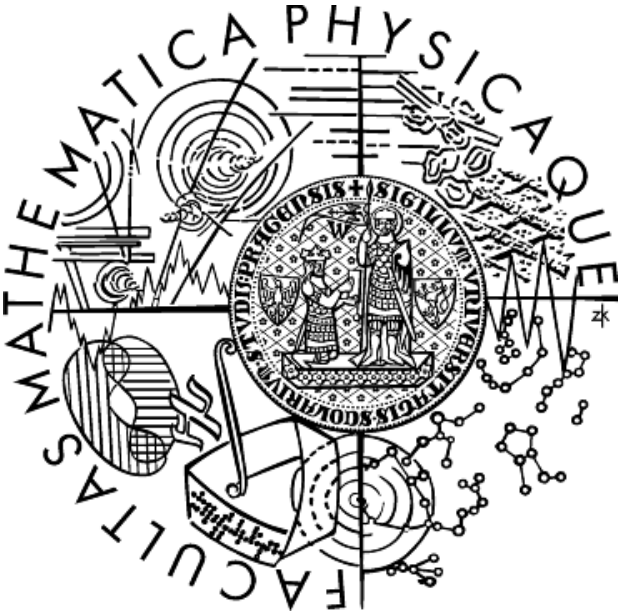
Charles University in Prague
Faculty of Mathematics and Physics

DIPLOMA THESIS



Dávid Džamba

Membrane potential measurements in *Saccharomyces cerevisiae* mutant strains deficient in various membrane transporters

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Study program: Biophysics and Chemical Physics

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I hereby certify that I wrote the thesis myself using only the referenced sources. I agree with publishing and lending of the thesis.

Prague, 16 April 2009 Dávid Džamba

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Abstract: Potassium transport across the cell membrane is supposed to contribute to the maintenance of membrane potential. In this work, the diS-C_3(3) assay was used to determine the contribution of three main potassium transporters of *S. cerevisiae* cells (Trk1p, Trk2p and Tok1p) to the membrane potential changes in three different growth phases. It was shown that deletions of these transporters cause hyperpolarisation of the cell membrane in exponential and early diauxic growth phases; no difference was detected in post-diauxic cells. Another contribution of this work is in a deeper study of the activity of ABC pumps which are crucial in multidrug resistance. It was proved that apart from Pdr5p and Snq2p pumps, there are also other active extrusion pumps (most likely Pdr10p and Pdr15p). It was also showed that Δpdr1, Δpdr3 mutant strain is not an equivalent of strain lacking the multidrug pumps Pdr5p, Snq2p and Yor1p. The results in this work indicate that dis-C_3(3) probe is probably a substrate of other pumps such as Pdr15p. Study of these transporters with our method is thus very suitable and could provide detailed information about their kinetics and help in finding their inhibitors.

Keywords: *Saccharomyces cerevisiae*, DiS-C_3(3), Membrane potential, Potassium transporters, Multidrug resistance pumps.

Názov práce: Meranie membránového potenciálu u mutantných kmeňov *Saccharomyces cerevisiae* deficientných v rôznych membránových transportéroch
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Abstrakt: Predpokladá sa, že draslíkový transport cez bunečnú membránu prispieva k udržovaniu membránového potenciálu. V tejto práci bol za použitia fluorescenčnej metódy využívajúcej sondu diS-C_3(3) zistený príspevok troch hlavných draslíkových transportérov buniek *S. cerevisiae* (Trk1p, Trk2p and Tok1p) k zmene membránového potenciálu, a to v troch rôznych rastových fázach. Bolo preukázané, že delecie týchto transportérov spôsobia hyperpolarizáciu bunečnej membrány v exponenciálnej a skorej diauxickej fáze; v post-diauxickej fáze nebola zaznamenaná žiadna zmena. Táto práca prispela tiež k štúdiu aktivity ABC púmp, ktoré hrajú hlavnú úlohu v mnohonásobnej liekovej rezistencii. Bolo dokázané, že okrem Pdr5p a Snq2p púmp existujú aj ďalšie aktívne pumpy (pravdepodobne Pdr10p a Pdr15p). Bolo tiež preukázané, že Δpdr1, Δpdr3 mutantný kmeň nie je ekvivalent kmeňa bez Pdr5p, Snq2p a Yor1p púmp. Výsledky tejto práce ukazujú, že sonda dis-C_3(3) je pravdepodobne substrátom ďalších púmp ako Pdr15p. Štúdium týchto transportérov našou metódou je preto veľmi vhodné a môže poskytnúť detailne informácie o ich kinetike ako aj pomôcť v hľadaní ich inhibítorov.

Kľúčové slová: *Saccharomyces cerevisiae*, DiS-C_3(3), Membránový potenciál, Draslíkové transportéry, Pumpy mnohonásobnej liekovej rezistencie.

1 Introduction

Multidrug resistance is a hot topic of current biomedical research since it is one of the major obstacles in cancer therapy. It is mediated by multidrug transporters (MDR transporters) which are present in bacterial, yeast as well as mammalian cells. The study of these transporters is crucial for deeper understanding of the problem which in the long run can lead not only to better treatment of cancer but also many other diseases like cystic fibrosis or malaria [1] [2].

Another important group of membrane transporters is a group of potassium transporters. They are responsible for potassium uptake into cells which is involved in lots of important physiological processes, for example pacemaker activity in heart, insulin release or potassium uptake in glial cells [3]. They also play a key role in regulating and maintaining membrane potential. Malfunction of potassium transporters leads to fatigue, muscle weakness, mood changes and also to many severe diseases like kidney hypertrophy or hypertension.

Yeast shares basic structural and functional organization with higher eukaryotes and has an advantage of easier manipulation. The aim of this work was to study the impact of different mutations in potassium and MDR transport systems of *Saccharomyces cerevisiae* including characterization of these strains in terms of growth and cultivation conditions. Our goal was to find out how these mutations influence membrane potential and the ability to extrude foreign substances from cytosol and thus to contribute to the general understanding of the problem.

This work was created under the intercollegiate agreement between the Rheinische Friedrich-Wilhelms-Universität in Bonn and the Charles University in Prague. The first part which was performed at Institut für Zelluläre und Molekulare Botanik der Uni Bonn was mostly focused on preparation of *S. cerevisiae* mutant strains using available molecular biology techniques. Most of the fluorescence

measurements were performed in the second part of this work at the Biophysics Division of the Institute of Physics of Charles University in Prague, where this measurement method was developed.

2 Theory

2.1 Saccharomyces cerevisiae

Saccharomyces cerevisiae, or so-called baker's yeast, is a single cell eukaryote and belongs to the Ascomycota phylum of the Fungi kingdom. It is used since ancient times in baking and brewing. It is a very good model of eukaryotic organisms so it is intensively studied in molecular and cell biology - it is the first eukaryotic organism with completely sequenced genome. Lots of processes present in *S. cerevisiae* are highly analogous to mammalian cells and therefore the study of yeast contributes to understanding basic mechanism present also in human cells.

2.1.1 Cell composition

Cells of *S. cerevisiae* are round to ovoid, 5-10 μm in diameter. Cell size and shape are determined by given yeast genus and are highly influenced by cultivation conditions. They also change during the growth, for instance in exponential growth phase cells are much smaller than in the subsequent growth phases. The cell envelope consists of cell wall and cytoplasmic membrane. The inside of the cell is filled with cytosol which contains vacuoles, nucleus, mitochondria, and other organelles.

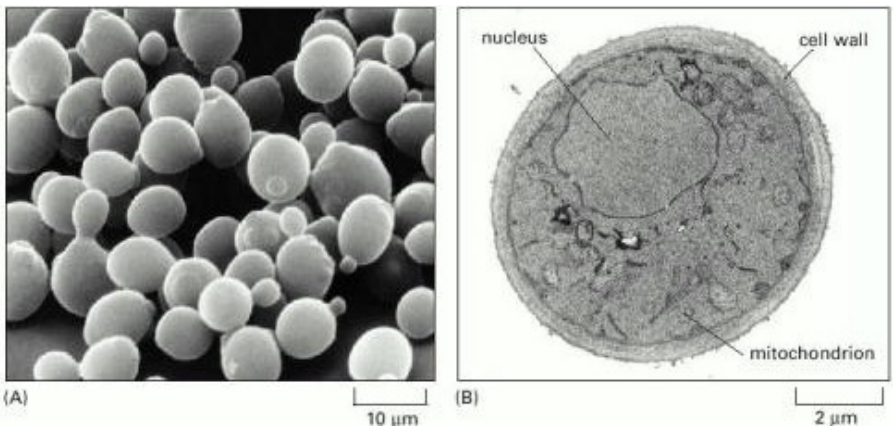

Figure 1: The yeast *Saccharomyces cerevisiae*. (A) A scanning electron micrograph of a cluster of the cells. (B) A transmission electron micrograph of a cross section of a yeast cell, showing its nucleus, mitochondrion, and thick cell wall. [4]

Cell wall

The main function of the cell wall is to provide structural support and protection from mechanical stress. It is 150 - 450 nm thick and is composed mainly of polysaccharides (80%) and proteins (6-10%). It also permits the creation of a stable osmotic environment for the cell.

Cytoplasmic membrane

Cytoplasmic membrane is a semipermeable lipid bilayer and it separates the inside of the cell from its surroundings. It contains a variety of specific proteins which enable transport through the membrane, ion channel conductance and cell signalling.

2.1.2 Reproduction

The most common mode of yeast reproduction is vegetative (asexual) budding. Here a small daughter cell, a bud, is formed on the parent cell. While the bud grows, the nucleus splits into two parts and one part migrates into the daughter cell. When the bud separates from a parent cell, a new daughter cell is created.

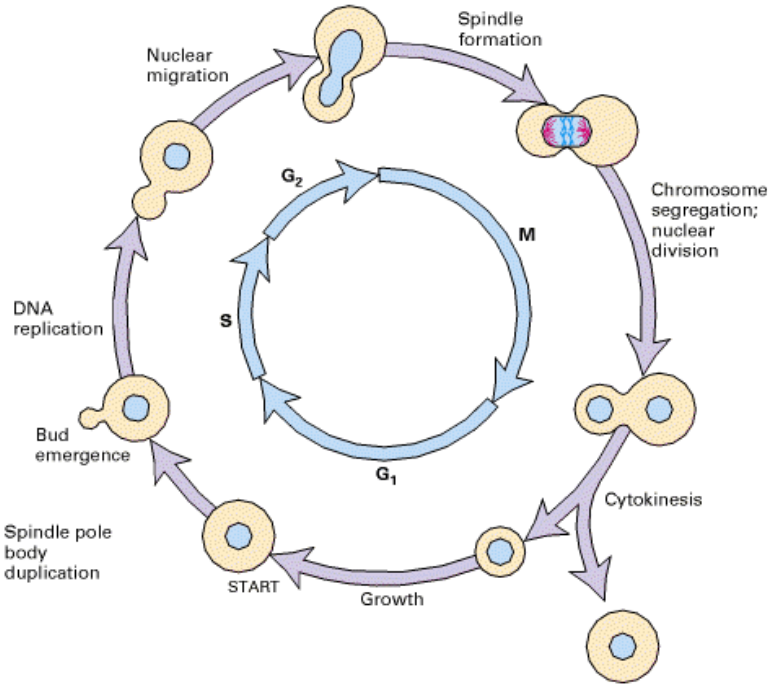

Figure 2: Main events in *S. cerevisiae* cell cycle. [5]

Under certain conditions, some yeast strains can undergo also sexual reproduction. In this process, two haploid cells conjugate and create a diploid zygote, which can either vegetatively reproduce or undergoes sporulation connected with nucleus meiotic division and results in two haploid cells.

Culture growth in liquid medium

For research purposes, *S. cerevisiae* culture is usually grown in a liquid medium before samples are prepared out of it. There are many different liquid media varying in substrate compositions. The overall growth can be expressed by a growth curve which demonstrates the dependence of optical density of culture suspension (which is proportional to the number of cells) on time. In medium with a fermentable substrate (e.g. glucose) we can distinguish 6 growth phases.

1. lag phase: during lag phase cells adapt to new growth conditions and acquire energy needed for reproduction.

2. exponential phase: in this phase the culture growth is the most rapid. Cells are smaller as they reproduce before reaching mature size. There is also the smallest percentage of dead cells in culture in this phase.

3. diauxic shift: after depletion of glucose from the medium cells alter their metabolism to a new carbon source (ethanol, glycerol). During this adaptation to oxidative metabolism the number of cells no longer increases but rather slightly decreases.

4. post-diauxic phase: when cells adapt to the new conditions the culture starts to grow again but much slower than in the exponential phase. Cells are more resistant to heat shock in this phase.

5. stationary phase: after about 4-7 days cells enter the stationary phase in which the number of cells does not change.

6. death phase: scarcity of nutrients causes gradual cells death.

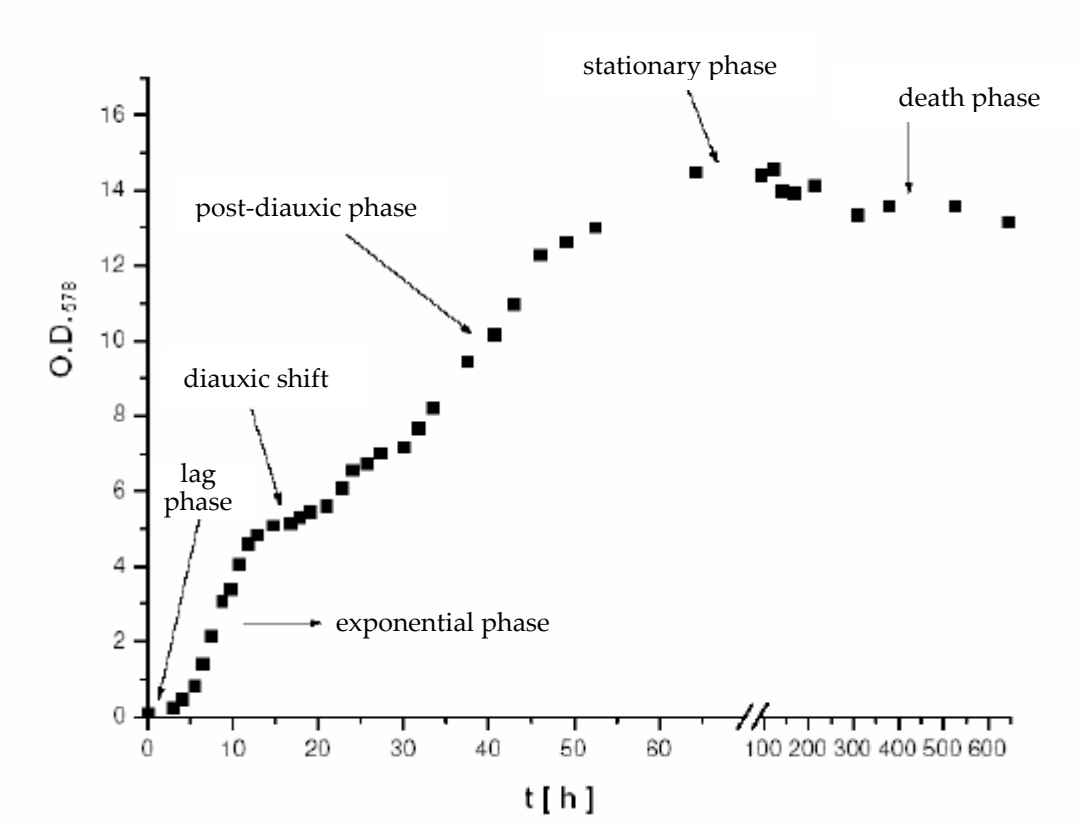

Figure 3: Typical growth curve of *S. cerevisiae.* [6]

2.1.3 Membrane potential

One of the essential conditions for cells to grow is sustained membrane potential. Membrane potential is an electrical potential difference between exterior and interior of a cell, where plasma membrane acts as a highly resistive electrical barrier.

Membrane potential is created as a result of distribution of ions which carry positive or negative charge. Ions move according to electric and concentration gradients. When these two gradients are equal we speak about dynamic equilibrium. Membrane potential at dynamic equilibrium is called equilibrium potential and is given by the Nernst equation:

$$\Psi = \frac{RT}{zF} ln \frac{c^{out}}{c^{in}}$$

where R is universal gas constant, T temperature, z number of elementary charges of given ion, F Faraday constant, c^{out} concentration of given ion outside the cell and c^{in} concentration of given ion inside the cell.

The difference between the electrical potential on the internal side of a membrane Ψ^{in} depicted as dipole potential (inside) in Figure 4 and the electrical potential on the outer side of a membrane Ψ^{out} – dipole potential (outside) is called resting membrane potential $\Delta\Psi = \Psi^{in} - \Psi^{out}$.

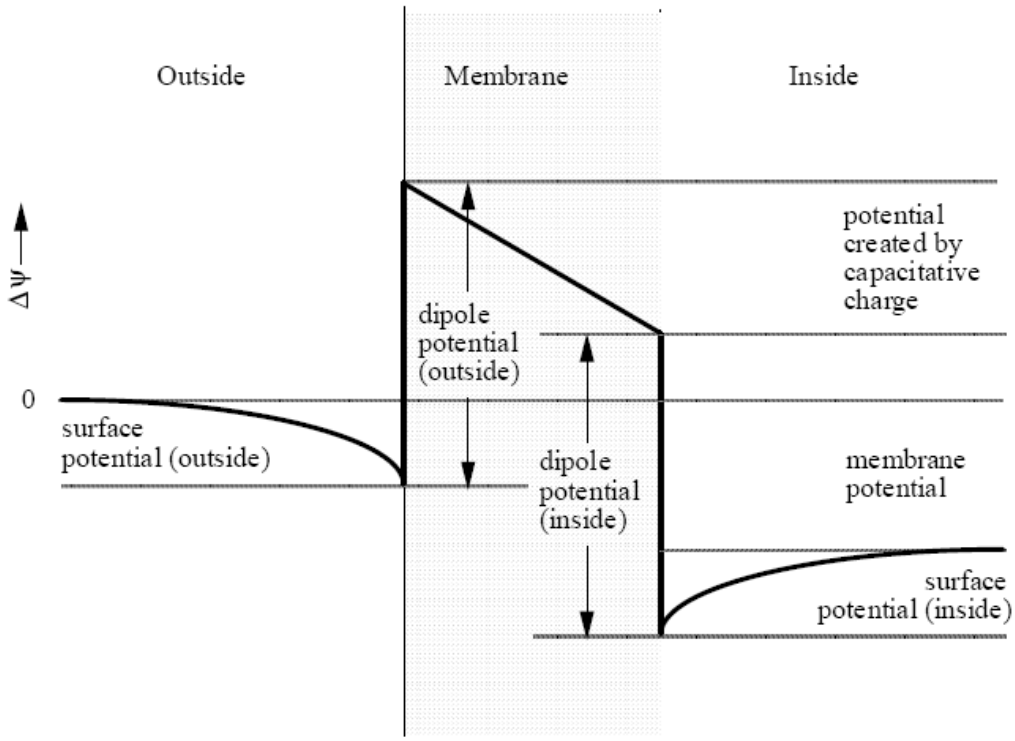

Figure 4: Components of the membrane potential. [7]

$\Delta\Psi$ is the sum of equilibrium potentials of all ions (e.g. Na^+, K^+, H^+ etc.) and is usually negative with values between -9 to -100mV. Reduction of membrane potential (in absolute value - it is less negative) is called membrane depolarisation and its increase (it is more negative) is called membrane hyperpolarisation.

The main regulator of the membrane potential in yeast is the proton pump H^+-ATPase. It transports protons out of the cell using energy stored in ATP molecules, changing thereby the pH in the cell. Its activity can be upregulated (e.g. by glucose)

but also downregulated (e.g. by lysosomotropic substances such as DM-11 etc. [8]). Other ions whose transport across the plasma membrane is believed to significantly contribute to the maintenance of membrane potential are potassium cations. [9]

2.1.4 Main membrane transport systems

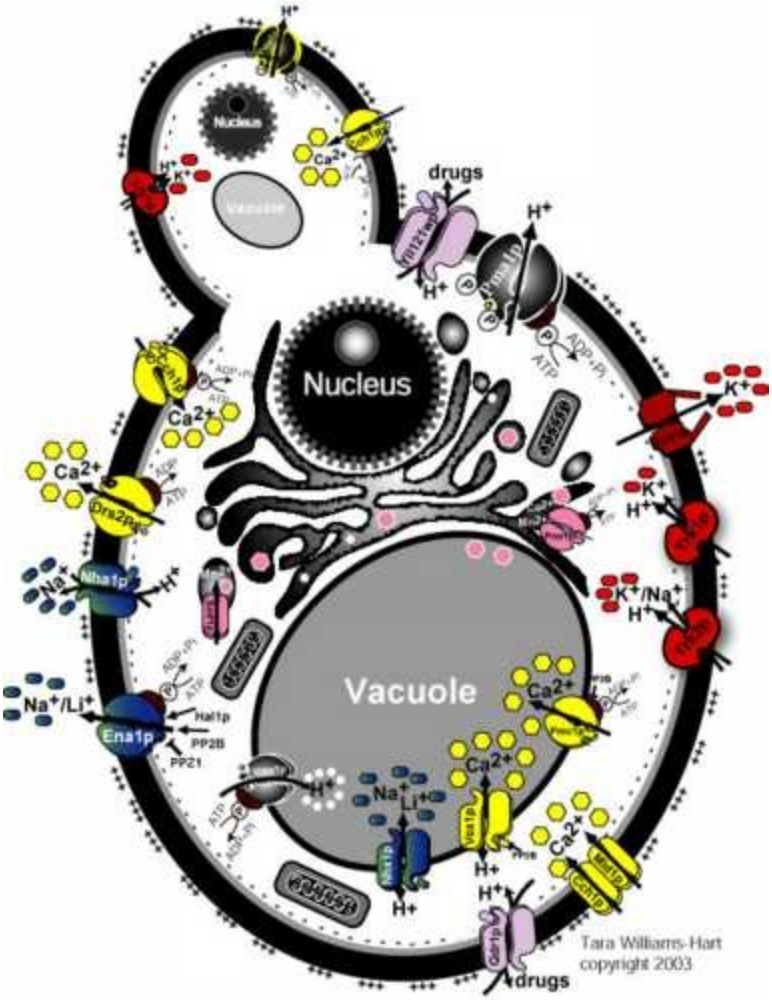

Figure 5: The main transport systems of *Saccharomyces cerevisiae*. [10]

Transport across the membrane can be either non-specific permeation or mediated transport which can be further divided to passive transport (from the region with higher concentration to the region with lower concentration) and active transport (from the region with lower concentration to the region with higher concentration). Active transport can be driven by ATP hydrolysis (primary active transport) or it

can be coupled with the transport of another substance, especially with H^+, which provides energy for transport (secondary active transport).

According to the transport mode we distinguish three main types of membrane transporters:

1.Channels/pores: passive transporters (e.g. Tok1p)

2.Carriers: secondary active transporters (e.g. Nha1p)

3.Pumps: primary active transporters (e.g. ABC pumps)

2.2 Multidrug resistance in yeast

Multidrug resistance plays a key role in organism resistance against a wide variety of chemicals, including drugs used to eradicate bacterial or tumour cells during chemotherapy or other kind of treatment. This resistance is the result of membrane multidrug resistance transporters (MDR transporters) which efflux foreign substances from cell cytosol. In yeast we speak about pleiotropic drug resistance transporters (PDR transporters).

2.2.1 Regulatory networks

As far as we know, multidrug resistance in *S. cerevisiae* is regulated by two main regulatory networks – PDR and YAP [11]. These contain genes encoding transcription factors - proteins which bind to promoters of genes of membrane proteins providing multidrug resistance and thus regulate their transcription. Both regulatory networks are functionally interconnected so they can influence or in some cases (e.g. heat shock) even substitute each other.

YAP regulatory network

YAP (Yeast Activator Protein) regulatory network is involved in mediating resistance to a variety of toxicants (e.g. cycloheximide) and in providing tolerance

to oxidative stress. Main transcription factors of this regulatory network are Yap1p and Yap2p coded by genes YAP1 and YAP2.

PDR regulatory network

PDR1 and PDR3 are two main genes of PDR (Pleiotropic Drug Resistance) regulatory network. They encode transcription factors Pdr1p and Pdr3p which are high homologues (36% identical amino acid sequence) and both regulate transcription of many drug extrusion pumps (e.g. Pdr5p, Snq2p, Yor1p etc.) as well as transcription of several transcription factors (e.g. Pdr1p positively regulates transcription of Pdr3p). Some of the transcription factors can self-regulate their own transcription, too (e.g. Pdr3p).

Another very important gene of this regulatory network is YRR1 gene. The expression of YRR1 is controlled by Pdr1p (and probably also Pdr3p) implying interconnection between regulatory circuits that control expression of the drug extrusion pumps in *S. cerevisiae*. Yrr1p is a self-regulating transcription factor which regulates transcription of YOR1 and SNQ2 genes.

2.2.2 Transporters

There are two superfamilies of multidrug efflux membrane transporters – MFS and ABC superfamily [12].

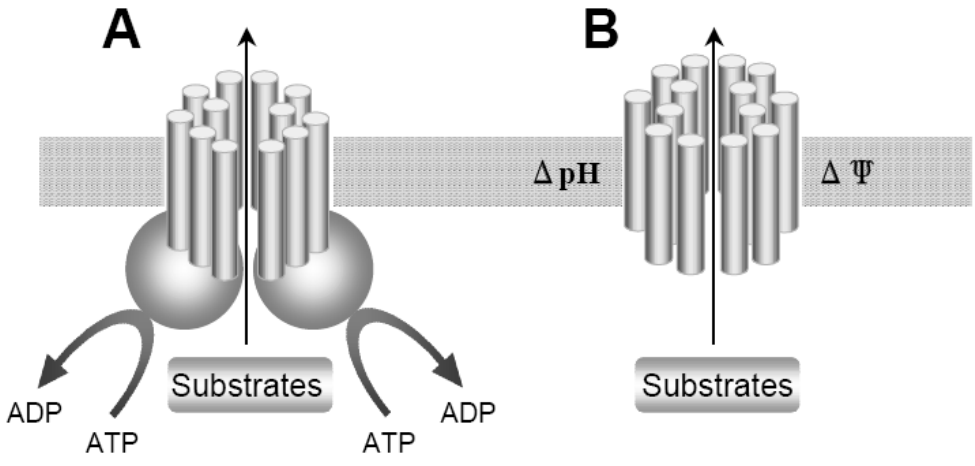

Figure 6: Topology of (A) ABC transporter and (B) MFS transporter. [13]

MFS membrane efflux transporters

The major facilitator superfamily (MFS) is one of the two largest families of membrane transporters found on the Earth [14]. It was originally defined as a superfamily of permeases. It consists of a large group of structurally and functionally homologous membrane transporters present ubiquitously in bacteria, archaea, and eukaryotes. Phylogenetic analyses revealed the occurrence of 17 distinct families within the MFS, each of which generally transports a single class of compounds (e.g. simple sugars, oligosaccharides, inositols, drugs etc.). All MFS transporters possess either 12 or 14 putative or established transmembrane alpha-helical domains usually segmented in two units. MFS proteins utilize a proton gradient to expel the substances.

ABC membrane efflux transporters

The ATP-binding cassette (ABC) transporters are also present in all organisms from bacteria to humans. These integral membrane pumps are responsible for the ATP-powered translocation of a wide variety of substrates across membranes. The molecular mechanism of these efflux pumps - the protein binding a structurally diverse range of compounds – is still unknown. ABC pumps are usually composed of two units, each containing one transmembrane and one ATP-binding domain. Transmembrane domain consists of several (usually 6) α-helixes with substrate binding region. There are several possible routes of ABC pump transport. According to the "vacuum cleaner" hypothesis, drugs may be extruded directly from the membrane (inner or outer leaflet) into the extracellular space. Flippase model proposes that drugs can be flipped from the inner to the outer leaflet. Another possibility is classical pump model where drugs are extruded directly from cytoplasm [2].

There are several ABC transporters in *S. cerevisiae*. One of the best-studied ones is **Pdr5p** whose 3D structure has been revealed by negative staining technique

(Figure 7, [15]). Pdr5p has a relatively short lifetime – half-time is ca. 60-90 minutes. PDR5 gene is not essential for the cell, however, it provides resistance to various inhibitors (e.g. cycloheximide, chloramphenicol etc.). It is positively regulated by Pdr1p, Pdr3p and Yap1p transcription factors and negatively regulated by Rdr1p transcription factor. Another very important ABC transporter is **Snq2p** which is a homologue to Pdr5p (37% identity) and so it has also similar substrate specificity. SNQ2 gene is not essential and it is present in cell genome in several copies. It is positively regulated by Pdr1p, Pdr3p, Pdr8p, Yrr1p and Yrm1p. **Yor1p** is another ABC transporter present in *S. cerevisiae*. It provides resistance against oligomycin, reveromycin A, aureobazidin A and it also detoxicates organic anions containing carboxyl groups. **Pdr10p, Pdr11p, Pdr12p** and **Pdr15p** are examples of ABC pumps which have not yet been studied to such an extent as the above 3 pumps. Pdr15p, in contrast to Pdr5p, is strongly induced by various stress conditions including heat shock, low pH, weak acids, or high osmolarity [16].

Figure 7: A, three-dimensional reconstruction of a Pdr5p dimer at 25-Å resolution in negative staining. [15]

The pleiotropic drug resistance network in yeast represents a very complicated problem which is still being very intensively researched. For better orientation in PDR transcription regulators and their ABC family target genes see Figure 8.

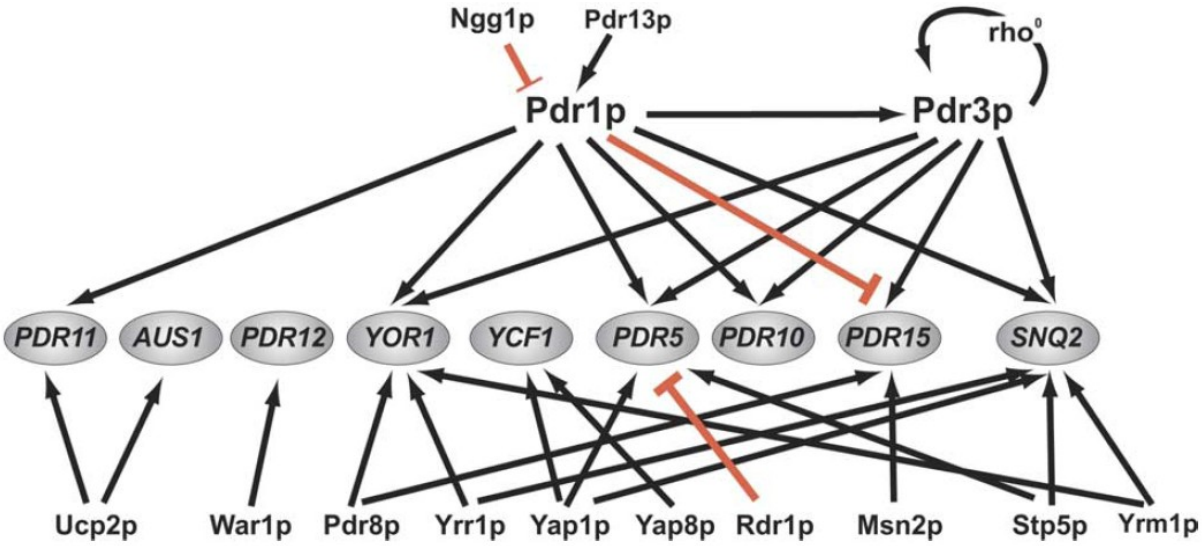

Figure 8: The PDR network in yeast. Genes in the centreline represent target genes of transcriptional regulators depicted above and below. Note that the cartoon lists only genes of the ABC family. The yeast PDR network also contains non-ABC genes whose function has not been established in many cases. Red lines indicate a negative regulatory impact, while black lines ending with an arrow indicate positive regulation. [17]

2.3 Potassium transporters

Proper potassium transport is essential for every living cell since it regulates and maintains its membrane potential. Malfunction of potassium transport leads to a variety of severe diseases. Most of the cells accumulate potassium against a large concentration gradient inside the cell using several potassium transporters. For review of potassium transporters in *S. cerevisiae* see Figure 9 [18].

2.3.1 Trk1p and Trk2p [19]

Trk1p and Trk2p are the most important potassium-specific transport systems present in *S. cerevisiae*. Trk2p is a weaker expressed homologue to Trk1p. They derive energy from the plasma membrane proton-motive force. Both transporters facilitate high-affinity potassium uptake into the cells and appear to be functionally redundant under a wide range of environmental conditions. The presence of both, Trk1p and Trk2p transporters, enables cells to grow at micromolar K^+ concentration. Yeast lacking TRK1 gene require millimolar K^+ concentration and the growth depends upon the activity of Trk2p. Cells carrying deletions in both

TRK1 and TRK2 genes require at least 50mM K^+ concentration where cells take up K^+ via poorly understood non-specific mechanisms (NSC1).

2.3.2 Tok1p [19]

Tok1p is a voltage-dependent potassium channel with strong outward rectification. The channel is open at positive and very low negative membrane voltages and its activity is modulated by external K^+ concentration. Deletion in TOK1 gene does not affect the growth phenotype and thus the physiological role of Tok1p is still unclear.

2.3.3 Nha1p [20]

S. cerevisiae cells possess an alkali metal cation antiporter encoded by the NHA1 gene. Nha1p is unique in the family of yeast Na^+/H^+ antiporters for its broad substrate specificity (Na^+, Li^+, K^+).

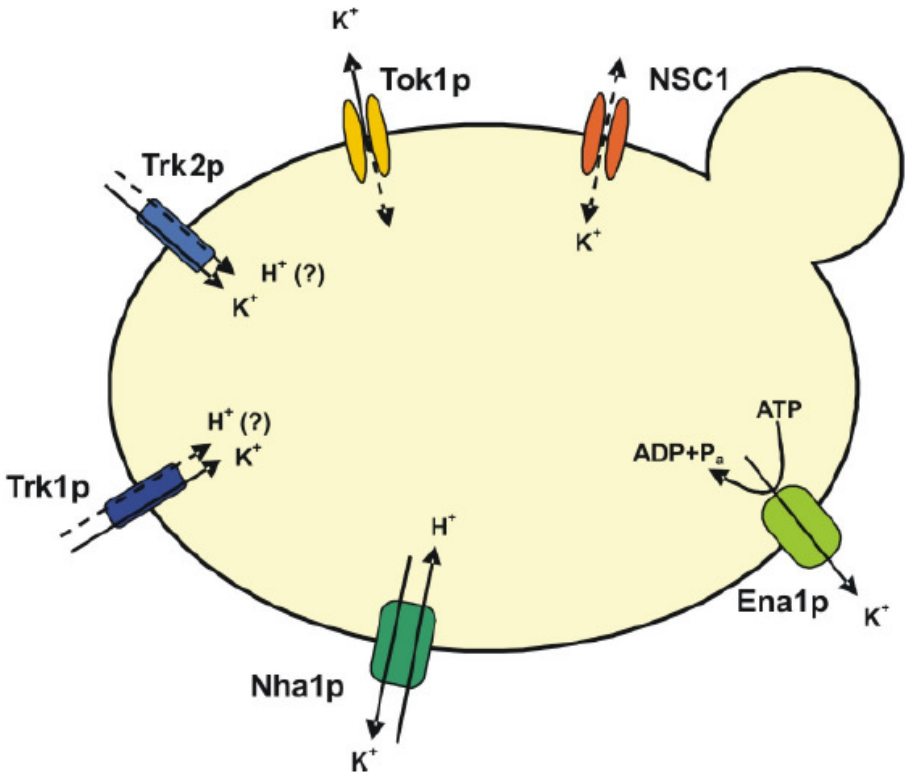

Figure 9: Potassium transport systems in *S. cerevisiae.* [18]

2.3.4 Ena1p [21]

Another transporter that transports also K^+ cations through *S. cerevisiae* plasma membrane is Ena1p. It is an ATPase and is primarily responsible for Na^+ and Li^+ efflux during salt stress. However, at high pH and high K^+ concentrations it effluxes also potassium cations.

2.3.5 mKir2.1p

Potassium transporter mKir2.1p belongs to inward rectifying K^+ subfamily of the potassium channel superfamily. It is a mammalian transporter originally isolated from a mouse. To study this channel, *S. cerevisiae* mutant strain carrying KIR2.1 gene was created. The phenotype of this mutant depended on the level of Kir2.1 expression and on the external pH. The activity of mKir2.1p in the yeast cells was almost negligible at pH 3.0 and the highest at pH 7.0. Kir2.1p was permeable for both potassium and rubidium cations, but neither sodium nor lithium were transported via this channel [3], [18], [22].

2.4 Membrane potential measuring methods

2.4.1 The most common methods

Classical method of membrane potential measurement is by means of a microelectrode inserted into the cell. The microelectrode is usually a glass capillary with 1 μm diameter tip on its end and is inserted into the cell with the help of microscope and micromanipulator. The advantage of this method is the possibility to measure also fast changes that are observed for example in action potentials in neurons. The biggest disadvantage is the impossibility to measure on small cells since the electrode damages the cell so it not very suitable technique for yeast.

Membrane potential can be also measured by using water soluble positively charged probes with large content of hydrophobic chemical structures (e.g. diS-C_3(3), TPP , TMRM, etc.). Thanks to these structures the probes are able to

penetrate through the hydrophobic centre of double lipid layer. There are three main possibilities how to take advantage of such probes. The first technique is based on measurement of increase or decrease of the probe concentration in the cell suspension using ion selective electrode, for example the TPP^+ electrode. This method makes it possible to measure time dependence of the membrane potential. The second type of probes, used especially in the past, are radioactively labelled probes. When added into the medium, they start to accumulate in the cells until the electrochemical potentials in medium and cells equalize. The medium is afterwards centrifuged and final membrane potential is determined from the relative radioactivity in the pellet and residual liquid (applying the Nernst equation). The biggest disadvantage of this method is its inability to measure membrane potential time behaviour and the necessity to handle radioactive matter. The third possibility is the technique I use in this work and it takes advantage of fluorescent probes. The main advantage of this technique is the possibility to measure on almost any kind of cells or organelles and many different modifications that can be made depending on requirements. On the other hand, when the cell volume in suspension is too large the light dispersion problem may occur. I present this method in more detail in the next chapter. For further information about membrane potential measurement methods see [23].

2.4.2 Measurement with diS-C_3(3) fluorescent probe

Fluorescent probe diS-C_3(3) (3,3'-dipropylthiacarboxycyanin iodide – Figure 10) which was chosen for our measurements is a lipophilic, positively charged fluorescent probe. As it is positively charged it is attracted to negative cell interior and thanks to its lipophilic character it can penetrate through the cell membrane. The probe redistribution between cell interior and exterior lasts until redistribution equilibrium is reached. This process takes seconds to tens of minutes and is described by the Nernst equation.

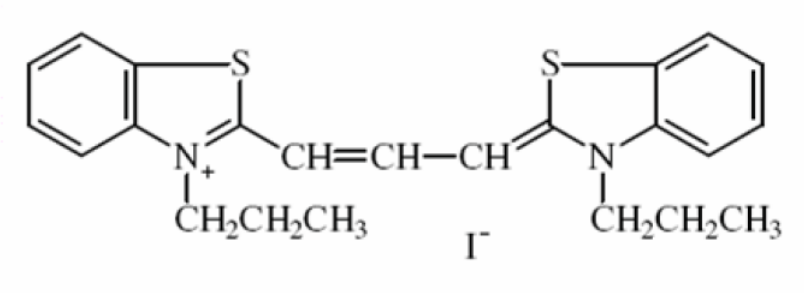

Figure 10: Fluorescent probe diS-C_3(3). [24]

Measurement method based on diS-C_3(3) fluorescent probe enables measurement of relative changes of its concentration in cells thanks to changes in probe spectroscopic properties, in particular of λ_{max} – the wavelength for which the intensity of the fluorescent signal I_{max} is maximal. When probe excited by light with a wavelength of 531 nm, λ_{max} of the emission spectrum is approximately 569 nm when the probe is unbound, or ca. 580 nm when the probe is bound to some intracellular components (e.g. albumin, RNA, lipids etc.). As seen in Figure 11, the overall fluorescent signal (red line) consists of the signal from free probe in medium (blue line) and the signal from probe in cells (purple line). Free probe in medium is mostly in unbound state; moreover, the intensity of the fluorescent signal during experiment remains almost constant because the difference in probe concentration in the medium is negligible (when taking into account volume ratio of cells and medium). The signal from the probe in cells is the sum of the signal of unbound probe in cells and the signal of bound probe in cells, which is actually the signal that shifts the λ_{max} of the overall spectrum to higher wavelengths.

The measurement with diS-C_3(3) fluorescent probe does not reflect only the magnitude of the cells' membrane potential. As found out at the Institute of Physics of the Charles University, diS-C_3(3) probe is a substrate of pumps responsible for multiple drug resistance and thus it reflects also their activity. This is a very important fact about diS-C_3(3) because it opens new possibilities of studying MDR transporters. This method enables to measure not only activities but also kinetics of these pumps under required conditions. It can therefore help to

understand how these transporters work, how they influence each other, how they behave under specific stresses as well as to find potential inhibitors of these pumps.

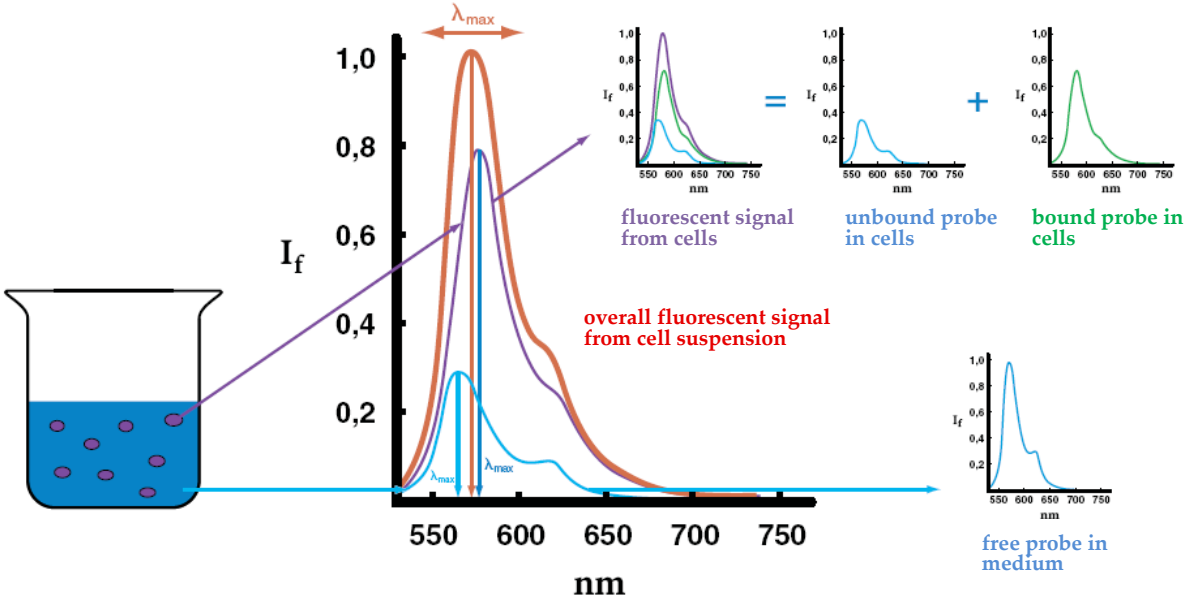

Figure 11: Composition of overall fluorescent signal from diS-C3(3) probe measured in cell suspension. [25]

3 Materials and methods

3.1 Materials

3.1.1 Chemicals

Tables 1-3 contain lists of all buffers, solutions, plasmid DNAs and primers used in the first part of this work for preparation of *S. cerevisiae* mutant strains.

compound name	compound composition
acid washed glass beads	glass beads (0.45 – 0.52 mm) are stirred in concentrated HCl for 1 h, repeatedly washed with distilled water until the pH of the water is almost neutral and finally exposed to 150 °C for 3 h.
DNA ladder	in this work: 1 kb GeneRuler™ from *Fermentas, St Leon-Rot*
10x lithium acetate	1 M lithium acetate, pH set to 7.5 with acetic acid
lithium acetate buffer (prepared freshly)	0.5 ml 10x TE buffer II, 0.5 ml 10x lithium acetate, 4 ml H_2O
lithium acetate buffer / glycerol solution (prepared freshly)	0.5 ml 10x TE buffer II, 0.5 ml 10x lithium acetate, 0.57 ml 87 % glycerol, 3.43 ml H_2O
P/C solution	1/1 (v/v) mix of phenol equilibrated with Tris-HCl pH 7.0 and chloroform
PEG solution	50 % (w/v) polyethyleneglycol (PEG) 4000 (5 g PEG filled up to 10 ml with H_2O), autoclaved
1x sample buffer	1 ml 10x sample buffer, 9 ml H_2O
10x sample buffer	50 % Glycol, 2 % 50x TAE buffer, 0.25 % bromophenol blue, 0.25 % xylene cyanol
1x TAE buffer	40 mM Tris-HCl, 1 mM EDTA dissolved in H_2O, pH set to 7.0 with NaOH
1x TE buffer I	1 ml 10x TE buffer I and 9 ml H_2O
10x TE buffer I	100 mM Tris-HCl ; 10 mM EDTA dissolved in H_2O, pH set to 7.5 with NaOH
10x TE buffer II	100 mM Tris-HCl ; 1 mM EDTA dissolved in H_2O, pH set to 7.5 with NaOH
TSN solution	2 % TritonX-100; 1 % SDS; 100 mM NaCl; 10 mM Tris-HCl; 1 mM EDTA dissolved in H_2O, pH set to 8.0 with NaOH
3 M NaAc	3 M sodium acetate, pH set to 4.8 with glacial acetic acid

Table 1: List of buffers and solutions used for preparation of *S. cerevisiae* mutant strains.

plasmid name	promoter	reference
pYEX-TOK1	P_{CUP1}	J. Ludwig, unpublished
pYEX-GFP-Kir2.1	P_{CUP1}	G. Hasenbrink et al., 2005
p77X-pma-TOK1	P_{PMA1}	J. Ludwig, unpublished
p77X-pma-Kir2.1	P_{PMA1}	J. Ludwig, unpublished

Table 2: List of plasmid DNAs used for preparation of *S. cerevisiae* mutant strains.

primer name	sequence 5′ → 3′	orientation
T187	aagagggccgctgctctctg	sense primer
T317	agttgggtaacgccagggttttcc	anti-sense primer
T242	atcatatagaagtcatcga	pYEX-sense
B281	gaatacaagagctcccaataacc	TOK1 antisense

Table 3: List of primers used for preparation of *S. cerevisiae* mutant strains.

Table 4 provides the list of chemicals used in the second part of this work.

chemical	producer	dissolving agent
yeast extract	Serva	distilled water
peptone	Oxoid	distilled water
agar	RNDr. Jan Kulich, s.r.o.	distilled water
glucose (anhydrous)	Penta	distilled water
disodium hydrogen phosphate	Lach-Ner, s.r.o.	distilled water
citric acid	Lachema, Brno	distilled water
yeast nitrogen base	Formedium	distilled water
complete supplement	Formedium	distilled water
ammonium sulphate	Sigma	distilled water
potassium chloride	Fluka	distilled water
glucose liquid 1000 agent	PLIVA-Lachema Diagnostica s.r.o.	distilled water
DM-11	[26]	distilled water
CCCP	Sigma	DMF
MRG-12	[26]	ethanol
FK506	LC Laboratories	ethanol
diS-C_3(3) probe	Molecular Probes	ethanol for UV spectr.
DMF	Fluka	
ethanol for UV spectroscopy	PLIVA-Lachema s.r.o.	

Table 4: List of chemicals used for culture cultivation and spectroscopy measurements

Cultivation media and buffer

- **40% glucose:** glucose 40 g
  distilled water 60 ml
- **1M potassium chloride:** potassium chloride 14.91 g
  distilled water 200 ml
- **YPD cultivation medium:** yeast extract 10 g
  peptone 10 g
  40% glucose (autocl. separately) 50 ml
  1M potassium chloride 50 ml
  distilled water up to 1000 ml
- **YNB cultivation medium:** yeast nitrogen base 1.9 g
  complete supplement 790 mg
  ammonium sulphate 5 g
  40% glucose (autocl. separately) 50 ml
  1M potassium chloride 50 ml
  distilled water up to 1000 ml
- **YPD solid medium for Petri dishes:** YPD cultivation medium 200 ml
  agar 2 g
- **C-P buffer:** sodium phosphate 1.9 g
  distilled water 1000 ml
  citric acid ~ ml (to set final pH to 6.0)

Storage of chemicals

1M potassium chloride, 40% glucose, C-P buffer, DM-11 and CCCP were stored at 4°C. MRG-11, FK-506 and diS-C3(3) were stored at -20°C. The rest of chemicals used for culture cultivation and spectroscopy measurements were stored at room temperature.

3.1.2 List of *S. cerevisiae* strains

strain	genotype	reference
AD1-3	*MATα, PDR1-3, ura3, his1, yor1::hisG, snq2::hisG, pdr5::hisG*	[27]
BWT	*MATa his3-Δ200 leu2-3,112 trp1-Δ901 ura3-52 suc2-Δ9*	[28]
BΔP	BWT Δpdr1, Δpdr3	IZMB, Bonn
BΔA	BWT Δsnq2, Δyor1, Δpdr5	IZMB, Bonn
BΔPK	BWT Δpdr1, Δpdr3, Δtrk1, Δtrk2, Δtok1	IZMB, Bonn
BΔAK	BWT Δsnq2, Δyor1, Δpdr5, Δtrk1, Δtrk2, Δtok1	IZMB, Bonn
B1TRK	BWT Δpdr1, Δpdr3, Δtrk1, Δtrk2, Δtok1, tok1::PCDR1-TRK1	IZMB, Bonn
B2TRK	BWT Δpdr1, Δpdr3, Δtrk1, Δtrk2, Δtok1, tok1::PCDR2-TRK1	IZMB, Bonn
BTOK	BWT Δpdr1, Δpdr3, Δtrk1, Δtrk2, Δtok1, tok1::pma-TOK1	this work
BKIR	BWT Δpdr1, Δpdr3, Δtrk1, Δtrk2, Δtok1, tok1::pma-Kir2.1	this work
BTOKE	BWT Δpdr1, Δpdr3, Δtrk1, Δtrk2, Δtok1, [pYEX-TOK1]	this work

Table 5: List of *S. cerevisiae* strains used in this work.

Strains storage

Strains were stored as glycerol stocks at -80°C (long-term storage) or at -20°C (short-term storage). Petri dishes used for pre-culture inoculation were stored at 4°C not longer than 1 month.

3.2 Methods

3.2.1 Yeast cultivation

Small amount (~μl) of culture from glycerol stock was spread on YPD Petri dish and cultivated for ca. 2 days at 30°C in incubator *Raven 2*. Using sterile metal loop one colony was transferred into 10.5 ml of YPD or 11 ml of YNB liquid medium and cultivated in shaking bath at 30°C for ca. 24 hours. . This pre-culture was used to inoculate main-culture to optical density (OD) of 0.2 (which corresponds to 5.10^6 cells/ml). The inoculation OD of cells used for measurement in the exponential phase was ca. 0.005. The main-culture was again cultivated in shaking bath at 30°C for certain time depending on the required growth phase and further used for sample preparation.

To prevent contamination, the inoculation process was performed in a flow box with burner.

3.2.2 Sample preparation

First of all, OD of the main-cultures was measured to check correct culture growth phase. Then the cells were pelleted by centrifugation (2800rpm, 1min) and washed twice with distilled water. Small part of these cells was resuspended in C-P buffer to OD 0.1 (on spectrophotometer *Novaspec II*) which was then distributed in 3 ml volumes into cuvettes.

3.2.3 Measurement of growth curves

To measure the strain growth curves, two sets of main cultures were prepared (one with a 12-hour delay). Approximately once in an hour the OD was measured. Finally, two sets of 12 hours long growth curves (0-12 hours and 12-24 hours) were obtained and combined to obtain the final strain growth curves.

3.2.4 Glucose test

The amount of glucose in growth medium provides an important information about the strain growth phases because when the culture depletes glucose the cells undergo diauxic shift and continue in post-diauxic growth phase.

The glucose concentration in the medium was measured in parallel with OD measurement during the growth curve measurements. This method is based on oxidation of glucose by glucose oxidase to hydrogen peroxide which reacts with phenol and 4-aminoantipyrine and forms a quinoneimine dye. The intensity of this dye measured by spectrophotometer is proportional to the glucose concentration [29].

The protocol for glucose test [30]:

1. Fill cuvette with 1 ml of the *glucose liquid 1000* agent.
2. Add 10 μl of examined medium to the cuvette and mix gently.

3. Incubate at 20-25°C for ca. 20 minutes.
4. Add 1 ml of distilled water to the cuvette.
5. Measure OD at 546 nm (use 1 ml *glucose liquid 1000* with 1 ml of distilled water as the reference).

3.2.5 Protocols for preparation of *S. cerevisiae* mutant strains

Agarose gel electrophoresis [31]

1. Make a 1% agarose solution in 100ml of 1x TAE buffer.
2. Heat the solution until the agarose dissolves (e.g. in a microwave oven).
3. Let the solution cool down to about 60 °C.
4. Put on gloves and wear them from here on (ethidium bromide is a mutagen).
5. Add ethidium bromide to a final concentration of 0.5 ug/ml.
6. Stir the solution to disperse the ethidium bromide, then pour it into the gel rack.
7. Insert the comb at one side of the gel, about 7 mm from the end of the gel.
8. When the gel has cooled down and become solid, carefully remove the comb. The holes that remain in the gel are the slots.
9. Put the gel, together with the rack, into a tank with 1x TAE buffer. Ethidium bromide at the same concentration can be added to the buffer. The gel must be completely covered with 1x TAE buffer, with the slots at the end electrode that will have the negative current.
10. Inject DNA ladder (molecular weight marker) diluted in 1x sample buffer (in a 1:6 ratio) in one control slot.
11. Inject samples diluted in 1x sample buffer (in a 1:6 ratio) into the next slots.
12. Connect the wires to apply the current. Set the voltage to ca. 85 V so that the current is ca. 100mA (it should not exceed 110mA).

13. Run the gel for ca. 20 minutes (until the DNA ladder reaches the end of the gel). The DNA moves toward the positive anode due to the negative charges on its phosphate backbone.
14. Put the gel under the UV light to make the bands visible.
15. Document the gel for further analysis.

Digestion of DNA with restriction endonucleases [32]

A reaction mix consisting of template DNA, reaction buffer, restriction endonuclease (enzyme) and distilled water is prepared in a suitable 1.5 ml tube. The reaction mix is then incubated at the temperature optimal for the enzyme used (often 37 °C) for a certain time (often 1 h). The total volume of such reaction mixes is usually between 5 and 50 µl. A typical reaction mix for final volume of 10 µl could for example be:

plasmid DNA (1 µg/µl)	1 µl
10 x endonuclease reaction buffer	1 µl
distilled water	7.5 µl
enzyme (10 U/µl)	0.5 µl

The total volume of a reaction mix is determined by the amount of enzyme needed to completely digest the template DNA. Because enzymes are usually supplied in a storage buffer containing 50 % glycerol and a final glycerol concentration of > 5 % can inhibit the reaction, the amount of enzyme must not exceed 10 % of the total reaction volume.

Phenol/chloroform extraction [18]

Phenol/Chloroform (P/C) extraction is used to remove proteins from nucleic acid (DNA and RNA) solutions.

1. Add distilled water to the nucleic acid solution (reaction) to a total volume of 100 - 200 µl.
2. Add an equal volume of P/C solution**.**
3. Stir rigorously ("vortex") for 1 - 2 min.
4. Centrifuge (≥ 10000 g, 1 - 2 min).
5. Transfer the upper (aqueous) phase containing nucleic acids to a fresh tube. Avoid carrying over any of the interphase or the organic phase!

Ethanol precipitation [18]

Ethanol precipitation is a method used to purify and/or concentrate RNA, DNA.

1. Take tube with nucleic acids solution. Add 1/10 volume of 3 M NaAc and 3x volume of at least 95% ethanol.
2. Incubate on ice for 15 minutes. In case of small DNA fragments or high dilutions incubate overnight.
3. Centrifuge (20000 g, 4°C, 30 min).
4. Discard supernatant, being careful not to throw out DNA pellet which may not be visible.
5. Rinse with 500 µl of 70% ethanol.
6. Centrifuge (20000 g, 5 min).
7. Discard supernatant and let the pellet dry (ca. 2 min at room temperature). Finally, dissolve in 25 µl of distilled water.

Preparation of competent cells [32]

20-50 ml of an exponentially growing liquid culture in YPD medium is transferred into a sterile 50 ml tube. Cells are pelleted by centrifugation (~1000 g, 5 min), the supernatant is discarded, the pellet is resuspended in 10 ml of 1x TE buffer I and

centrifuged again (~1000 g, 5 min). The supernatant is again discarded, the cells are resuspended in 5 ml lithium acetate buffer and incubated at 30 °C for 1 h. After another round of centrifugation the cells are resuspended in 5 ml lithium acetate / glycerol solution and kept on ice. This suspension of competent yeast cells can either be used immediately for transformation or stored at -80° C for later use.

Transformation of yeast [31]

1 µg of DNA (in max. volume 10 µl), 2 µl of carrier DNA (herring sperm DNA – XIV type, 0.5 µg/µl, denatured by boiling for 10 min) and 5 µl of ethanol are combined in a sterile 1.5 ml tube and 100 µl of competent yeast cell suspension is added. After 5 min incubation at room temperature 250 µl of PEG solution is added. The mixture is incubated for 1 h at 30 °C on a shaker (~ 250 rpm) and after that a heat shock (46°C, 15 min) is applied. Cells are allowed to cool to room temperature (5 - 10 min), plated on appropriate selection agar plates and incubated at 30 °C. Single colonies should appear after 2-5 days.

Isolation of DNA from yeast [31]

Liquid cultures (5 ml) of transformed yeast cells are grown overnight in selective media. 2 x 1.5 ml are pelleted using a benchtop centrifuge (~ 14000 g, 20 sec). The supernatant is removed completely and the cells are resuspended in 50 µl of distilled water by vortexing. 0.2 ml TSN solution, 0.2 ml P/C solution and 0.3 g acid washed glass beads are added. The mixture is vortexed vigorously for 3 min and centrifuged (~ 14.000 g, 5 min). The upper aqueous phase is transferred to a fresh tube and again mixed with 0.2 ml P/C solution. After vortexing and centrifugation as above, the aqueous phase is transferred to another tube and 1/10 volume 3 M NaAc and a 3-fold volume of ethanol are added. After 5 min on ice the mix is centrifuged at 20000 g at 4° C for 3 min. The supernatant is discarded and the pellet is washed with 500 µl of 70 % ethanol in water (v/v). After another brief centrifugation the supernatant is removed completely and the pellet (containing

DNA and RNA) is dried for 2 min at room temperature and finally dissolved in 25 µl H_2O. Aliquots of this preparation are used as templates in the analytic PCR.

Analytic PCR

Tubes, water and buffers are UV irradiated for some minutes before use. UV radiation causes nicks and thymidine-dimerisation that both prevent the ability of contaminating DNA to serve as a template in the PCR and lead to a false positive result.

First, we prepare a premix which consists of:

10x reaction buffer	3.5 µl
$MgCl_2$(25 mM)	2.1 µl
dNTPs (10mM)	0.7 µl
Taq Polymerase (5 U/µl)	0.18 µl
Primer 1 (100 pmol / µl)	0.18 µl
Primer 2 (100 pmol / µl)	0.18 µl
distilled water	up to 33 µl

33 µl of premix per reaction is transferred to PCR tubes and 2 µl of template DNA is added. Using a thermocycler the PCR is run using the following standard protocol:

1x	2 min	94 °C	initial denaturation
20-35x	20 sec	94° C	denaturation
	30-50 sec	X° C (see below)	annealing
	(length of product in bp/40) sec	72° C	elongation
1x	3-5 min	72 ° C	final elongation

An estimate of the annealing temperature X can be calculated using the following formulas that approximate the melting temperature of primer DNA duplexes and subtract between 3 and 10 °C from the resulting temperature:

primers shorter than 25 bp: $T_m = 2(A+T) + 4(C+G)$

primers longer than 25 bp: complicated formula (usually not needed)

Glycerol stock preparation

1. Centrifuge (~1000 g, 5 min) 1.5 ml of culture.
2. Discard 800 μl of supernatant.
3. Resuspend pellet in 700 μl of left supernatant.
4. Add 300 μl of 87% glycerol.
5. Mix properly and place on ice (after glycerol addition cells are heat sensitive).
6. Store at -80°C.

3.3 Apparatus and description of measurements

Optical density measurement

The optical density (OD) was measured on spectrophotometer *Novaspec II* at 576 nm. The measured culture sample was diluted (usually in the ratio 1:10) to lower the reading value for more precise measurement.

Fluorescent measurements with diS-C_3(3) probe

The fluorescent measurements were performed on spectrofluorometer *FluoroMax 3* (from *Jobin Yvon-Spex* company) with xenon lamp as the light source. It communicates with computer via *DataMax* software which enables also reading the λ_{max} and I_{max} values directly from the measured spectrum. The excitation wavelength was set to 541 nm and emission spectrum was detected in the range from 560-590 nm. Orange emission filter (cut off 540 nm) and green excitation filter were used. One measurement took ca. 20 seconds.

The samples were prepared in 10 mm PMMA cuvettes (from *P-Lab*) according to the chapter 3.2.2 Sample preparation. At the beginning of the measurement diS-C_3(3) probe was added to the sample to a final concentration of 2.10^{-8} M. The fluorescent spectra were repeatedly measured until the values stabilized. Afterwards, tested chemical compounds were added to the cuvettes – FK-506, CCCP, DM-11 and MRG-11 in the concentrations 15 μM, 33 μM, 10 μM and 20 μM, respectively. After values stabilized again the second addition was performed.

4 Results and discussion

4.1 Preparation of S. cerevisiae mutant strains

Most of the *S. cerevisiae* mutant strains used in this work have been prepared in the Institut für Zelluläre und Molekulare Botanik der Uni Bonn and I could utilize them (see Table 5). They can be divided into two sets. The first set contains two strains: BΔA and BΔAK. They are both deficient in main ABC transporters Snq2p, Yor1p and Pdr5p. BΔAK is moreover deficient in the major yeast K^+ transporters Trk1p, Trk2p and Tok1p. The strains of the second set BΔP, BΔPK, B1TRK and B2TRK are all deficient in transcription factors pdr1 and pdr3. BΔP contains all major K^+ transporters, BΔPK contains none of them and B1TRK and B2TRK contain only Trk1p on a strong and weak promoter, respectively. To complete the second set, strain containing only Tok1p potassium transporter, BTOK, was needed. Moreover, if we wanted to compare yeast potassium transporters with the mammalian Kir2.1, BKIR strain needed to be prepared.

The aim was therefore to prepare 4 strains: BTOK and BKIR based on BΔPK strain with directly integrated TOK1 and Kir2.1 genes into the genome and BTOKE and BKIRE based on the same strain with TOK1 and Kir2.1 genes carried on shuttle plasmids (episomal expression).

4.1.1 Preparation of competent cells

The first step in the transformation process was the modification of *S. cerevisiae* cells – preparation of competent cells. In this work, the lithium acetate method for preparation of competent cells was used according to the *Transformation of yeast* protocol (3.2.5 Protocols for preparation of *S. cerevisiae* mutant strains).

4.1.2 Transformation of *S. cerevisiae* cells

Before I started with the transformation I checked the quality of the plasmid DNAs. I ran agarose gel electrophoresis according to the protocol (chapter 2.3.5) with DNA ladder and all four plasmid DNAs (Figure 12). The gel showed that all four plasmid DNAs are suitable for transformation process.

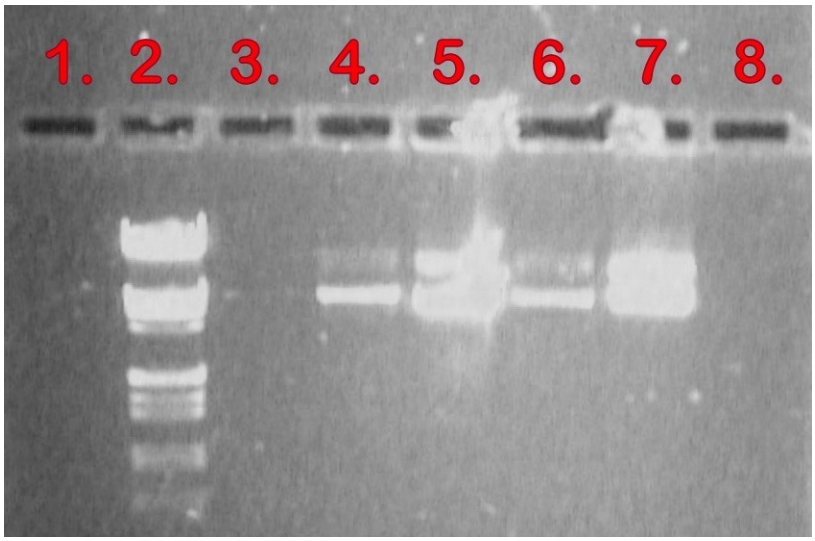

Figure 12: Plasmid DNA quality control gel. Lines: 1. empty, 2. DNA ladder, 3. empty, 4. pYEX-TOK1, 5. pYEX-GFP-Kir2.1, 6. p77X-pma-TOK1, 7. p77X-pma-Kir2.1, 8. Empty

Episomal expression

The first type of transformation was episomal expression of circular plasmid DNAs. The resultant strains should carry pYEX-TOK1 plasmids (strain BTOKE) and pYEX-GFP-Kir2.1 plasmids (strains BKIRE). Both the plasmid DNAs contain gene for uracil and leucine production (see Figure 13) in order to allow verification of transformation. I performed the transformation according to the protocol and plated the transformed cells on Petri dishes with solid selective medium with 100mM KCl and without uracil and leucine. The cells were able to grow on such a selective medium only if the transformation was successful since uracil is one of the essential amino acids and non-transformed cells were not able to produce it on their own.

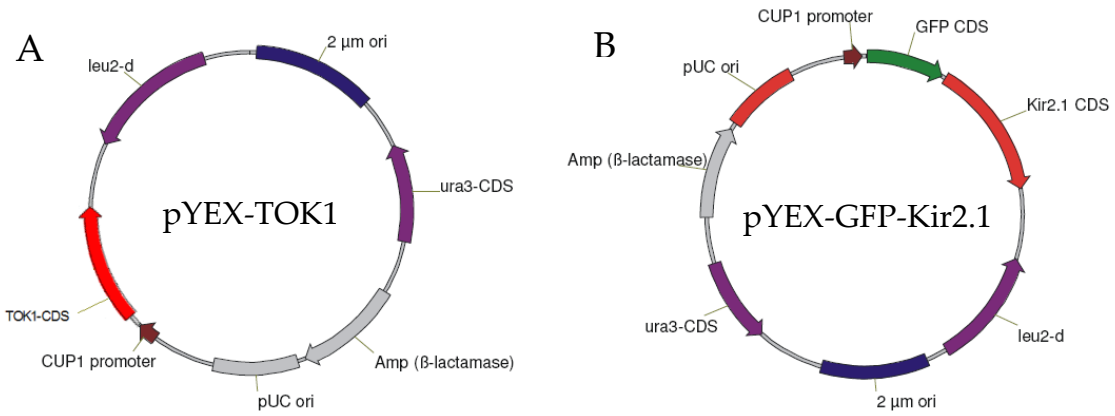

Figure 13: Schematic maps of (A) pYEX-TOK1 and (B) pYEX-GFP-Kir2.1

Directed integration by homologous recombination

The second type of transformation was a directed integration of plasmid DNA into the *S. cerevisiae* genome by homologous recombination. This transformation method required linearization of circular plasmid DNA so it could be pasted to the place of the original gene. In our case, the linearization of both the plasmids (p77X-pma-TOK1 and p77X-pma-Kir2.1) was performed with NotI restriction endonuclease according to the *Digestion of DNA with restriction endonucleases* protocol. As seen in Figure 15, after successful linearization the plasmids should contain the pre-tok part at the beginning and post-tok part at the end so that they could integrate into the tok1 locus by homologous recombination. The linearization was verified by gel electrophoresis (Figure 14).

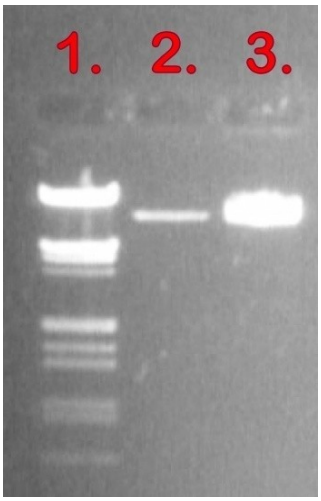

Figure 14: Verification of plasmid DNAs linearization. Lines: 1. DNA ladder, 2. p77X-pma-TOK1 after linearization, 3. p77X-pma-Kir2.1 after linearization

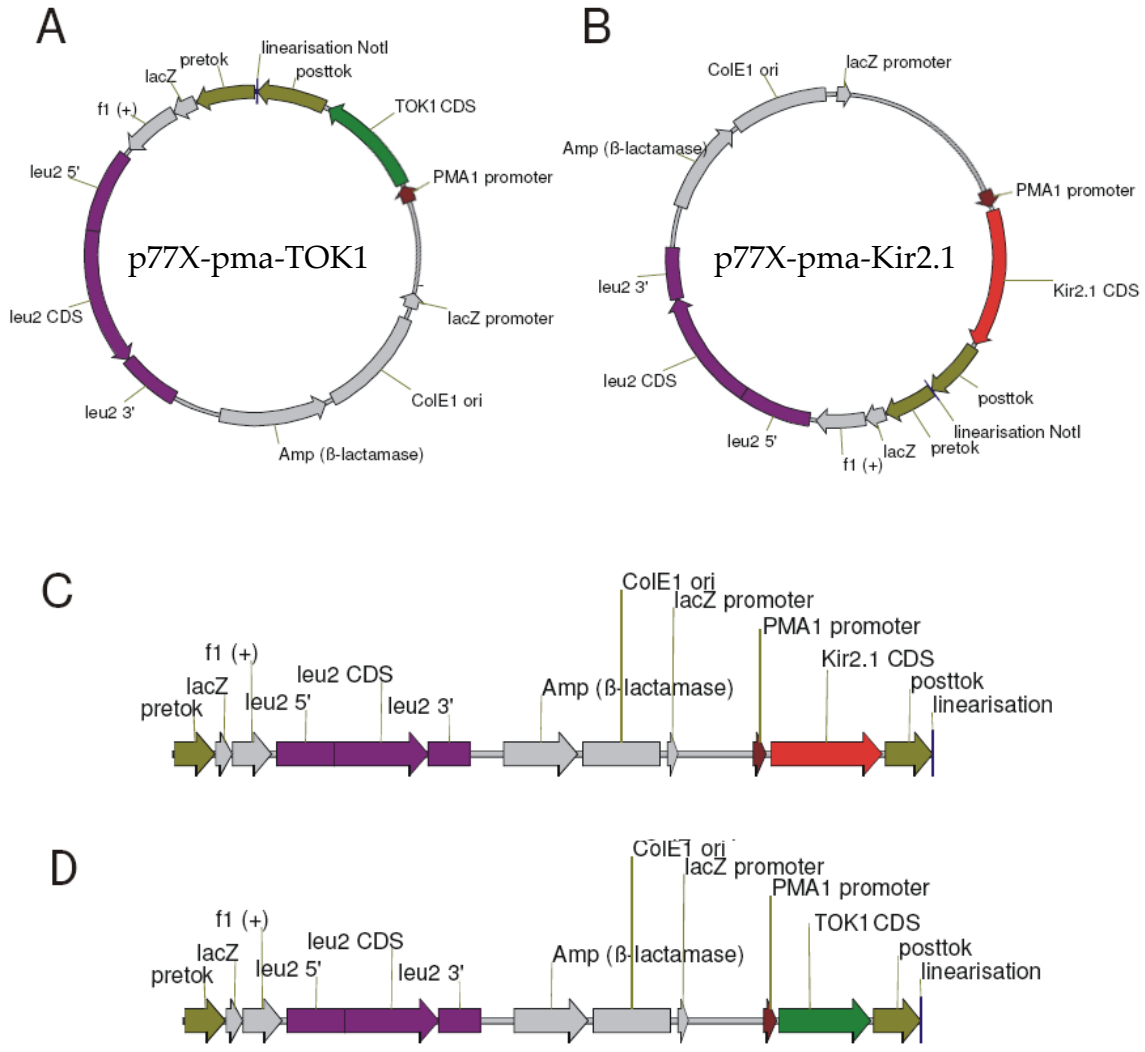

Figure 15: Schematic map of p77X-pma-TOK1 (A) before and (B) after linearization and schematic map of p77X-pma-Kir2.1 (C) before and (D) after linearization.

After the successful plasmid DNA linearization I performed phenol/chloroform extraction and ethanol precipitation according to the corresponding protocols, to prepare DNA for transformation. The transformation was performed together with episomal expression transformation and transformed cells (BTOK and BKIR strains) were plated on Petri dishes with solid selective medium containing 100mM KCl without leucine, which was the selection marker for transformation with p77X-pma-TOK1 and p77X-pma-Kir2.1 plasmid DNAs.

4.1.3 Verification of transformations

Three days after the transformation, the cultures BTOK, BKIR and BTOKE were clearly grown on Petri dishes. BKIRE strain did not create any colonies, presumably due to unsuccessful transformation. I inoculated one culture of host strain BΔPK and 2 cultures from each transformed strain to liquid selective media (Δleu,Δura for BTOKE and Δleu for BTOK and BKIR) containing 100mM KCl. One of the two BTOK cultures did not grow, so there were 6 cultures for further verification process: BΔPK, BTOK, BKIR(1), BKIR(2), BTOKE(1) and BTOKE(2).

Isolation of DNA

Isolation of DNA from yeast strains was the first step in the verification of transformations. It was performed according to the corresponding protocol and its purpose was to isolate the whole genetic information of given strains (genomic DNA together with possible plasmid DNA) and prepare it for further analysis.

PCR

PCR played a key role in verification of transformation. It was used to prove the correct integration of the p77X-pma-TOK1 and p77X-pma-Kir2.1 constructs into the BΔPK genome in BTOK and BKIR strains as well as to check the episomal expression of pYEX-TOK1 plasmid in BTOKE strain. The PCR was performed in two sets according to the protocol for all 6 cultures listed above.

In the first set, T187 and T317 primers were used to prove transformation by direct integration. T187 primer binds upstream (5') of the deletion/replacement cassette within the TOK1 5' non-coding region. The second primer, T317 binds within p77X at a vector sequence not present in the yeast genome (see Figure 16). Thus, a fragment could only be amplified when the deletion/replacement cassette was integrated in the correct genomic locus, which should be in the case of BTOK,

BKIR(1) and BKIR(2). The BΔPK, BTOKE(1) and BTOKE(2) served as negative control.

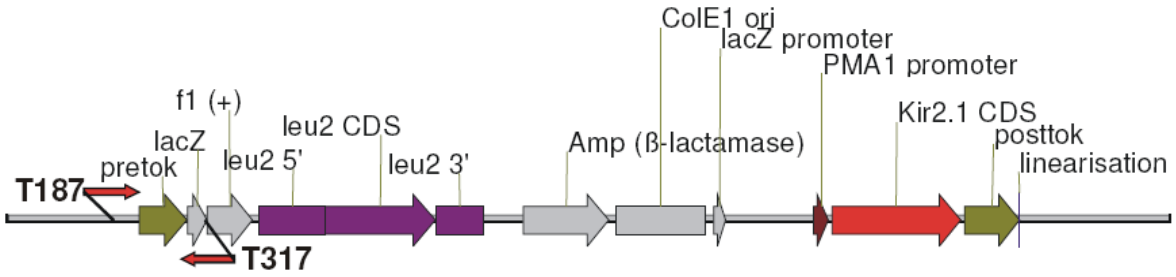

Figure 16: Schematic representation of p77X-pma-Kir2.1 integrated in the tok1 locus and the annealing positions of the PCR primers T187 and T317.

In the second PCR set, T242 and B281 primers were used. These primers bind only to the pYEX plasmid DNAs so the amplified fragment refers to the presence of the pYEX plasmid in the isolated DNA. Positive results were thus expected with DNA isolated from BTOKE(1) and BTOKE(2) strains.

Gel electrophoresis

The final step in verification of transformations was the gel electrophoresis which displayed amplified fragments of the PCR products. As seen in Figure 17, the pattern was exactly as expected – PCR with T187 and T317 primers amplified fragments of DNA isolated from BTOK, BKIR(1) and BKIR(2) (lines 2,3 and 5); and PCR where T242 and B281 primers were used amplified fragments of DNA isolated from BTOKE(1) and BTOKE(2) (lines 12 and 13). The rest lines were, as expected, empty. We can thus conclude that the verification process confirmed correct transformation of BTOK, BKIR and BTOKE strains.

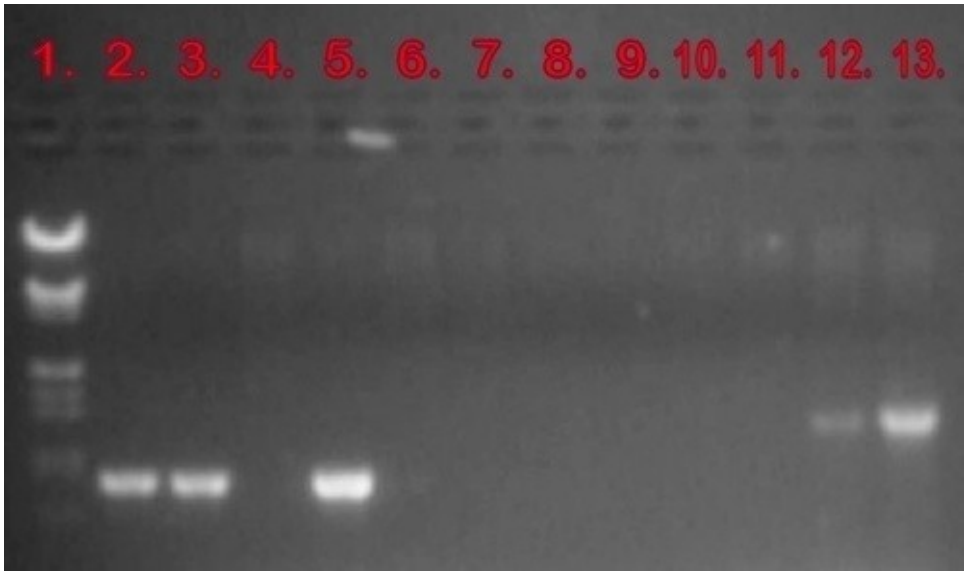

Figure 17: Verification of transformations. Lines: 1. λ DNA ladder, 2. BTOK, 3. BKIR(1), 4. BΔPK, 5. BKIR(2), 6. BTOKE(1), 7. BTOKE(2), 8. BTOK, 9. BKIR(1), 10. BΔPK, 11. BKIR(2), 12. BTOKE(1), 13. BTOKE(2); Products of PCR with T317 and T187 primers are in lines 2-7 and with T242 and B281 primers in lines 8-13.

Glycerol stock preparation

After the verification process confirmed correct transformations of BTOK, BKIR and BTOKE strains, glycerol stock conserves were prepared and stored for further measurements.

4.2 Characterisation of S. cerevisiae mutants by growth profile and fluorescence measurements

The second part of this work consisted in fluorescence measurements with diS-C_3(3) probe and gives answer to the question: **How does deletion of K^+ transporters influence the membrane potential of *S. cerevisiae*?**

The measurements were performed on two sets of *S. cerevisiae* isogenic mutant strains. The first set contained BΔA and BΔAK strains and the second BΔP, BΔPK, BKIR, BTOK, B1TRK and B2TRK strains. The measurements were also performed on BWT (wild type strain). All the cells were measured in three growth phases in YPD medium and in one growth phase in YNB medium.

4.2.1 Characterisation of strains by means of growth curves

One of the most important conditions influencing physiological state of yeast cells is their growth phase. It influences membrane potential, cell wall thickness as well as the activity or presence of MDR pumps and other transporters. Therefore, it is very important to prepare samples from the cells which are all in the same growth phase, so we could compare the results of different mutant strains.

Because of the mutations which were performed on the strains, their growth characteristics differ. Thus, we had to measure the growth curves of each of them as described in chapter *3.2.3 Measurement of growth curves*. Together with the measurement of glucose concentration (chapter *3.2.4 Glucose test*) we had enough information to define growth phases (exponential, early-diauxic and post-diauxic) of each strain. Since the cultures were grown in two different media, the growth characteristics had to be determined for both of them.

YPD medium

The growth curves in YPD medium were almost the same for all the strains except for the BΔA strain (see Figure 18). This strain grew approximately twice as much as the rest. The shapes of growth curves were typical – we could clearly see the lag phase (first 1-2 hours), the exponential phase (until ca. 9-10 hour), the diauxic shift and then the diauxic phase.

The glucose test graph (Figure 19) completely corresponds to the growth curves. We can see that the strains deplete glucose from the medium after 16-20 hours of growth (BΔA strain after 9 hours). Approximately at this time comes the diauxic shift and the cell metabolism changes.

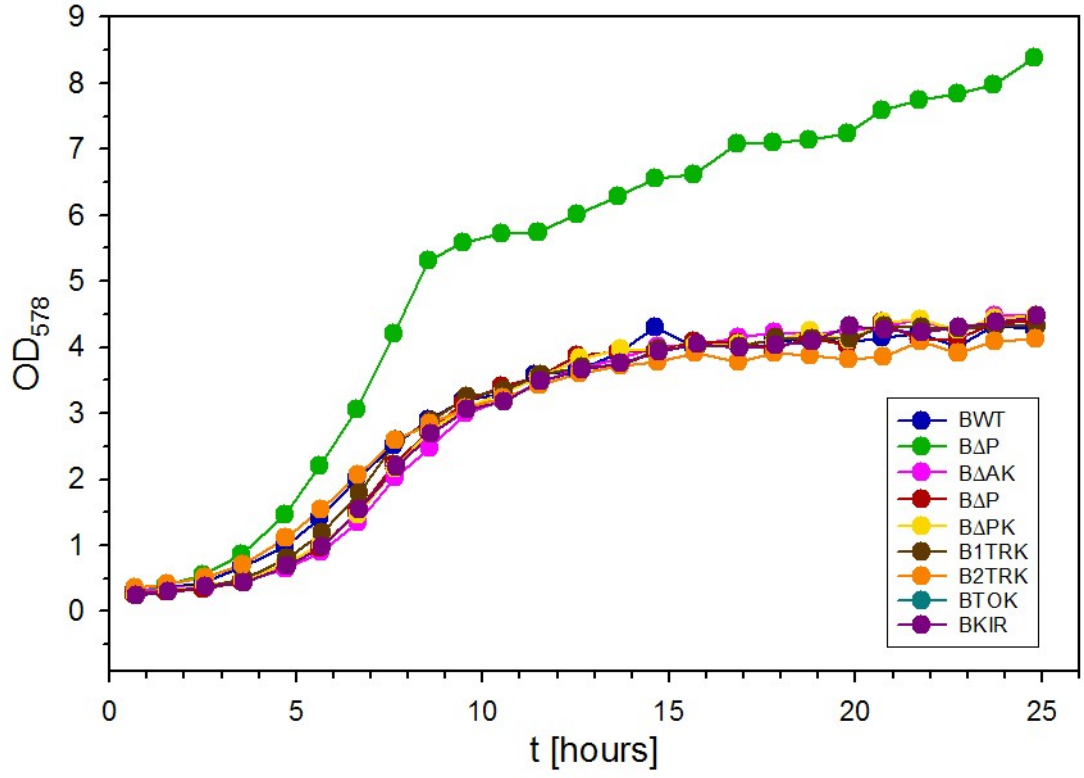

Figure 18: Growth curves of individual strains (see inset) in YPD medium.

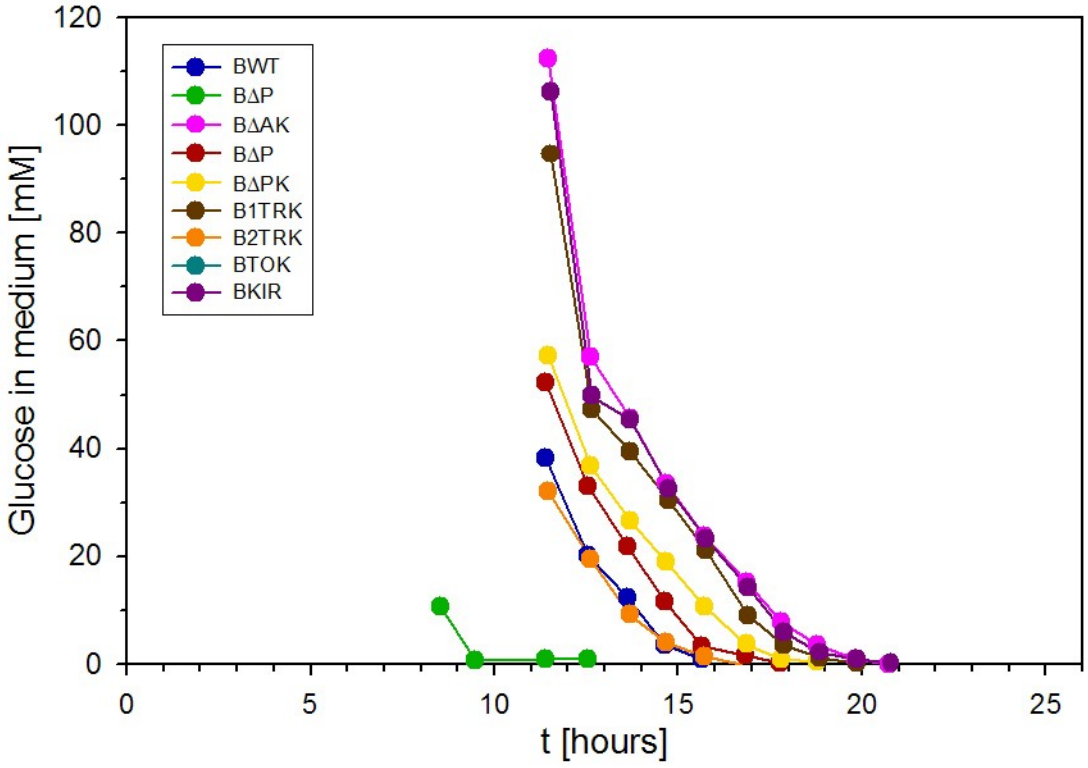

Figure 19: Glucose concentration measurement of individual strains (see inset) in YPD medium.

Using growth curves and glucose test curves we selected three growth phases in YPD medium:

1. *exponential phase* – 5 to 6 hour-old cultures
2. *early-diauxic phase* – 20 hour-old cultures (10 hour-old in the case of BΔA strain)
3. *post-diauxic phase* – 40 hour-old culture

YNB medium

The growth curves in YNB medium (Figure 20) divided the strains into two groups. BWT, BΔP, B1TRK and B2TRK strains showed similar growth characteristics as in the YPD medium. However, the growth curves of BTOK, BKIR, BΔA, BΔAK and BΔPK strains showed very unusual growth behaviour - even after 25 hours their OD was low (between 1.0 and 2.3).

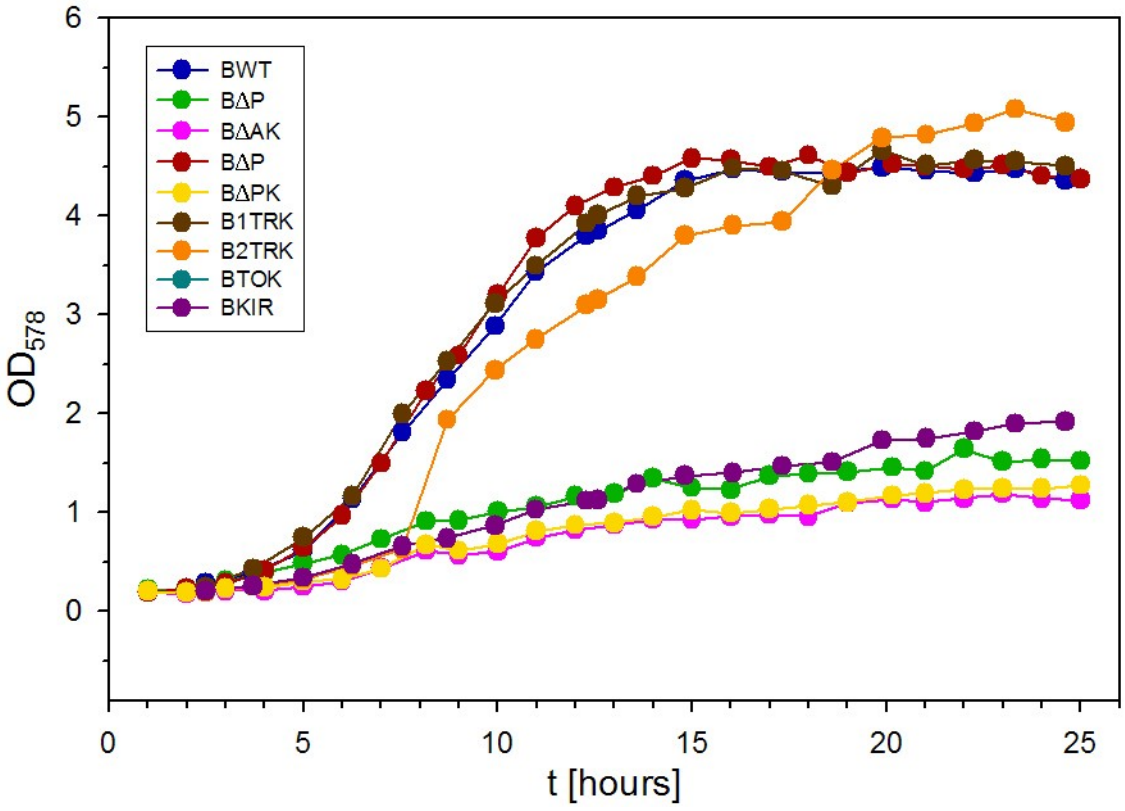

Figure 20: Growth curves of individual strains (see inset) in YNB medium.

This slow growth of BTOK, BKIR, BΔA, BΔAK and BΔPK strains was also visible in the glucose test graphs (Figure 21). After 25 hours of growth there was 50-120 mM glucose present in the media and even after 50 hours there was ca. 50 mM glucose concentration in the media of BΔA, BΔAK and BΔPK (data not shown). The BΔP, BWT, B1TRK and B2TRK strains depleted glucose from medium after 16, 19, 19, and 23 hours, respectively.

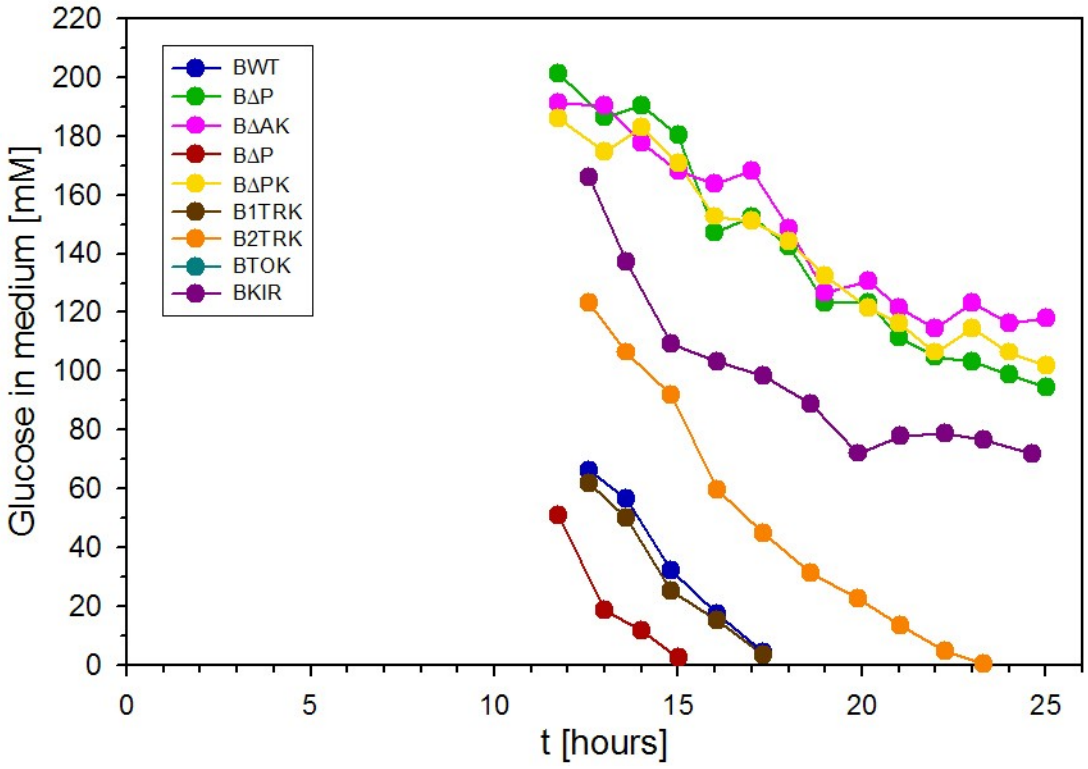

Figure 21: Glucose concentration measurement of individual strains (see inset) in YNB medium.

Because of the huge difference in the growth characteristics of given strains in YNB medium, the fluorescence measurements could be performed only in one growth phase:

1. *exponential phase*
   - 6 to 9 hour-old cultures (BWT, BΔP, B1TRK and B2TRK)
   - 18 to 20 hour-old cultures (BTOK, BKIR, BΔA, BΔAK and BΔPK)

Here I would like to emphasize importance of glucose concentration measurement during the measurement of growth curves. It is necessary for correct determination

of culture growth phases. Even in many published articles it is assumed that isogenic strains have the same growth characteristics; this can cause severe result discrepancies.

4.2.2 Wild type strain measurements

Wild type strain (BWT) has no genetic modification in ABC pumps, transcription factors or potassium transporters. Because this strain served as the parent strain of all the above mutants, it was desirable to use it as a reference strain showing the standard impact of growth phase on wild type strain staining [33], [34]. In addition the response to 3 previously tested chemical stressors (immunosuppressant FK506, protonophore CCCP and lysosomotropic compound MRG-12) provided information on the activity of Pdr5p alone (FK506 [35]), and of Pdr5p plus Snq2p (CCCP, MRG-12). The combination of the last 2 stressors was used because CCCP, apart from inhibiting the 2 ABC pumps, also activates the H^+-ATPase (membrane hyperpolarisation [35]) in contrast to MRG-12, which also inhibits the 2 pumps but inhibits the H^+-ATPase (unpublished data).

As seen in Figure 22 the staining is the lowest at exponential cells, higher at early diauxic cells and the highest staining was shown by post-diauxic cells. This result is caused by the ABC pumps (Pdrp5, Snq2p) which extrude diS-C_3(3) probe out of the cytosol. The presence of these pumps is the highest in exponential growth phase and gradually declines in the following growth phases [34].

In the wild type strain, all the ABC pumps are present. As far as we know, addition of FK506 should inhibit only one of them - Pdr5p and this addition should have no other influence on the cells [35]. As seen in Figure 23, addition of FK506 clearly increases the staining (compared to the curves without FK506 addition), which was due to the Pdr5p inhibition. This increase is the highest in exponential cells, lower in early diauxic cells and in the post-diauxic phase no change in staining is visible. This corresponds with the occurrence of Pdr5p in different growth phases.

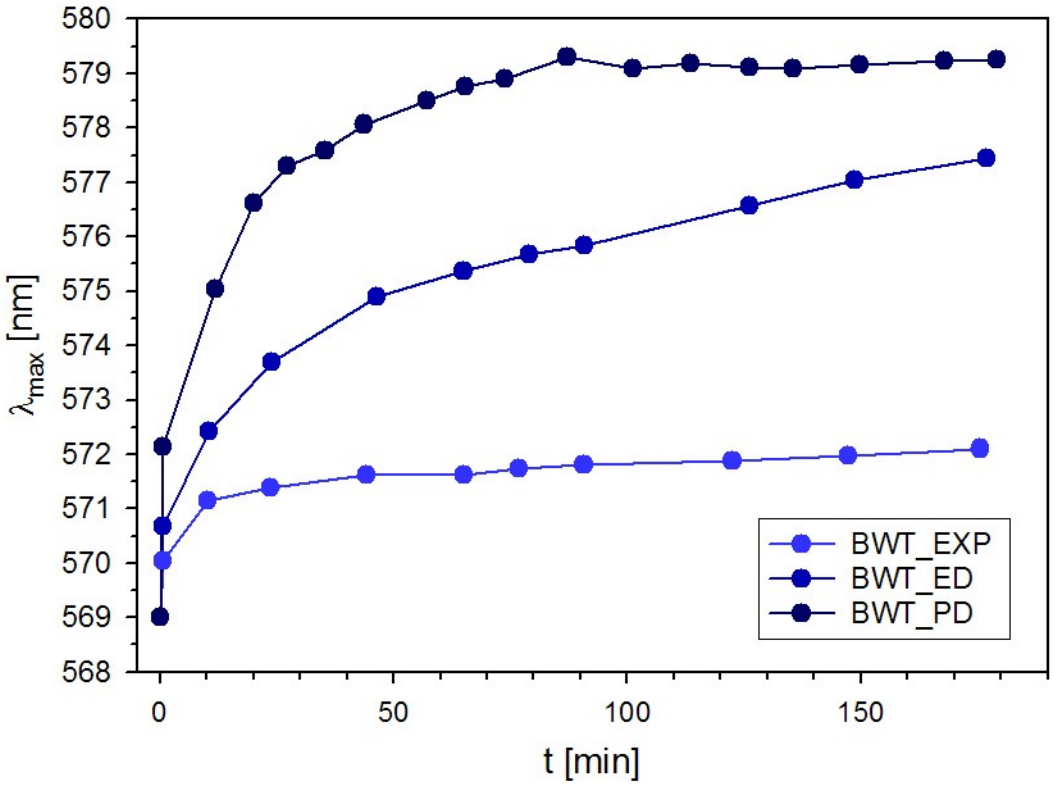

Figure 22: Staining curves of BWT strain in exponential, early diauxic and post-diauxic growth phases.

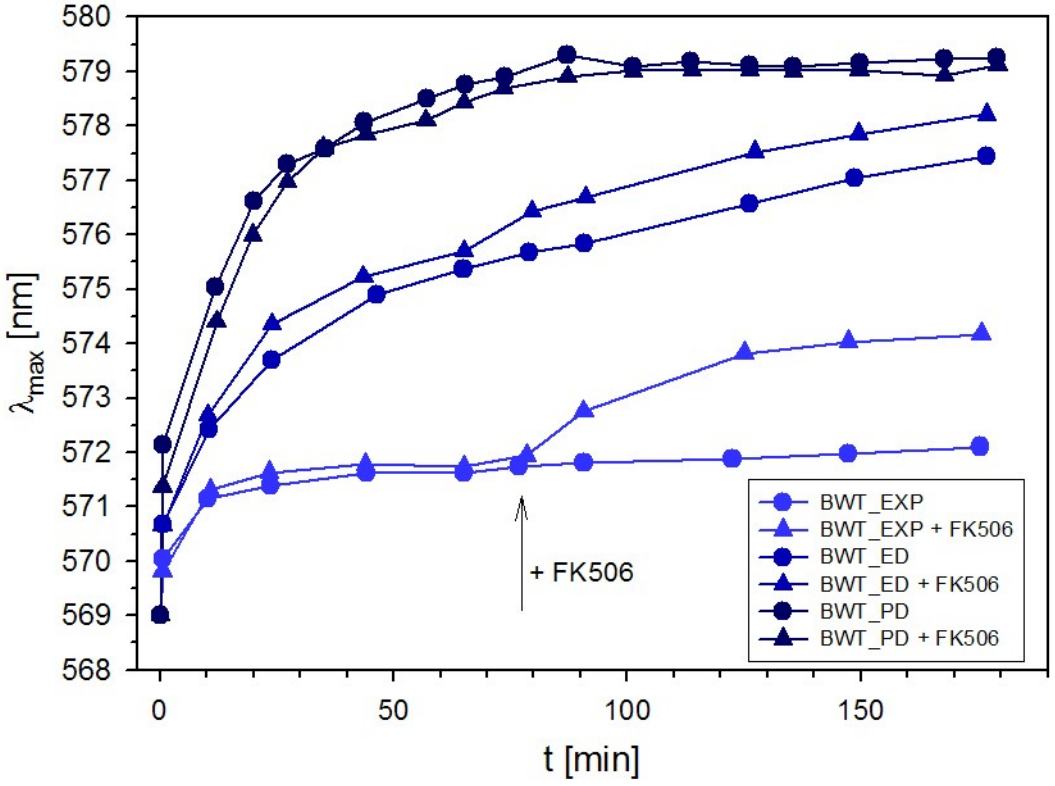

Figure 23: Staining curves of BWT strain in exponential, early diauxic and post-diauxic growth phases with and without FK506 addition.

To prove the presence of both Prd5p and Snq2p, MRG-12 and CCCP were added. MRG-12 is a substrate of ABC pumps and inhibits the H^+-ATPase which can cause a small depolarisation of the cell membrane. CCCP is also a substrate of Pdr5p and Snq2p but activates the H^+-ATPase; this results in a hyperpolarisation of the cell membrane [35]. As seen in Figure 24, addition of MRG-12 increased the staining and so did CCCP but the staining level is in this case even higher due to the hyperpolarisation of the membrane (unlike the MRG-12 addition which causes slight depolarisation). The staining level corresponding to the actual membrane potential is therefore somewhere between these two staining levels (provided that both pumps are inhibited).

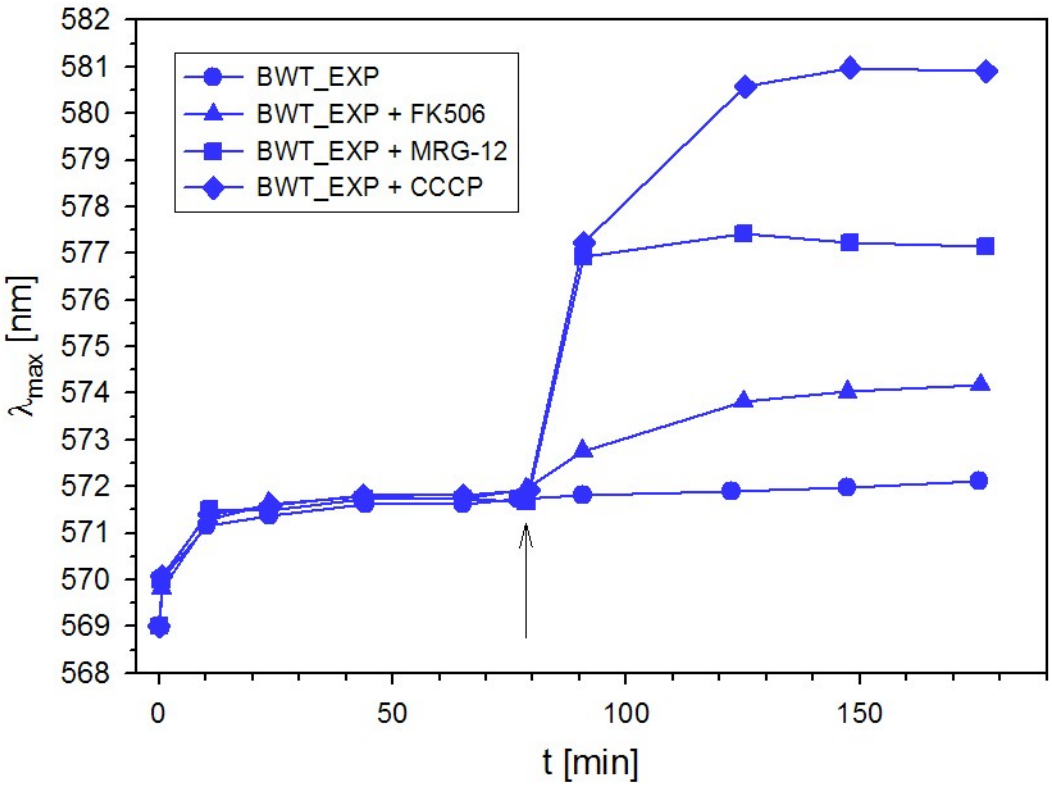

Figure 24: BWT strain in exponential growth phase without any addition, with FK506, MRG-12 and CCCP addition.

The use of these 3 stressors is a very useful diagnostic tool for evaluating the activities of individual pumps exporting the probe, and judging the magnitude of membrane potential under different conditions.

4.2.3 Impact of deletion of K^+ transporters on membrane potential

The main task of this work was to find out how does the deletion of main K^+ transporters influence the cell membrane potential. As suggested in [9], hampered K^+ transport should hyperpolarise the membrane since K^+ cannot efflux from the cell. To verify this thesis, two isogenic strain sets were employed.

1st isogenic set - BΔA and BΔAK

Figure 25 shows comparison of the staining curves of BΔA and BΔAK strains grown in different media and to different growth phases. As these two strains have the same deletions in ABC pumps and no deletions in transcription factors, the staining level (the final λ_{max} value of the staining curve) should correspond only to the value of their membrane potential. Part A (YPD medium, exponential phase), part B (YPD medium, early diauxic phase) and part D (YNB medium, exponential phase) of Figure 25 show a higher value of membrane potential in BΔAK strain compared to the BΔA strain. Staining curves of the post-diauxic cells grown in YPD medium (Figure 25, part C) show almost no difference in staining. We can thus conclude that deletion of the main K^+ transporters hyperpolarises the membrane of exponential and early diauxic cells. In the case of post-diauxic cells the membrane potentials of these two mutant strains are equal.

Even though the BΔA and BΔAK strains have deleted the three main ABC pumps (Snq2p, Yor1p and Pdr5p) the staining of exponential cells grown in YPD medium is very low compared to older cells (Figure 26). It is known that exponential cells have to have high membrane potential needed for reproduction so the low staining indicates the presence of some other ABC pumps (e.g. Pdr15p, Pdr10p) for which the diS-C_3(3) probe could also be a substrate. Figure 26 shows that the staining level of early diauxic cells is also a little bit lower than the staining level of post-diauxic cells. This can be due to already mentioned probe extrusion pumps as well as to lower membrane potential during the diauxic shift [34].

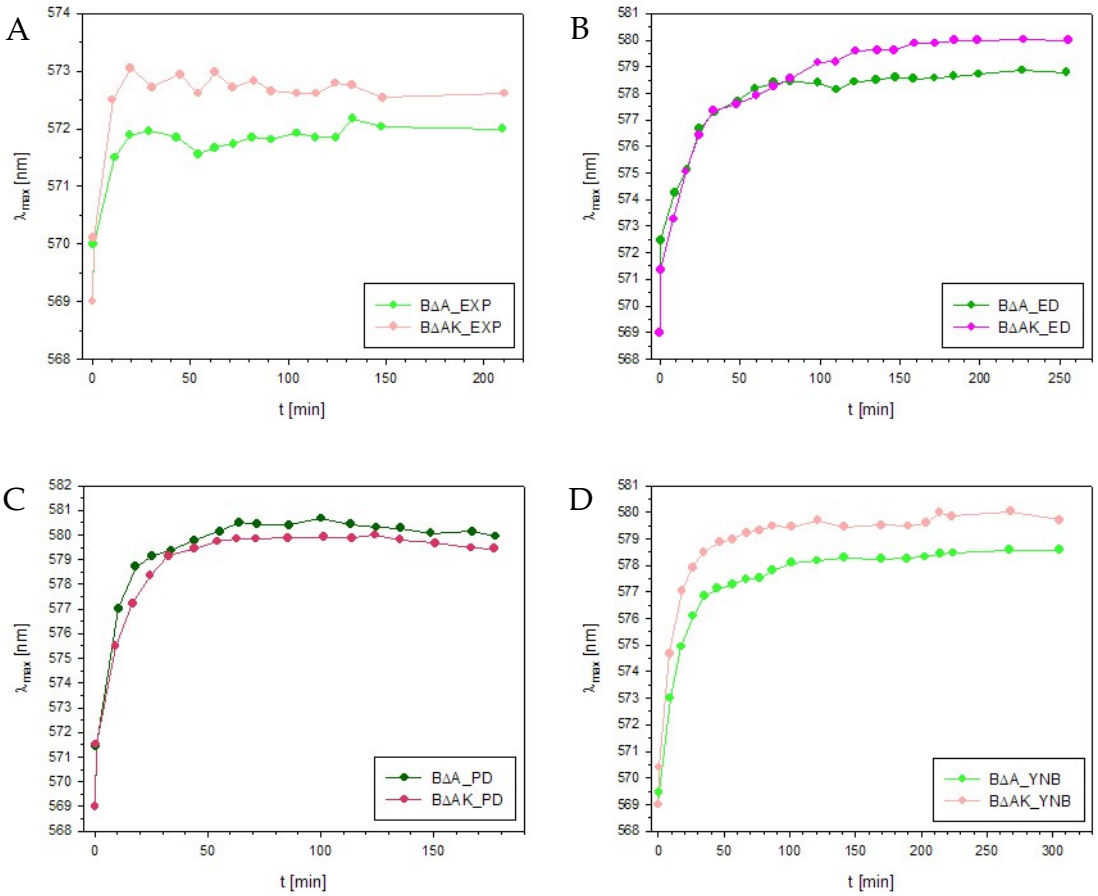

Figure 25: Comparison of the staining curves of BΔA and BΔAK strains. (A) YPD medium, exponential phase, (B) YPD medium, early diauxic phase, (C) YPD medium, post-diauxic phase, (D) YNB medium, exponential phase.

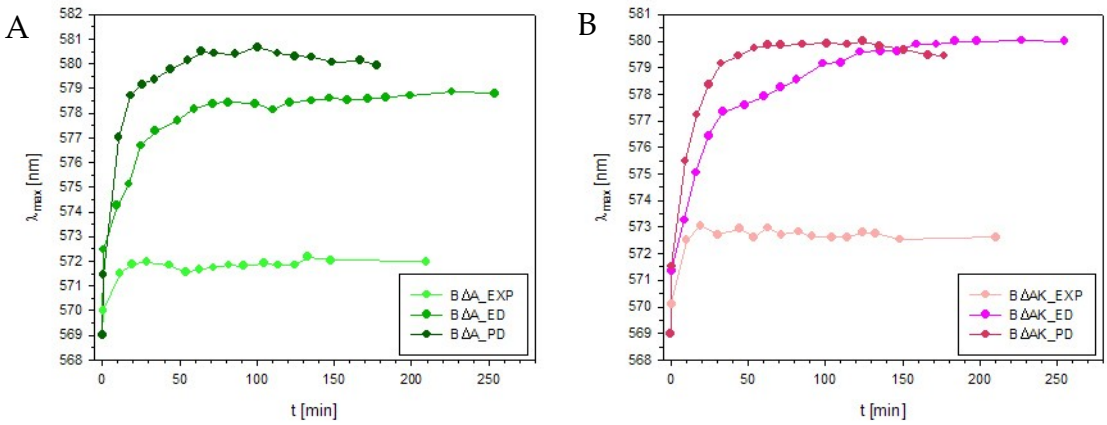

Figure 26: Staining curves of (A) BΔA and (B) BΔAK strains in exponential, early diauxic and post-diauxic growth phases.

So are there any pumps which efflux diS-C_3(3) probe out of the cytosol in BΔA and BΔAK strains? FK506, MRG-12 and CCCP should give answer to this question. Figure 27 shows the influence of these substances. FK506 addition does not cause any change since there is no Pdr5p present in the cells. Addition of both MRG-12 and CCCP increased the staining level – CCCP by ca. 7.5 nm and MRG-12 by ca. 2.5 nm. The different increase in staining is caused by the fact that MRG-12 slightly depolarises and CCCP slightly hyperpolarises cell membrane. Since neither Pdr5p nor Snq2p is present in these cells, this result shows that both compounds are probably substrates of additional ABC pumps, for which diS-C_3(3) is also a substrate (competitive inhibitor). The measurement with BΔAK strain (data not shown) gives the same result.

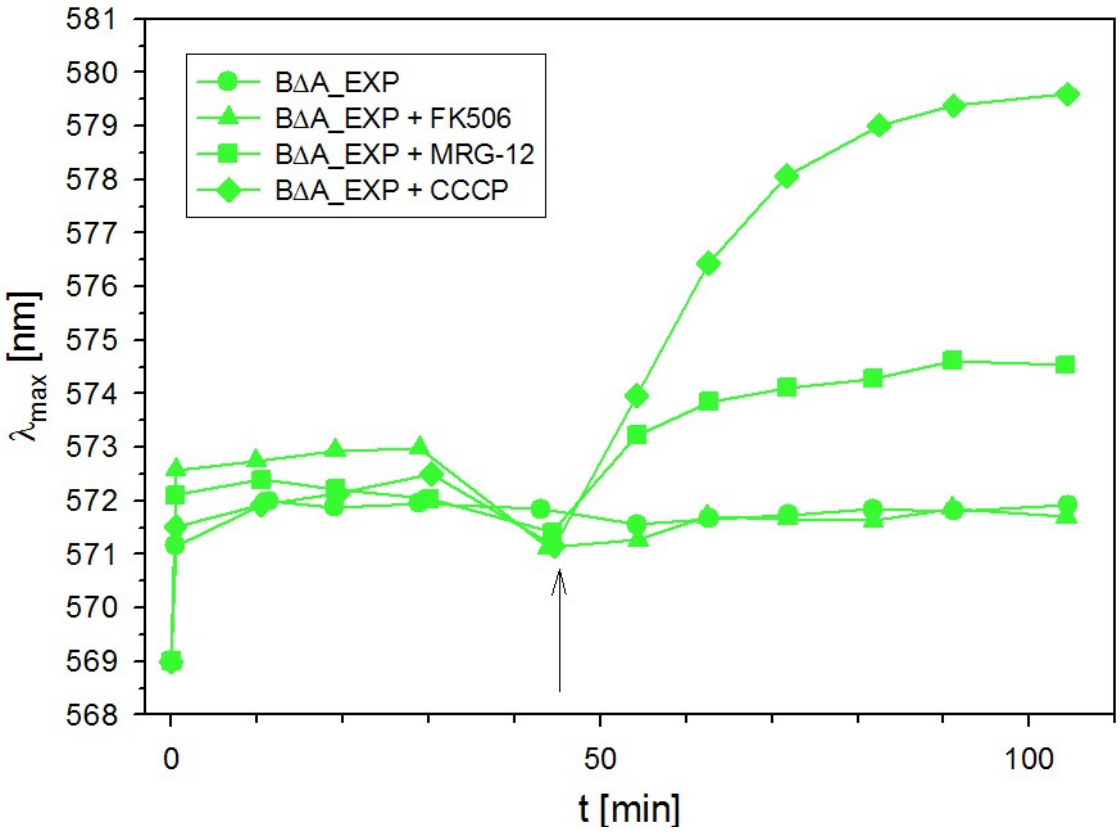

Figure 27: BΔA strain in exponential growth phase without any addition, with FK506, MRG-12 and CCCP addition.

I also compared the staining curves of exponential BΔA and AD1-3 strains. As seen from Figure 28, the staining level of AD1-3 strain was much higher than the staining level of BΔA strain. Even though the same three ABC pumps (Snq2p,

Yor1p and Pdr5p) were deleted in both strains, AD1-3 strain has a mutation in the gene for transcription factor PDR1 leading to its overexpression. Pdr1p has a negative regulation impact on transcription of Pdr15p (Figure 8), which results in the higher staining of AD1-3.

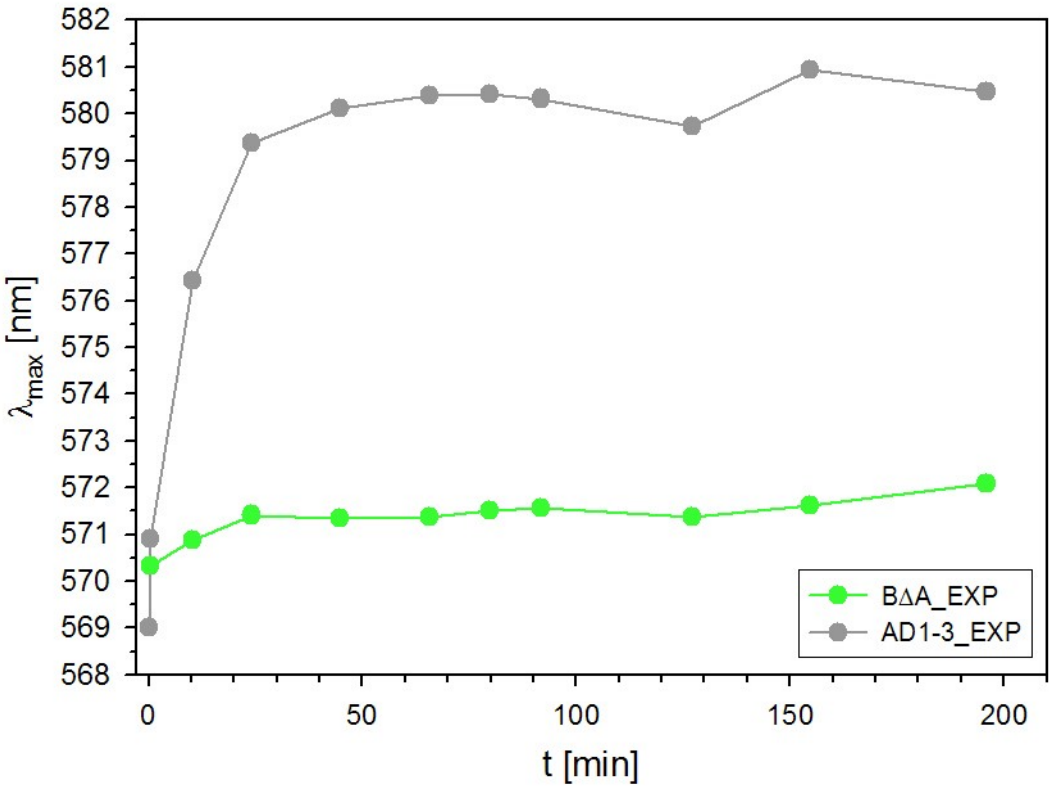

Figure 28: Staining curves of exponential BΔA and AD1-3 strains.

2nd isogenic set - BΔP and BΔPK

The second set of strains with the same genetic multidrug resistance background (deletion of transcription factors pdr1 and pdr3 and no deletions in ABC pumps) are BΔP and BΔPK strains. We asked the same question: How does the deletion of K^+ transporters influence the cell membrane potential?

Figure 29 shows that early diauxic cells grown in YPD medium (part B) lacking three main K^+ transporters have hyperpolarised membrane compared to those which possess them. In the case of post-diauxic cells (part C) the result is similar as in 1st isogenic set of strains BΔA and BΔAK – we see no evident difference. Cells

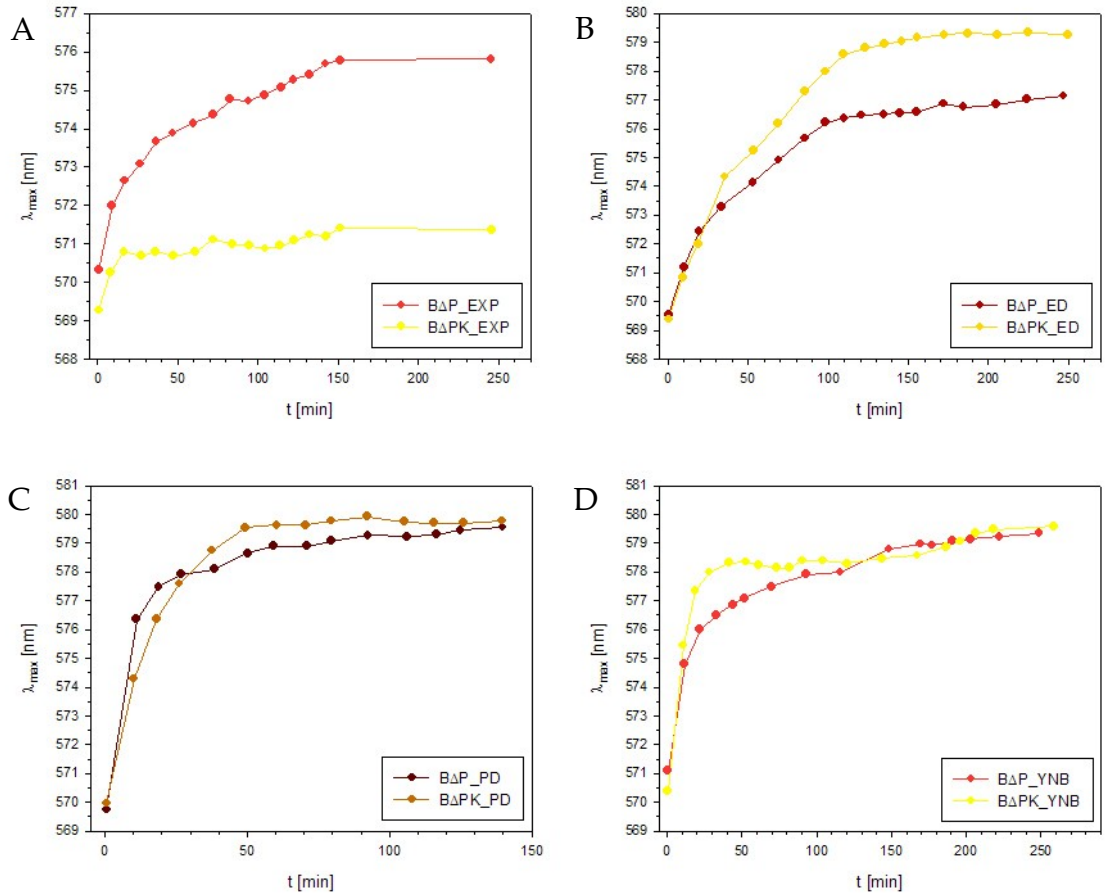

Figure 25: Comparison of the staining curves of BΔP and BΔPK strains. (A) YPD medium, exponential phase, (B) YPD medium, early diauxic phase, (C) YPD medium, post-diauxic phase, (D) YNB medium, exponential phase.

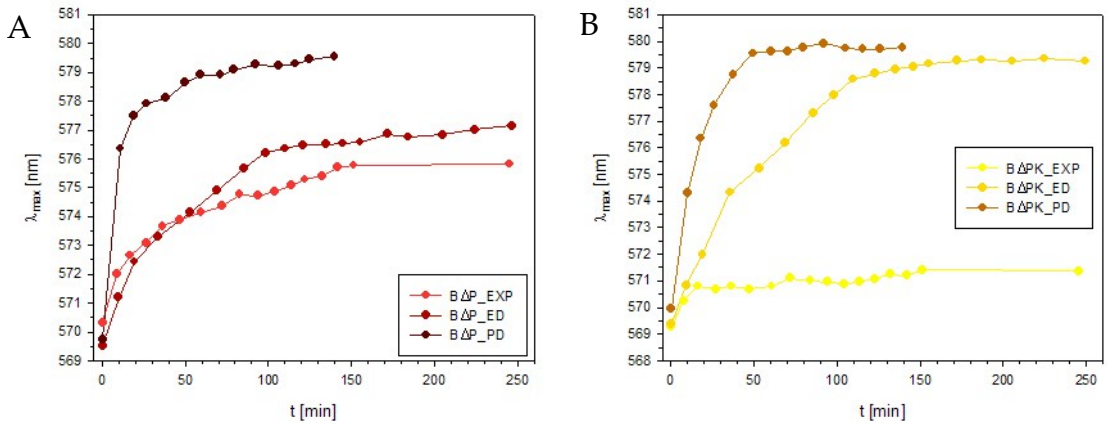

Figure 26: Staining curves of (A) BΔP and (B) BΔPK strains in exponential, early diauxic and post-diauxic growth phases.

grown in YNB medium show no difference as well. What is a real mystery is part A of Figure 29 – exponential cells grown in YPD medium. The staining of the BΔP strain is much higher than the staining of BΔPK strain (4 nm difference). Because of this unexpected result this particular experiment was repeated 2 more times with the same result. Giving satisfactory answer to this phenomenon would require deeper analysis and it is beyond the scope of this work. However, it is probably somehow connected with the deletion of transcription factors pdr1 and pdr3 which regulate many cell processes, including the composition of the membrane and thickness of the cell wall and having possible impact on the membrane potential.

Deletion of pdr1 and pdr3 transcription factors resulted in lower activities of ABC pumps. Nevertheless, some pumps are still present, as seen in Figure 30. The staining levels of exponential cells are again the lowest, followed by early diauxic and post-diauxic cells. When we look at the known ABC pumps (Figure 8) we see that for example Pdr10p is only under the control of Pdr1p and Pdr3p transcription factors. Pdr5p is also under the control of both of these transcription factors. However, there are some pumps which are probably still active – Snq2p which is under the control of another 4 transcription factors (especially Yrr1p and Yrm1p) as well as Pdr15p which is negatively controlled by Pdr1p.

The same stressors were used for deeper analysis of activity of the ABC pumps after deletion of pdr1 and pdr3 transcription factors (Figure 31). Addition of FK506, which, as far as we know, is a substrate only of Pdr5p, resulted in ca. 0.7 nm staining increase. When compared to BΔA (Figure 27) where it did not cause any difference we can suppose that Pdr5p pump is present in BΔAK mutant in some residual activity. When compared to BWT strain (Figure 24) where it caused a 2 nm increase in staining we can estimate the contribution of this residual activity. Addition of MRG-12 and CCCP showed similar results as in BΔAK strain, manifesting the activity of other pumps, not only of Pdr5p.

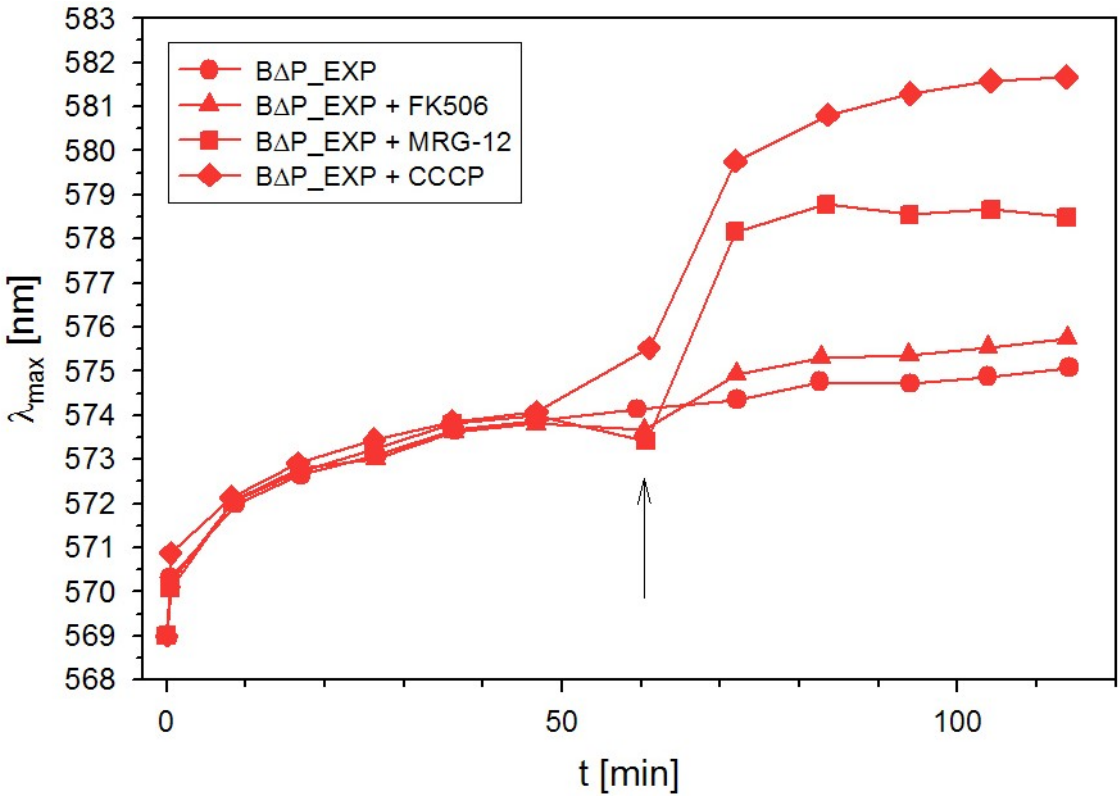

Figure 31: BΔP strain in exponential growth phase without any addition, with FK506, MRG-12 and CCCP addition.

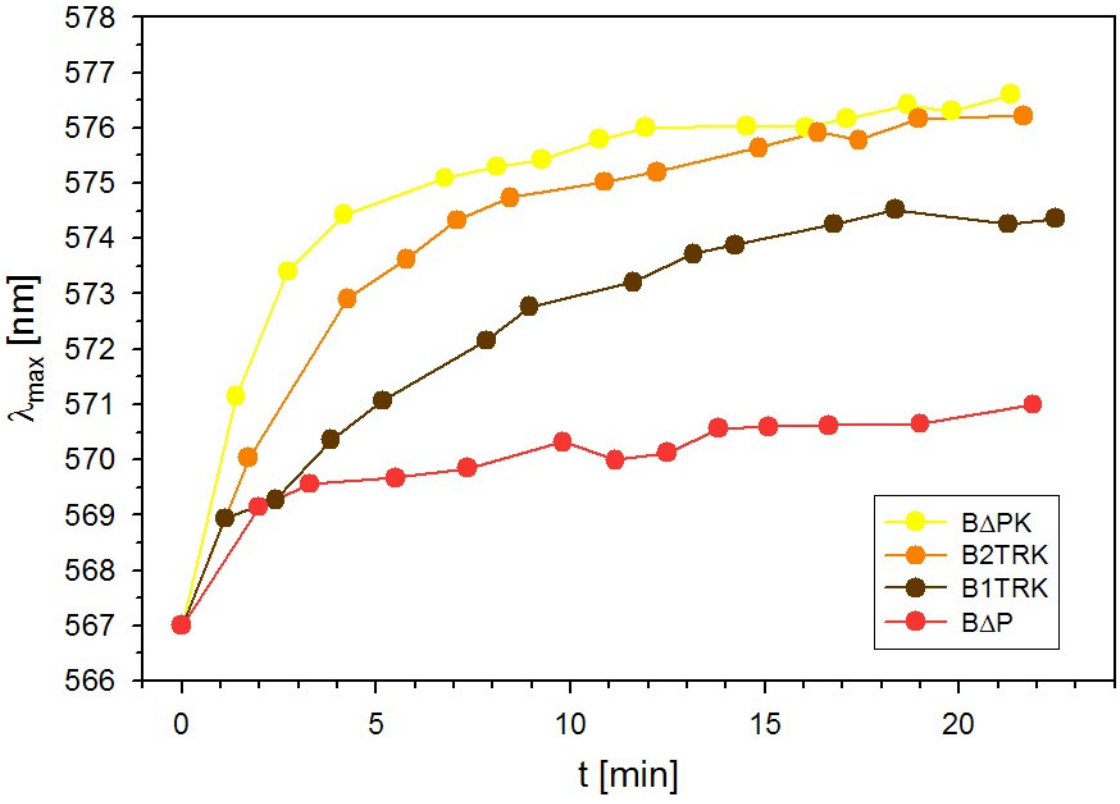

Figure 32: Influence of single potassium transporter on cell membrane potential. BΔP contains Trk1p, Trk2p, Tok1p K^+ transporters, BΔPK contains none of these transporters, B1TRK contains only Trk1p on a strong promoter and B2TRK contains only Trk1p on a weak promoter.

The purpose of this part of the work was to see the contribution of individual potassium transporters (Tok1p, Trk1p and mKir2.1p) to cell membrane potential. The preliminary measurements made during the first part of this work indicate the influence of introducing the K^+ transporter Trk1p on a strong and weak promoter into the BΔP strain on membrane potential (Figure 32). As seen in Figure 32, this introduction of the Trk1p on a weak promoter does not have any substantial effect on membrane potential (the staining curve for B2TRK is practically identical with that for BΔPK) whereas introduction of the same transporter on a strong promoter causes a significant dump in membrane potential towards the BΔP level. More of these measurements are underway. Nonetheless, this measurement method makes it possible to measure such results when all the conditions are optimal.

5 Conclusion

- The first thing I want to point out in conclusion of this work is the importance of measurement of glucose concentration in growth medium during the measurement of growth curves. As was shown in this work, various mutants have different growth characteristics. Glucose test is thus a very helpful tool for precise determination of growth phases, which is essential for comparison of results obtained with different strains.
- The main conclusion of this work is confirmation of expected hyperpolarisation of cell membrane in the absence of main K^+ transporters (Tok1p, Trk1p and Trk2p). The results of [9] were not only verified by many independent measurements but this hyperpolarisation was manifested by a 2 nm difference in staining (Figure 29, part B) – the difference which has as yet never been published. This work also shows how the cell membrane hyperpolarisation caused by the lack of K^+ transporters depends on the culture growth phase.
- Another contribution of this work is in a deeper study of the activity and presence of ABC pumps in different mutants. It was shown that not only Pdr5p and Snq2p are the main multidrug transporters but there are also other active extrusion pumps in BΔA mutant lacking Yor1p, Pdr5p and Snq2p. Comparison of BΔA and AD1-3 strains suggests Pdr10p and Pdr15p pumps. This work also showed that Δpdr1, Δpdr3 mutant strain is not an equivalent of strain without any multidrug pumps.
- New mutant strains would be required for further study of multidrug resistance in *S. cerevisiae*. Mutants lacking PDR10 and PDR15 genes would be of special interest since there is not much information about these two multidrug transporters even though their contribution to yeast multidrug resistance is indisputable. The results of this work indicate that dis-C_3(3) probe is probably a substrate of other pumps such as the Pdr15p. Study of these transporters with

our method is thus very suitable and could provide detailed information about the kinetics of these pumps and help in finding their inhibitors.

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