A., Castillo, R., Sun, E. I., & Saier, M. H. (2012). The major facilitator superfamily (MFS) revisited. *FEBS Journal*, *279*(11), 2022–2035. https://doi.org/10.1111/j.1742-4658.2012.08588.x

Sá-Correia, I., dos Santos, S. C., Teixeira, M. C., Cabrito, T. R., & Mira, N. P. (2009). Drug:H+ antiporters in chemical stress response in yeast. *Trends in Microbiology*, *17*(1), 22–31. https://doi.org/10.1016/j.tim.2008.09.007

Saier Lab Bioinformatics Group. (2020). *https://tcdb.org/*. http://www.tcdb.org/search/result.php?tc=2.A.1

Smith, F. D., Harpending, P. R., & Sanford, J. C. (1992). Biolistic transformation of prokaryotes: Factors that affect biolistic transformation of very small cells. *Journal of General Microbiology*, *138*(1), 239–248. https://doi.org/10.1099/00221287-138-1-239

Tenreiro, S., Nunes, P. A., Viegas, C. A., Neves, M. S., Teixeira, M. C., Cabral, G., & Sá-Correia, I. (2002). AQR1 gene (ORF YNL065w) encodes a plasma membrane transporter of the major facilitator superfamily that confers resistance to short-chain monocarboxylic acids and quinidine in saccharomyces cerevisiae. *Biochemical and Biophysical Research Communications*, *292*(3), 741–748. https://doi.org/10.1006/bbrc.2002.6703

Tsai, S. P., Hartin, R. J., & Ryu, J. (1989). Transformation in restriction-deficient Salmonella typhimurium LT2. *Journal of General Microbiology*, *135*(9), 2561–2567. https://doi.org/10.1099/00221287-135-9-2561

Velasco, I., Tenreiro, S., Calderon, I. L., & André, B. (2004). Saccharomyces cerevisiae Aqr1 is an internal-membrane transporter involved in excretion of amino acids. *Eukaryotic Cell*, *3*(6), 1492–1503. https://doi.org/10.1128/EC.3.6.1492-1503.2004

Walker, G. M. (1998). *Yeast Physiology and Biotechnology* (1st ed.). John Wiley & Sons Ltd.

Zimmerman, U., and J. V. (1982). Electric field-induced cell-to-cell fusion. J. Membr. Biol. *J. Membr. Biol.*, *67*, 165–182.

Żymańczyk-Duda, E., Brzezińska-Rodak, M., Klimek-Ochab, M., Duda, M., & Zerka, A. (2017). Yeast as a Versatile Tool in Biotechnology. In *Yeast - Industrial Applications*. InTech. https://doi.org/10.5772/intechopen.70130

6 Appendices

6.1 APPENDIX A

6.1.1 Culture Media

6.1.1.1 Stock Solutions for media preparation

Table 6-1 Stock solutions for media preparation.

VITAMINS FOR YEAST (MG) FOR 1 LITER OF BI-DISTILLED WATER	Biotin	250
	Calcium Panthotenate	200
	Inositol	1000
	Pyridoxine	100
	Thiamine	100
TRACES (METAL BASED) (MG) FOR 1 LITER OF BI-DISTILLED WATER	Boric Acid (H_3BO_3) in 100 mL of bi-distilled water	10
	Cooper Sulphate ($CuSO_4.5H_2O$)	1
	Potassium Iodide (KI)	2
	Sodium Molybdate ($Na_2MoO_4.2H_2O$)	4
	Zinc Sulphate ($Zn\ SO_4 . 7H_2O$)	14
	Ferric Chloride ($FeCl_3. 6H_2O$)	5000
	Manganese Sulphate ($Mn\ SO_4.H_2O$)	400
	Citric Acid H_2O ($C_6H_8O_7. H_2O$)	10000

The solution was sterilized in the autoclave for 20 minutes. The stock solution was preserved away from light and at room temperature.

6.1.1.2 Media composition

Table 6-2 Media composition.

		YNB (YEAST NITROGEN BASE)	*164*	*165*
SALTS (MG/L)	Monopotassium phosphate (KH_2PO_4)	1000	1000	1000
	Magnesium Sulphate $7H_2O$ ($SO_4Mg.7H_2O$)	500	700	700
	Sodium Chloride ($NaCl$)	100	500	500
	Calcium Chloride $2H_2O$ ($CaCl_2.2H_2O$)	100	400	400
VITAMINS (MG/L)	Biotin	0,002	0,002	0,002
	Panthotenate	0,4	2	2
	Folic Acid	0,002		
	Inositol	2	10	10
	Niacin	0,4		
	PABA (4-Aminobenzoic acid) $C_7H_7NO_2$	0,2		
	Pyridoxine	0,4	1	1
	Riboflavin	0,2		
	Thiamine	0,4	1	1
TRACES (METAL BASED) (MG/L)	Boric Acid (H_3BO_3)	0,5	0,01	0,01
	Cooper Sulphate ($CuSO_4.5H_2O$)	0,04	0,001	0,001
	Potassium Iodide	0,1	0,02	0,02
	Ferric Chloride ($FeCl_3$. $6H_2O$)	0,2	5	5
	Manganese Sulphate ($Mn\ SO_4.H_2O$)	0,4	0,4	0,4
	Sodium Molybdate ($Na_2MoO_42\ H_2O$)	0,2	0,004	0,004
	Zinc Sulphate ($Zn\ SO_4$. $7H_2O$)	0,4	0,014	0,014
	Potassium Sulphate ($K_2\ SO_4$)			1000
BUFFERS (ML)	Citric Acid H_2O ($C_6H_8O_7$. H_2O)		10500	10500
	Potassium Hydroxide 10 M pH=6,1 (KOH)		16100	16100

*pH value must be approximately 6.15 at the time of sterilization.

	863 (YEAST-SPECIFIC)	*853 (E. COLI SPECIFIC)*
YEAST EXTRACT (G/L)	10	5
BACTO PEPTONE (G/L)	10	
BACTO TRYPTONE (G/L)		10
Dipotassium phosphate (K_2HPO_4) (G/L)		0,7
Monopotassium phosphate (KH_2PO_4) (G/L)		0,3
Sodium Chloride (NaCl) (g/L)		5
Glucose (g/L)	20	1

The culture medium was sterilized in the autoclave for 20 minutes and preserved away from light and at room temperature.

6.1.1.3 Antibiotics

Ampicillin

Ampicillin was used in cultures of *E. coli MC1061 and XL1Blue strains* as a selection pressure, considering that the plasmids that were used for *E. coli* transformation carried the gene responsible for ampicillin resistance integrated in their sequence.

The stock concentration was 100 mg/mL, and working concentration was 100 µg/mL (1mL of the stock solution was added per liter of media)
The stock solution was prepared by dissolving 1g of sodium ampicillin in H_2O to reach a final volume of 10 mL and was stored in aliquots at -20°C (for 1 year or at 4°C for 3 months).

Geneticin (Antifungal)
Geneticin is used in cultures of yeast, in this case *S. cerevisiae 23344c strain,* as a selection pressure, considering that at the time of transformation, the fragment used to transform carries the gene conferring resistance to this antibiotic *(KanMX).*
Working concentration: 200mg/L.

Nourseothricin (Antifungal)

Nourseothricin is used in cultures of yeast, in this case, *S. cerevisiae* 23344c strain as a selection marker, considering that at the time of transformation, the fragment used to transform confers a resistance to this antibiotic.

Working concentration: 100 mg/mL.

All antibiotics used were acquired from Sigma Aldrich, Belgium

In order to prepare 500 mL of minimum media containing amino acids to complement auxotrophic mutations, 50 mL of Yeast Nitrogen Base (10x concentrated) without ammonium was added 50 mL of Glucose at 30% (w/v) and the required amino acid. Following are the quantities used and the concentration of each amino acid that complements auxotrophic mutations:

20 mL Glutamine (2,5%, w/v)
5 mL Proline (10%, w/v)
5 mL Uracil 0, (25 %, w/v)
5 mL Tryptophan (0,25 %, w/v)
2,5 mL Leucine (1%, w/v)
5 mL Adenine (0,25 %, w/v)
2,5 mL Lysine (1%, w/v)
1 mL Histidine (1%, w/v)
1,25 mL Methionine (1%, w/v)
1,25 mL Cysteine (1%, w/v)
Complemented the volume of medium with sterile water.

6.1.2 Solutions

- Lithium Acetate (+ Polyethylene glycol 4000 or PEG 4000)

0,1M Lithium Acetate

(11, 4mM PEG 4000)

- Blue loading buffer 5x for proteins

312 mM Tris pH 6, 8

50% Glycerol

10% SDS (Sodium Dodecyl Sulphate)

0, 5% bromophenol blue

5% β-mercaptoethanol (add at the time of use)

- Blue Loading Buffer 10x for DNA

0, 25% bromophenol blue

25mM EDTA

50% glycerol

- Phenylmethanesulfonyl fluoride (PMSF) 0,2M
  1g of PMSF in 30 mL of Isopropanol

- Ponceau Red

200mg/mL Ponceau Red

3% TCA

- Sodium Acetate +EDTA Buffer Solution (AE)

50mM sodium acetate pH 5, 2

10mM EDTA

- Tris-borate-EDTA buffer 10x (TBE)

0, 9M Tris

0, 9M boric acid

32mM EDTA

- Tris Buffer Saline (TBS)

50mM Tris

150mM NaCl

Adjust the pH to 7, 5 with HCl 1M

- Tris Buffer Saline Tween (TBST)

Dissolve 1mL of Tween 20 into 1L of TBS solution

- Lysis Solution for DNA isolation

2% Triton X-100

1% SDS

100 mM NaCl

10 mM Tris pH 8.0

1 mM EDTA

- NuPAGE® MOPS Buffer SDS 20x

50mM MOPS

50mM Tris Base

0,1% SDS

1mM EDTA

pH 7,7

To prepare 500mL of 20X NuPAGE® MOPS SDS Running Buffer, the following reagents were dissolved in 400mL ultrapure water:
MOPS 104,6 g; Tris Base 60,6 g; SDS 10 g EDTA 3,0 g. The components were mixed well and the volume adjusted to 500mL with ultrapure water.
The Buffer was stored at +4°C. This buffer is stable for 6 months when stored at +4°C. (*NuPAGE ® Technical Guide General information and protocols for using the NuPAGE ® electrophoresis system*, 2010)

- NuPAGE® Transfer Buffer 20x

25 mM Bicine

25 mM Bis-Tris (free base)

1 mM EDTA

pH 7.2

To prepare 125 mL of 20X NuPAGE® Transfer Buffer, the following reagents were dissolve to 100 mL ultrapure water:

Bicine 10,2 g: Bis-Tris (free base) 13,1 g; EDTA 0,75 g.

The components were well mixed and the volume adjusted to 125 mL with ultrapure water and stored at +4°C. The buffer is stable for 6 months when stored at +4°C. 4. (Invitrogen, 2009; *NuPAGE ® Technical Guide General information and protocols for using the NuPAGE ® electrophoresis system*, 2010).

For the working solution 25 mL of the transfer buffer 20x and 50 mL of methanol 99.9% were transferred into 500 mL of sterile water.

- Blocking Solution

Also, 2 g of blocking agent were dissolved in 100 mL of TBST.

- Lysis Solution (500mL):

25 mL 1M Tris pH8
250 µL 1%NP40
15 mL 5M NaCl
5 mL EDTA 1 mM
@ 500mL with water

6.1.3 Antibodies

The quantity used of each antibody in western blotting test differs, the solution in which the antibody was introduced was 10mL TBST+ blocking reagent.

Anti-His6 (Monoclonal Anti-polyHistidine produced in mouse)

200 µg/ml; 10 µL in solution

Reference number: 11922416001 from Roche Life Science (Belgium)

PGK1 (3-PhosphoGlycerate Kinase)

250µg at 1µg/µL; Use 100x dilution and 2 µL in solution

From Invitrogen/Life technologies (Belgium)

Goat anti-mouse IgG-HRP (secondary antibody, Horseradish peroxidase conjugate, antibody produced in goat)

200µg/0,5mL; Use 1000x dilution and 10 µL in solution

From GE Healthcare/Fisher Scientific (Belgium)

Penta-His Antibody BSA-free (3 µg) (mouse monoclonal IgG1)

5 µL in solution

From Qiagen© (Belgium)

RGS-His Antibody BSA-free (3 µg) (mouse monoclonal IgG1)

5 µL in solution

From Qiagen©(Belgium)

Tetra-His Antibody BSA-free (3 µg) (mouse monoclonal IgG1)

5 µL in solution

From Qiagen©(Belgium)

6.1.4 Molecular Weight Ladders

Table 6-3 Molecular Weight Ladders.

Page Ruler™ Prestained Protein Ladder	Reference number: 26616	From Thermo Scientific™ (Belgium)
6xHis Protein Ladder	Reference number: 34705	From Qiagen© (Belgium)
GeneRuler 1kb Plus DNA Ladder, ready-to-use	Reference number: SM1333	From Thermo Scientific™ (Belgium)