School of Doctoral Studies in Biological Sciences

University of South Bohemia in České Budějovice

Faculty of Science

Studies to determine the structure and function of Trk - K^+ translocating system

Ph.D. thesis

Deepika Uttam Kale

Supervisor: PD. Dr. Jost Ludwig

Center for Nanobiology and Structural Biology,
Institute of Microbiology,
Academy of Sciences of the Czech Republic

České Budějovice 2020

This thesis should be cited as:

Kale, D.U. 2020: Studies to determine the structure and function of Trk proteins- K^+ translocating system. PhD Thesis Series, No. 6, University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic, 161 pp.

Annotation

This thesis is focused on the functional and structural analysis of potassium translocation proteins (Trk) in the plasma membrane of yeast (*S. cerevisiae* and *Pichia pastoris*). In *S. cerevisiae*, Trk1 and Trk2 are the sole specific potassium uptake systems and required for growth in potassium limiting conditions. *P. pastoris* has only one Trk protein PpTrk, which had not yet been functionally analysed. All Trk proteins possess a huge cytosolic part of unknown role, the "long hydrophilic loop" (LHL). In order to investigate the role of LHL, functional studies were performed. Comparative functional analysis of Trk1 from *S. cerevisiae* and Trk from *P. pastoris* (PpTrk) revealed differences in the selectivity for monovalent cations. Furthermore, as a first step towards structure elucidation by X-ray diffraction crystallography, production of Trk proteins and parts of Trk proteins was attempted using different expression systems (*E. coli*, *S. cerevisiae* and *P. pastoris*).

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č.111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to - v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou – elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice

10.2.2020 Deepika U. Kale

This thesis originated from a partnership of the Faculty of Science, University of South Bohemia and the Center for Nanobiology and Structural Biology, Institute of Microbiology of the Czech Academy of Sciences, supporting doctoral studies in the Molecular and Cell Biology and Genetics study programme

Financial support

University of South Bohemia, GAJU years 2016 – 2018: 149/2016/P
and, years 2019: 04-017/2019/P.
Grant agency of Czech Republic (GAČR): GA16-19221S
Czech Ministry of Education Grant, Czech Research Infrastructure for Systems Biology (C4SYS)- LM2015055.

Acknowledgements

This Ph.D. journey has been incredible and one of the most wonderful experience of my life! I didn't do it alone. Therefore, I would like to express my gratitude to dear ones, who shared this journey with me ♥.

I am very grateful to my supervisor, Dr. Jost Ludwig, who gave me an opportunity to work with him, you have taught me so many things. Thank you for all the suggestions for the thesis, I could not imagine finishing it by myself. Thanks for your support in many experiments and also to attend conferences & courses ☺.

I thank Dr. Rainer Schindl, for hosting my internship at Med. Uni. Graz and for initiating the collaboration with Dr. Roland Malli on potassium sensors. Graz internship was one of the best experience during my Ph.D. studies. I am grateful to everyone from the Biophysics Dept ☺.

This would not have been possible without my family. I thank my parents, my true inspirations- my mom (Lata Kale) and dad (Uttam Kale), thanks for the unconditional love and care, for believing and cheering my every decision. I am thankful to my sisters, Uma and Uttara, and my adorable brother- Vishal. Thanks for staying by me and for sharing immense love. I thank my Akka (Grandma), for showing me what to do when I was lost! I thank Advika for bringing a lot of happiness and love to our family. Thanks to all my relatives whom I can't mention as I have so many ☺, love you all ♥.

I thank my Prof. "Dr. Neelima D. and Dr. Mukund D.", who introduced me to the world of Science and without there guidance, I wouldn't have started this journey.

Shruthi, Alka and Poonam S. it's being more than 12 years that we have known each other ♥. I don't have words to show gratitude to you guys. Thank you, for the amazing conversations and trips we had together. Lots of love ♥.

Ajay, I wouldn't have come here, without your help, thank you for being there when I needed you the most. One thing I really missed was our "Chai time"♥.

Karin and Pavel S., thanks a ton. You guys are family to me ♥. Thanks for taking care in many situations; I am glad that I could attend your wedding. Thanks, for showing me the Czech culture. I can truly say that Czech is my 2nd home ☺.

Binny and Samar, thank you for all the amazing trips, fun parties and movie nights. Taking time out from work and being with you guys, just felt like home♥.

Vrushali, Ankita, and Apurva thanks for the amazing trips around Europe ♥.

Daniel B., thank you for all the shared moments, and for everything ☺.

I thank Dr. Rudiger E., for initiating my internship with Dr. Schindl and for my 1st skiing trip. I thank Dr. Babak M. for establishing our new collaboration and for your constant support. Prof. Jannette C., Dr. David R., and Dr. Josef L. thank you for your constant motivation.

I thank all my friends from the castle who made Nove hrady much livelier and happening place. I thank my friends from CB University, for their support and for involving me in different fun activities. I thank all the lab members, and summer school students for sharing the work. I am thankful to everyone from the administration. Finally, once a week playing some game was great fun! Thanks to all my basketball and badminton friends ☺.

List of papers and author's contribution

The thesis is based on the following papers:

1. **Kale D**., Spurny P., Shamayeva K., Spurna K., Kahoun D, Ganser D., Zayats V and Ludwig J. *The S. cerevisiae cation translocation protein Trk1 is functional without its"long hydrophilic loop" but LHL regulates cation translocation activity and selectivity*.
Biochimica et Biophysica Acta - Biomembranes, 2019;1861:1476–88. DOI: 10.1016/j.bbamem.2019.06.010 (5 year IF- 3.807)

Deepika Kale carried out experiments, contributed in analyzing data and preparing figures and helped in writing the manuscript.

2. **Kale D**., Kulik N. Kahoun D. and Ludwig J. *Functional characterization of the Pichia pastoris high affinity K^+ translocation system PpTrk (Manuscript in preparation)*

Deepika Kale carried out the experiments, analyzed the results, prepared figures and helped in writing the experimental part of the manuscript.

Jost Ludwig, the corresponding author of mentioned paper, approves the contribution of Deepika Kale in this paper as described above.

……..……

PD Dr. Jost Ludwig

Dedicated to my Grandmother "Saalu Khadke" and Aunt "Kalavati Dhage"

माझ्या प्रिय आजीस "साळू खडके" आणि आत्या "कलावती ढगे" यांना समर्पित !

Content

List of Abbreviations

aa - amino acid(s)
ACU - Alkali Cation Uptake
Amp - Ampicillin
BiFC - Bimolecular fluorescence complementation
BMGY - Buffered complex medium containing glycerol
BMMY - Buffered complex medium containing methanol
C- tail - C- terminus
cf.- confer (compare)
CM- Chloramphenicol
cPCR - Colony PCR
DDM - n-Dodecyl β-D-maltoside
DMSO - Di-methyl sulfoxide
DNA - Deoxyribonucleic Acid
DSP - Dithiobis (succinimidyl propionate)
e.g. - exempli gratia / example given
EDC - 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
ENA - Efflux of Natrium
Fig. - Figure
FLISE - Flux measurement using ion selective electrode
GFP - Green fluorescent protein
i.e. - id est
IPTG - Isopropyl β-D-1-thiogalactopyranoside
Kan - Kanamycin
kD - Kilo Dalton
LHL- Long Hydrophilic loop
MFS - Major Facilitator Superfamily
MPM -Transmembrane helix - pore helix - Transmembrane domain
N-tail - N- terminus of Trk1
NHA - Na^+/H^+ Antiporter
NHS - N-Hydroxysuccinimide
Δ - Deleted/(Lack of)
OD - Optical density
PAGE - Polyacrylamide Gel Electrophoresis
pH - Reverse logarithm of H^+ concentration
PMA - Plasma membrane ATPase
RCK - Regulator of K^+ Channel Conductance
RT - Room Temperature
SDAP - Synthetic dextrose arginine phosphate medium
SDS - Sodium dodecyl sulfate
SKT - Superfamily of K^+ transporters
TM - Transmembrane
Tris - Tris-(Hydroxymethyl)-amminomethane
Trk - Transporters of Potassium (K^+ transporters)

Introduction

Transport across biological membranes is one of the essential processes, involved in many aspects of metabolism and signaling in all species. In cells, K^+ plays many important physiological roles; e.g., the intracellular concentration of K^+ plays a pivotal role in maintaining pH and membrane potential (Evans and Wildes, 1971), and in protein synthesis (Lubin and Ennis et al., 1964). K^+ is also required for the activation of key metabolic processes, such as pyruvate synthesis, for compensation of negative charges e.g. DNA and RNA, and also in regulation of enzyme activities (Page and Di Cera, 2006; Leigh and Jones, 1984). It has been shown that in *Saccharomyces cerevisiae* lack of K^+ activates oxidative stress response and the expression of genes required cell cycle progression is reduced (Barreto et al., 2012). In yeast the cellular K^+ concentration is 200 - 300 mM. However, yeast can grow from 10 μM to 2.5 M, of external K^+ concentrations and maintains a minimal amount of internal K^+ ~30 mM to survive (*for review see:* Arino et al., 2019; Arino et al., 2010).

This thesis describes experiments directed towards the understanding of the structure and function relationships Trk proteins that mediate translocation of ions across the plasma membrane of yeast (*Saccharomyces cerevisiae* and others). In order to understand terminology and the principle concepts, it starts with a brief description of the role of biological membranes and the proteins responsible for the translocation of molecules. Also, the basic concepts and terminology of different types of translocation/membrane transport will be described.

Transport across biological membrane

The biological membranes are dynamic, flexible, fluid structures that form a boundary around the cell composed of phospholipid bilayer, lipids and proteins. Each phospholipid molecule has a hydrophilic head and a hydrophobic tail. These macromolecules are in constant motion which the membrane a fluid character. In 1972, a model was proposed "fluid mosaic model" which describes the structure of biological membranes (Singer and Nicolson, 1972).

The biological membrane functions as a barrier to separate the contents of cells from the environment. It does not allow every substance to pass through. Small uncharged substances, like oxygen and carbon dioxide, can cross the cell membrane by simple diffusion. The membrane is also relatively permeable for water molecules; low-capacity bidirectional movement of water can take place via diffusion. However, high capacity faster transport takes place through water channels; Aquaporins (Agre 2006). In case of big and charged molecules (sugars, amino acids, peptides) the translocation across the plasma membrane takes place by two mechanisms: active and passive transport.

The molecules that cannot pass freely through the lipid bilayer can be translocated (transported) either through channels or with the help of carrier proteins. This process is called passive transport/facilitated diffusion. Passive transport does not require energy for translocation; it allows molecules to move along their electrochemical gradient (consisting of the chemical (concentration) gradient and the membrane potential that is usually inside negative).

If substances are transported against their electrochemical gradient (movement of a charged substance is influenced by both concentration gradient and membrane potential) the process is called active transport. Facilitated diffusion and active transport share many features. Both usually involve proteins, and show specificity for ions, sugars or amino acids.

Active transport, the transport of ions or molecules against the (electrochemical) gradient needs energy. In most cases, energy is obtained from the hydrolysis of ATP but it can also be directly driven by external energy, e.g. by light (light-driven H^+-pump-bacteriorhodopsin). Integral membrane proteins mainly carry out this process.

The second form of active transport is dubbed "secondary active transport". In this process the driving force for the movement of one molecule is obtained from the electrochemical gradient of another molecule that simultaneously crosses the membrane in energetically

favorable direction. Such transport mechanism is also known as cotransport. Therefore, in this process the energy for translocation of a substance is not derived directly from ATP nor light.

According to the direction of movement, secondary active transport can be classified as symport and antiport. In symport, the molecules are moved in the same direction, for example Na^+ and amino acid transporters in mammalian cells use the inwards directed electrochemical Na^+ gradient to transport amino acids against their gradient. The proton-sugar transporter in bacteria and the Na^+ sugar transporters use either inwardly directed proton or Na^+ gradients. Antiport moves two molecules in opposite directions, for example Na^+ in and Ca^{2+} out (Lodish et al., 2003; Murray et al., 1996; Berg et al., 2002).

Membrane transport proteins are proteins that are involved in the movement of ions, molecules such as proteins across biological membrane. In general, these transport proteins can be classified into ion channel/pore proteins, active transporters, group translocator and electron carriers (Lodish et al., 2003).

Ion channels are proteins that enables the translocation of molecules or ions across the membrane by a central pore. These pores/channels are specific for ions or molecules. The size and charge of the "substrate" and the structure of the protein determine their specificity. Ion channels translocate with high velocity of up to 10^8 ions per second. Channels can be in open state or in closed state. The opening (gating) of channels involves usually only a small conformational change. (Hille et al., 2001, Berg et al., 2002).

Transporters are integral membrane proteins, which mostly transport nutrients such as amino acid, sugars, peptides and vitamins (e.g. Bacterial permeases) (Lodish et al., 2003). They also play roles in cell metabolism, ion homeostasis, signal transduction, osmoregulation (Benedito et al., 2010). A more detailed description for the terms mentioned above is existing in many textbooks (e.g. Berg et al., 2002. Lodish et al., 2003, Alberts et al., 2002).

Saccharomyces cerevisiae

S. cerevisiae is a eukaryotic microbe belonging to the kingdom of fungi, often used as model organism in research to discover the functional relationships between sequence and gene products in higher eukaryotes. *S. cerevisiae* has a short generation time (doubling time under ideal growth conditions can be as low as 2 hours) and can be easily cultured. In 1996, the complete yeast genome (as the first genome of a eukaryote) was available to the public (Goffeau et al., 1996). The genome is made up of 12,068 kilobases contained in sixteen linear chromosomes. *S. cerevisiae* can be easily transformed; it has an excellent homologous recombination system enabling easy addition of new genes or deletions. It also has the ability to grow as a haploid what simplifies the creation of gene knockout and mutant strains. All together these properties make *S. cerevisiae* a versatile model organism to study eukaryotic systems.

In the following chapters, cation homeostasis in *S. cerevisiae* will be discussed. Because of the outstanding role of potassium ions, an overview on potassium translocation in the yeast will also be given.

Cation homeostasis and cation translocation proteins in S. cerevisiae

Cations are important for many metabolic processes within cells. They facilitate different biochemical reactions such as transport and ATP synthesis. However, at higher concentrations cations can be toxic, their concentration must be regulated and to regulate the concentration of these cations, cells have developed homeostasis mechanisms. *S. cerevisiae* cells maintain appropriate intracellular concentrations of Na^+ and K^+ and other cations by uptake, storage, and efflux mechanisms, which allow the cells to survive at extreme situations i.e., either limited availability or high concentration (Arino et al., 2010; Cyert et al., 2013). How these cations translocation systems help to maintain homeostasis in *S. cerevesiae* is described briefly below (see **Fig. 1** for an overview on the different cation-translocating membrane proteins). The main cation translocation systems in *S. cerevesiae* plasma membrane are Pma1, Ena1, Nha1 (Villalobo et al.,

1981; Haro et al., 1991, Prior et al., 1996**)** and the specific K^+ translocation systems are Trk1 Trk2 and Tok1 (Gaber et al., 1988; Ko et al., 1991; Ketchum et al., 1995).

Pma1 is a P_2-type H^+-ATPase made up of a single 100-kD polypeptide that pumps one proton across the plasma membrane per hydrolysed ATP molecule (Villalobo et al., 1981). Pma1 is one of the most essential cellular proteins. It generates an electrochemical proton gradient a "proton motive force", consisting of membrane potential (inside negative) and an inwardly directed proton concentration gradient. It plays an essential role in the maintaining neutral pH in the cytosol (Ambesi et al., 2000). There is also an isoform *S.c*. Pma2 (Schlesser et al., 1988) whose gene is expressed at lower level.

Ena1 and Nha1 are responsible for outward translocation of surplus cations. Ena1 (Efflux of Na) (Haro et al., 1991) is a primary active system (P-type ATPase) which pumps surplus of Na^+ but also K^+ and other monovalent cations out of the cell. ENA expression is induced by alkaline external pH. Thus, this protein is largely responsible for salt tolerance under alkaline conditions. Studies in *S. c.* revealed that the role of the Ena ATPase is essential for sodium detoxification at high external pH. Under these conditions the Nha1 antiporter system cannot effectively exchange Na^+ for protons (Ramos et al., 2011). Nha1 (Prior et al., 1996) is an alkali-metal (K, Na, Li)-proton antiporter, that is constitutively expressed at a low level. Its main role seems to be maintaining intracellular pH and K^+ constant.

Tok1 is a K^+ selective outward rectifying channel (Ketchum et al., 1995, Reid et al., 1996) and will be described together with other specific K^+-translocation systems in some more detail below. The existence of another cation translocation system, Nsc1 (for non-specific cation channel), was proposed from electrophysiological recordings, in which K currents were detected in *trk1 trk2* mutants. These K^+ currents can be blocked by Ca^{2+} and other divalent cations. Nsc1 was suggested to be responsible for the low affinity K^+ transport, but the specific protein

responsible for that has not yet been identified (Bihler et al., 1998; Madrid et al., 1998).

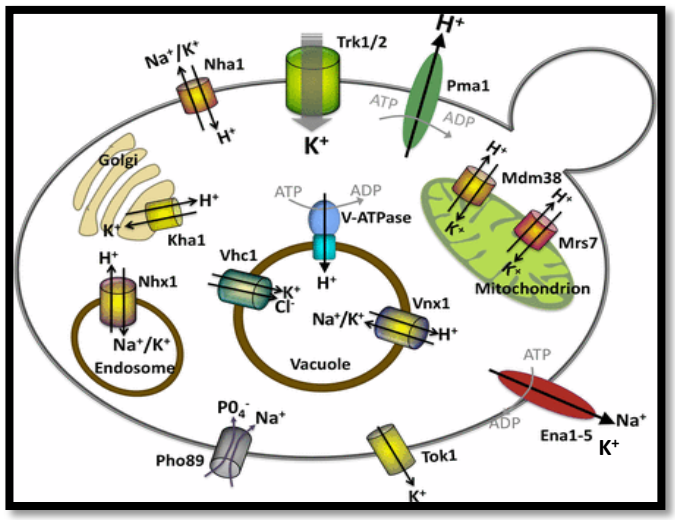

Fig. 1: The main transport systems that are responsible for transport of alkali metal ions in *S. cerevisiae*. (taken from Cyert et al., 2013, modified).

Additionally, there are a number of cation translocation systems that are localized in intracellular membranes. Proton/(Na^+/K^+) antiporters Vnx1 in vacuoles (Cagnac et al., 2007), Kha1 in the golgi apparatus (Ramirez et al., 1998) and Nhx1 in endosomes (Nass et al., 1997) and Mkh1/Mdm38, a protein essential for the exchange of K^+/H^+ across the inner mitochondrial membrane (Novikovsky et al., 2004). K^+ uptake in *S. cerevisiae* is mediated by two translocation systems Trk1 and Trk2 (Gaber et al., 1988; Ko et al., 1991). Trk1 is required for growth in low [K^+].

Potassium Translocation systems in various yeast

Types of K^+ uptake systems

Three different types of plasma membrane transporters mediating potassium influx have been described in yeast and other fungi: Trk (Transporters of K^+), HaK (High Affinity K^+ transporter) and ACU (Alkali Cation Uptake) ATPases. These transporters share the same function (i.e. K^+-translocation), but they differ by translocation mechanism and also in their abundance.

Trk's (Transport of K^+) are widely distributed in all yeasts. K^+ transport in *S. cerevisiae* depends mainly on Trk1. In the presence of Trk1 the role of Trk2 protein in K^+ supply is very low and its transport activity is undetectable (Arino et al., 2019; Arino et al., 2010). Thus, the role of Trk2 is not clearly known. However, it has been shown that Trk2 plays role in the maintenance of plasma membrane potential (Petrezselyova, et al., 2011). Hak symporters are a class of K^+ transporters encoded by HAK genes. HAK is present in many yeast such as *Hansenula polymorpha, Debaryomyces* and *Candida albicans* (Rodriguez-Navarro, 2000; Arino et al., 2010, Ramos et al., 2011). It is also found in *N. crassa* a mycelial fungus (Haro et al., 1999). The last type of K^+ uptake systems are the ACU ATPases which belongs to subfamily of P-type ATPases involved in high-affinity K^+ or Na^+ uptake. Yeast which have ACU ATPase, are e.g. *Ustilago maydis* or *Pichia sorbitophila* (Benito et al., 2004, Ramos et al., 2011).

Tok1, Trk1 and Trk2: Specific systems for K^+ translocation across the plasma membrane of S. cerevisiae

Tok1

Tok1 is a 691 amino acid long integral membrane protein. It is an outwardly rectifying K^+ selective channel in the plasma membrane of *S. cerevisiae* (Bertl et al., 1993, Ketchum et al., 1995, Reid et al., 1996). *Candida, Zygosaccharomyces, Yarrowia or Pichia* yeast species contain a gene homologous to the *S. cerevisiae* TOK1, whereas *S. pombe* and *D. hansenii* lack a similar system. TOK1 activity in *S. cerevisiae* might contribute to the maintenance of a stable plasma membrane potential (for review see, Arino et al., 2019; Arino et al., 2010). Tok1p belongs to two-pore domain potassium channel family and reported to be a "molecular target" of the yeast viral killer toxin K1 (Ahmed et al., 1999), but it was shown that Tok1p may be a target but it is not essential for toxin action (Breinig et al., 2002; Baev et al., 2003). The cytosolic carboxy terminal tail of Tok1 (160 amino acid long) strongly interacts with the central core

of the channel and regulates its gating (Loukin and Saimi 2002). Cells lacking Tok1 gene can grow at low potassium concentration <1 mM (Bertl et al., 2003). Deletion of TOK1 results in plasma membrane depolarisation while hyperpolarisation occurs in cells over expressing TOK1 (Maresova et al., 2006).

Potassium uptake: Trk1 and Trk2

Trk1 and Trk2 are specific K^+ uptake systems in *S. cerevisiae* plasma membrane. In the year 1988 the *TRK1* gene was cloned and shown that it is required for high-affinity potassium transport (uptake) (Gaber et al., 1988). *TRK2*, identified by Ko et al., 1991, was considered as second transporter of potassium in *S. cerevisiae*. Cells lacking gene for Trk1 and Trk2 are hypersensitive to low pH; they are severely limited in their ability to take up K^+ (Ko et al., 1991). The uptake of potassium by Trk2 is lower than Trk1 in the cells that are in exponential phase (Ramos et al., 1994). The presence of two *TRK* genes in *S.c.* that encodes for potassium uptake might accomplish similar function as *PMA1* and *PMA2* that encode for H^+ ATPases. In both cases, the second has lower expression and protein with slightly different properties (Haro et al., 1999, Ramos et al., 1994). Most *Saccharomyces* species contain a high-affinity K^+ transporter gene highly similar to *TRK1* in *S. cerevisiae.* A *TRKI* homologue (u-*TRK1*) was identified in *S. uvarum,* which encodes a membrane protein of 1241 amino acid that is 78 % identical and 86 % similar to *S.c.* Trk1 (Anderson et al., 1991).

Regulation of Trk proteins

In potassium limiting conditions, the *ACU* (Benito et al., 2004) and *Hak* (Su et al., 2002) gene expression is triggered. Both genes are transcriptionally regulated. However, this is not true for *TRK*. However, there are several proteins that might regulate Trk function at post-translational levels (for review see; Arino et al., 2019 and table 1.1 below).

Table 1.1: Some regulators of S. cerevisiae Trk proteins (adapted from Arino et al., 2019).

Gene/complex	**Description of the Gene**
ARL1	G protein of the Ras superfamily
BMH1,2	14-3-3 proteins involved in signalling processes during potassium starvation
CNB1/CRZ1	Calcineurin regulatory subunit/transcription factor
HAL4,5	Protein kinases involved in regulation of Trk
PMA1,2	Plasma membrane ATPase
PMP3	Plasma membrane protein involved in the regulation of membrane potential
PPZ1,2/HAL3	Serine/threonine protein phosphatase (PPZ)/Regulatory subunit of PPZ
SKY1	Protein kinase. Mutants show altered potassium transport
SNF1	Protein kinase. Mutants show defective potassium uptake
TPS1	subunit of trehalose-6-P synthase/phosphatase complex
TORC1	Target of Rapamycin complex involved in the regulation of potassium fluxes

Structure of Trk1

Bioinformatical analysis of the *S.c. TRK1* gene indicated that it encodes an integral membrane protein of 1,235 amino acids. The structure of Trk1 is not yet known, based on genetic information and solved structures of related proteins, several studies were done in order to characterize the structure of Trk1. Initially, the structure of Trk1 was assumed to be similar to those of the major facilitator superfamily (MFS) e.g. GLUT transporters, consisting of 12 transmembrane (TM) domains domains and a large hydrophilic loop which is located either intra- or extracellular. It was also assumed, that transport is secondary active (K^+/H^+-symport) in analogy to many members of the major facilitator superfamily (MFS) (Gaber et al., 1988). Durell and colleagues (Durell *et al.,* 1999) revealed by sequence analysis a possible evolutionary relationship between *S. cerevisiae* Trk (and the related prokaryotic Ktr, Trk and plant Hkt) and K^+ channels **(Fig. 2)**. The basis for this was the similarity within the putative selectivity filter region (see below), where they identified a single almost invariant glycine residue. Therefore, they proposed a different transmembrane topology, which is similar as in K^+ channels.

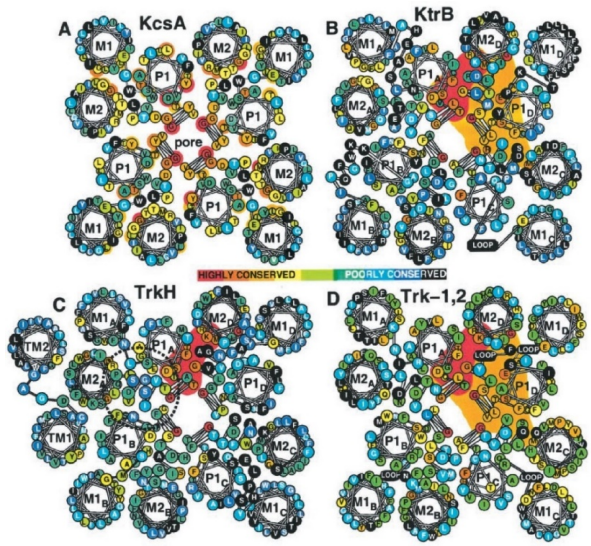

Fig. 2: Helical wheel representation of structural models of potassium translocation proteins: (A) KcsA, (B) KtrB, (C) TrkH, (D) Trk1, 2. The color code of residues indicates the level of conservation from high to low. Red background indicates regions with residues conserved among all of these translocation systems, the orange background indicates residues conserved among only the KtrB and Trk-euk (i.e., Trk1, 2) families (taken from Durell and Guy, 1999).

Potassium channels have a tetrameric structure (Mackinnon 1991, Doyle *et al.,* 1998). The crystal structure confirmed that channel is made up from a transmembrane helix - pore helix - transmembrane domain (MPM or M1PM2) motif. KcsA channels (as all K^+ channels) contain a "selectivity filter" with conserved amino acid sequence (TVGYG in KcsA) and a special arrangement of oxygen atoms from backbone carbonyl groups **(Fig. 3)** which plays an important role to differentiate between K^+ and Na^+.

The potassium channel is highly specific for K^+ ions, Na^+ and Li^+ permeate only very rarely. Only a single sodium ion is allowed to pass through the channel for every 10,000 potassium ions (Doyle et al., 1998). The selectivity for K^+ over Na^+ is mainly achieved by the difference in dehydration energies (Doyle et al., 1998, MacKinnon, 2003). At the

extracellular and internal ends of the filter, potassium ions are surrounded by water molecules. As K^+ enters the filter, it interacts with the carbonyl backbone of glycine residues within the selectivity filter and the hydration shell is replaced. Na^+ do not interact favorably with the oxygen atoms of the carbonyl backbone due to their smaller size (Doyle et al., 1998, Gouaux & Mackinnon, 2005). The free energy for dehydrating of K^+ (55 kCal/mol), is less than for dehydration of Na^+ (72 kCal/mol) (Berg et al., 2002). This difference is the main reason for selectivity of K^+ over Na^+.

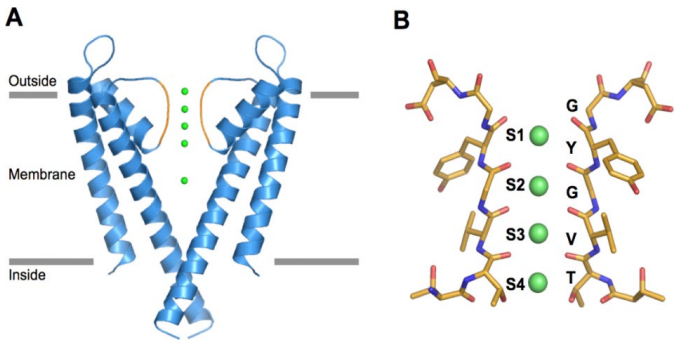

Fig. 3: KcsA potassium channel structure. **Panel A** shows two MPM motives oriented in membrane. The selectivity filter is shown in brown. **Panel B** shows the close view on the selectivity filter. S1-S4 represents the binding sites for K^+ ion. Green balls represent K^+ ion. Atoms are colored: carbon in brown, oxygen - red and nitrogen – blue (taken from Lockless et al., 2007).

Additionally, the similarity between KcsA-related K^+ channels and at that time presumed "symporters", allowed building first three-dimensional models of Trk/KtrB/HKT (superfamily of K transporters, SKT) proteins (Durell et al., 1999). In structural models of, they are similar to inward rectifying K^+ channels. As said above, each of the four K^+ channel forming α-subunits consists of two transmembrane (TM) helices (M1 and M2) connected by a short pore helix and a "selectivity-filter" region, the latter two subsumed as pore (P) region (MPM). In SKT proteins, the four repeats of the MPM motifs are all together on a single peptide chain. Four conserved glycine residues are placed within the selectively filter and were the starting point for the alignments used to build the models (Durell et al., 1999).

In 2011, the crystal structure of TrkH from *Vibrio parahaemolyticus* was solved (Cao et al., 2011) They gave a first insight to the structural organization of SKT proteins. TrkH crystallized as a homodimer as shown on **(Fig. 4)**. Each monomer consists (as predicted 3B the Durell and Guy models) of four MPM motifs arranged around the central pore. As expected, this architecture in general resembles the structure of KcsA, however, only the selectivity filter, pore helices and adjacent part of the transmembrane helices is really similar to potassium channel. The part below that together with the internal pore significantly differs from KcsA. In three out of four MPM repeats one transmembrane helix is bend in opposite direction of the internal pore. In addition, the internal pore is filled by a loop. It is a new structural feature, which was not seen before. Together with the loop the side chain of positively charged residue arginine 468 is located right inside of the pore forming the constriction on a way to the selectivity filter. Experimental results showed that mutation of the Arg 468 to alanine lead to the increased rate of K^+ influx (Cao et al., 2011).

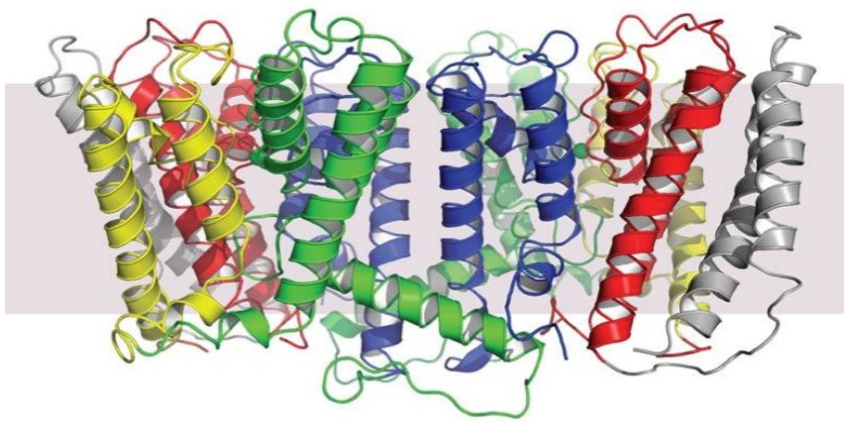

Fig. 4: The crystal structure of TrkH from *Vibrio parahaemolyticus.* TrkH has a dimeric structure. Every domain within each monomer is colored differently (taken from Cao et al., 2011).

Two years later the crystal structure KtrB, another member of the SKT family, together with its associated regulatory subunit KtrA protein,

was released. The crystal structure of the KtrAB complex was shown to consist of a homodimeric KtrB and a cytoplasmic octameric KtrA ring. Electrophysiological measurements showed that the regulatory subunits KtrA play an important role in gating mechanism of the protein (Vieira-Pires et al., 2013). The KtrB structure is very similar to the architecture of the TrkH channel described above. In both, TrkH and KtrB, the conserved glycine residues are located within the selectivity filter (Cao et al.,2011; Vieira-Pires et al., 2013). This all supports the earlier hypothesis about relationship between Trk/KtrB/HKT proteins.

Several studies were carried out in order to analyze the role of the conserved glycine residues in the selectivity filter in SKT proteins. For example, all four putative glycine residues from KtrB *Vibrio alginolyticus* were mutated in order to analyze whether these residues indeed form selectivity filter. The results showed that mutations of each of glycine to serine reduced the K^+/Na^+ selectivity. Also, these results support the prediction that glycine residues are located within the selectivity filter (Tholema et al., 2005).

The structure of *S. cerevisiae* Trk1 is not yet solved. However, homology between Trk1 and related proteins (TrkH and KtrB) allowed creating a rather detailed Trk1 structural model (Zayats et al., 2015). In this work the roles of residues conserved throughout the transporter's families were studied experimentally. As already mentioned above, the key residue that enabled to relate Trk1, TrkH and HKT potassium transporters to a common ancestor is the invariant glycine residue in the selectivity filter. The evidence for conserved glycine residues belong to the selectivity filter was given for TrkH and HKT proteins, but not yet for Trk1.

Therefore, the experimental and theoretical studies were focused on analysis of predicted conserved glycine residues. The results of this work indicated that location of glycine residues within the selectivity filter is also true for Trk1. Furthermore, the experimental results based on the Trk1 model showed that Trk1 as well as TrkH and KtrB possess a

conserved salt bridge between Asp 79 and Lys 1147. These results strengthen the hypothesis of evolutionary relationship between Trk1 and bacterial potassium transporters TrkH and KtrB, and in turn also indicate the validity of the model (Zayat et al., 2015). However, this model lacks information about some parts (C-tail, N-tail and long hydrophilic loop) of the proteins.

Regulatory Domains or Subunits

K^+ channels and SKT proteins can possess "regulator of K^+ channel conductance" (RCK) domains that play important role in the gating of the systems. These RCKs can be part of the channel protein and encoded on the same polypeptide chain. This is the case e.g. in Ca^{2+} activated channels where the RCK domain is located at the C-terminal end of the polypeptide (see e.g. Jiang et al., 2002). Alternatively, additional subunits can act as RCKs e.g. TrkA (with TrkH) and KtrA (with KtrB) are shown to regulate the gating of the complexes. However, they are not necessary for the principal channel function. TrkH and KtrB can mediate K^+ translocation alone (Cao et al., 2011, Vieira-Pires et al., 2013). For the closest homologue of eukaryotic Trk proteins, the bacterial KtrB, it has been shown that the KtrA (Nakamura et al., 1998) regulates cation translocation via the binding of ATP. I.e., conductivity (or open probability) is highest when ATP is bound, decreased when ATP is replaced by ADP and even lower in the absence of KtrA (Vieira-Pires et al., 2013). Similarly, the ion translocation activity of TrkH is enhanced by TrkA (Bossemeyer et al., 1989, Cao et al., 2011) when ATP is present (Cao et al., 2013). A representation of the composition including RCK domains for SKT protein is given below in **(Fig. 5)**. *S. cerevisiae* do not possess any additional RCK elements but contain a long hydrophilic loop (LHL). The LHL in *S. c.* comprises of ~50% of the total protein. LHLs of Trk1 and Trk2 are 633 aa and 317 aa long. There are no known proteins homologous to either Trk1 or Trk2 LHL. Also, the similarity between the Trk1(LHL) and Trk2(LHL) is very low (Kale et al., 2019) **(Fig. 5)**.

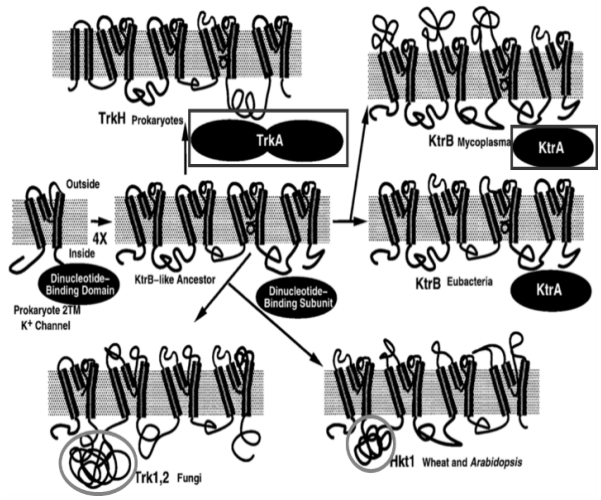

Fig. 5: The additional domain/subunit of TrkH (TrkA) and KtrB (KtrA), both are highlighted with the box. The fungal Trk's (long hydrophilic loop) and the loop (dinucleotide binding domain) of plant HKT are highlighted with a circle, (modified from Durell et al., 1999).

Transmembrane parts of Trk

As already described, the MPM elements of SKT proteins are linked together on one polypeptide chain and are designated as repeats A, B, C and D **(Fig. 6)**. Due to the similarity of the principal channel forming subunits it was predicted that SKT proteins have evolved from an ancestral "K^+ channel" through two-fold gene duplication. Therefore, the question was whteher it is possible overexpress and purify a single domain of Trk protein and can a domain alone form a functional tetramer in *S. cerevisiae*?.

Multimerization/subunit composition: As mentioned, a difference between K^+ channels and SKT (Ktr/Trk/HKT) proteins is that for the former the functional unit is a tetramer (corresponding to a SKT monomer, **cf. Fig.3D**). In principle, also SKT proteins could be fully functional as monomers. However, the structures of KtrB/KtrA and TrkH/TrkA indicate that their "functional" unit is composed of two channel forming (KtrB, TrkH) and eight regulatory subunits (KtrA, TrkA, see above; Cao et al., 2013; Vieira-Pires et al., 2013). Since Trk1 is more closely related to SKT

proteins than to KcsA, KtrB and TrkH were used as templates for homology modeling and thus also a dimeric composition was assumed (Zayats et al., 2015). However, the $M2_D$ subunit of fungal Trk1 contains several charged residues that could not be all modeled in a way that charged side chains are hidden from hydrophobic environment (Durell and Guy 1999). Therefore, a tetrameric composition where charged residues are located within the protein has been proposed. Here it is noteworthy to mention that fungal Trks cannot only mediate K^+ translocation but are also able to promote the flux of Cl^- and other anions across the plasma membrane (Kuroda et al., 2004; Rivetta et al., 2005; Rivetta et al., 2011). Thus, Trks might also play a role in maintaining intracellular Cl^- levels within an acceptable range, i.e. counteract the activity of Cl^- influx systems (Kuroda et al., 2004). The presence of an additional pore formed at the monomer interface(s) would be inconsistent with a monomeric functional unit. It is also unlikely in a dimer in which the monomer interface is tightly packed (as in TrkH, KtrB and our Trk1 model). Thus, a tetrameric composition in which a central metapore is formed at the monomer interface **(Fig. 7)** seems possible. Hence, a question was, what is the "normal" multimeric state of Trk protein?

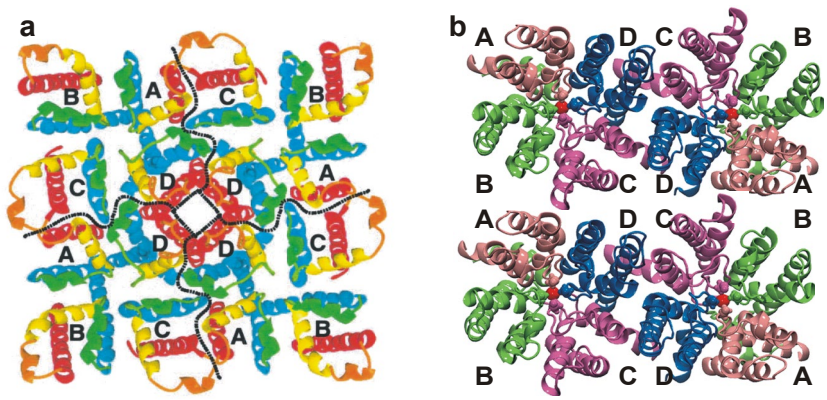

Fig. 7: a) Possible tetrameric arrangement of Trk monomers. A central pore could be formed by the four $M1_D$ helices (taken from Durell et al., 1999). **b)** A dimerisation of dimers as in our model would not lead to a central symmetrical pore.

Thesis overview

This thesis describes experimental work dedicated to the analysis of structure and function of Trk, the main K-translocation system in yeast. K^+ is one of the most essential ions for all organisms including fungi. In *S. cerevisiae*, Trk proteins allow cells to survive and grow in potassium limiting environments (a few μM K^+), while maintaining the homeostasis. As mentioned previously there are two specific K^+ uptake systems in *S. cerevisiae*: Trk1 and Trk2. For past three decades, studies related to fungal Trk proteins were focused on understanding the physiological role of the protein as well as to gain the structural information of the protein. Recently, in our lab an atomic scale model for Trk1 was generated using two bacterial K-uptake proteins (TrkH and KtrB as templates (Zayats et al., 2015). However, there is still a lot of structural information about Trk proteins and their role in yeast other than *S. cerevisiae* missing.

1. Trk proteins possess a long hydrophilic loop between $M1_B$ and $M2_A$ domains, and the role of LHL was unknown and no experiments were performed to address this question.
2. *Pichia pastoris* (correct *Komagataella phaffii*) is methylotropic yeast, it possess only one K^+ uptake system: PpTrk, that has not yet been studied in any detail. Hence, a question was, does PpTrk share similar function as ScTrk's?
3. The full structure of *S. cerevisiae* Trk1, as the model lacks information about cytosolic parts of the proteins (C-tail, N-tail, and LHL) as well as the multimeric state of the protein is not known. Can MPM domain alone be functional? And is it possible to produce and purify them as a single protein?

The results obtained during this study have been divided into three parts:

Part 1: The S. cerevisiae cation translocation protein Trk1 is functional without its "Long Hydrophilic Loop" but LHL regulates cation translocation activity and selectivity

To address the first question about the role of LHL in Trk proteins, comparative analysis of Trk1 and Trk1ΔLHL was carried out. growth tests, fluorescence microscopy, and ion flux measurements (FLISE) were used to determine the role of LHL. It was demonstrated that even after the deletion of LHL, Trk1 is functional and LHL plays a role in selectivity and in regulating the ion translocation activity *(published in Kale et al., 2019).*

Part 2: Characterization of the Pichia pastoris K^+ translocation system PpTrk

Pichia pastoris has a wide range of industrial applications, i.e. it is used in production of recombinant proteins, in vaccines, and biofuels. *Pichia pastoris* possesses PpTrk. K^+ plays an important role in regulating cell growth, nutrient uptake as well as maintaining homeostasis during the fermentation process (Lam et al., 2014). Experimentally, it was demonstrated that Trk1 in *S. cerevisiae* plays an important role in maintaining homeostasis during the ethanol fermentation. Therefore, a characterization of PpTrk was carried out. This included the cloning of the gene, its heterologous expression in *S. cerevisiae* and basic functional characterization by growth tests. It turned out that PpTrk seems to have a reduced K^+ selectivity compared to *S. c.* Trk1 that might be caused by a Glycine to Alanine exchange in the selectivity filter *(manuscript in preparation)*.

Part 3: Production of full length and parts of Trk proteins

The second part is divided into 3 parts A and B.

A) Heterologous overexpression of TRK1 in E. coli and P. pastoris and its homologous overexpression.

As the crystal structure of Trk1 is unknown an attempt was made to overexpression full length protein in *E. coli*. Because of failure to overexpress full length protein in *E. coli*, eukaryotic expression systems, *S. cerevisiae* and *Pichia pastoris* were used. The expression of both, *TRK1*

and *TRK1ΔLHL* was attempted. The experiments and results obtained are described in this section *(unpublished data)*.

B) Overexpression and functional studies of transmembrane parts (A and D), regulatory domain (LHL) and cytosolic parts (C- and N-tail) of S. cerevisiae TRK1.

A Trk1 monomer resembles a fully functional K^+ channel. Hence the idea was to overexpress and purify single MPM domains of TRK1, each corresponding to a K^+ channel alpha subunit. The production of A and D domains in *E. coli* was attempted. However, only low protein production was observed. Thus, further experiments focused on the targeting and possible function of these domains when the genes were expressed *S. cerevisiae*. Furthermore, the heterologous expression of (GFP-fusion) genes encoding LHL, C-terminal cytosolic "tail" (C-tail) and N-terminal cytosolic part (N-tail) in *E. coli* and their purification was carried out. These results are discussed in this section *(unpublished data)*.

Part 1

The *S. cerevisiae* cation translocation protein Trk1 is functional without its "Long Hydrophilic Loop" but LHL regulates cation translocation activity and selectivity

Kale D., Spurny P., Shamayeva K. Spurna K., Kahoun D., Ganser D., Zayats V., Ludwig J.

BBA - Biomembranes

2019

The *S. cerevisiae* cation translocation protein Trk1 is functional without its "Long Hydrophilic Loop" but LHL regulates cation translocation activity and selectivity.

Deepika Kale [a, b], Pavel Spurny [a], Katsiaryna Shamayeva [a], Karin Spurna [a], David Kahoun [c], Daniel Ganser [d], Vasilina Zayats [e] and Jost Ludwig [a *]

[a] Center for Nanobiology and Structural Biology, Institute of Microbiology, Academy of Sciences of the Czech Republic, Zamek 136, 373 33 Nove Hrady, Czech Republic
[b] University of South Bohemia in Ceske Budejovice, Faculty of Science, Branisovska 1760, 370 05 Ceske Budejovice, Czech Republic
[c] University of South Bohemia in Ceske Budejovice, Faculty of Science, Institute of Chemistry, Branisovska 1760, 370 05 Ceske Budejovice, Czech Republic
[d] Institute of Cellular and Molecular Botany, University of Bonn, Kirschallee 1, 53115 Bonn, Germany
[e] Centre of New Technologies, University of Warsaw, Stefana Banacha 2c, 02-097 Warsaw, Poland

Deepika Kale: kale@nh.cas.cz, Pavel Spurny: spurny@nh.cas.cz, Katsiaryna Shamayeva: shamayeva@nh.cas.cz, Karin Spurna: spurna@nh.cas.cz, David Kahoun: dkahoun@prf.jcu.cz, Daniel Ganser: daniel.ganser@web.de, Vasilina Zayats: v.zayats@cent.uw.edu.pl

* Corresponding Author: ludwig@nh.cas.cz, Jost Ludwig, Zamek 136, 373 33 Nove Hrady, Czech Republic, +420 389 033 844

This part is comprised of 33 pages (24-57) published data, which is present in the original thesis deposited at the Faculty of Science, University of South Bohemia

Abstract

In *Saccharomyces cerevisiae*, K^+-uptake under K^+-limiting conditions is largely mediated by the cation translocation systems Trk1 and Trk2 belonging to the family of SKT proteins. They are related to two-transmembrane-domain (inward rectifying K-) channels but unlike the symmetrical tetrameric structure of K-channels, a single Trk contains four pore-forming domains (A-D) encoded on one polypeptide chain. Between domains A and B Trks contain large cytosolic regions dubbed "long hydrophilic loop" (LHL). LHLs are not homologous/similar to any other identified protein (domain) and also show little similarity between Trk1 and Trk2. Here we demonstrate that Trk1 is functional without LHL. However, in growth experiments NaCl sensitivity of Trk1[ΔLHL] expressing cells is increased under K^+-limiting conditions compared to full-length Trk1. Non-invasive ion flux measurements showed that K^+-influx through Trk1 and Trk1[ΔLHL] is decreased in the presence of surplus Na^+ due to permeability of the proteins for both cations and competition between them. Trk1[ΔLHL] is less affected than full-length Trk1 because it is more selective for K^+ over Na^+. Furthermore, K^+ re-uptake after starvation is delayed and decreased in Trk1[ΔLHL]. Thus, a role of LHL is regulating cation fluxes through Trk1 by (i) allowing also Na^+ to pass if monovalent cations (mainly K^+) are limiting and (ii) by accelerating/enhancing a switch from low to high affinity ion translocation. We propose that LHL could modulate Trk1 transport properties via direct influence on a transmembrane helix ($M2_A$) which can switch between bent and straight conformation, thereby directly modifying the radius of the pore and selectivity filter.

Part 2

Characterization of the *Pichia pastoris* K^+ translocation system PpTrk

Kale D., Kulik N., Kahoun D., and Ludwig J.

(manuscript in preparation)

This part is comprised of 31 pages (59-90) unpublished data, which is present in the original thesis deposited at the Faculty of Science, University of South Bohemia

Abstract

According to the genomic sequence, the biotechnologically widely used yeast *Pichia pastoris* (syn. *Komagataella* spp.) has only one specific K^+ uptake protein, PpTrk. In this study, we report the first functional characterization of *PpTrk* heterologously expressed in *Saccharomyces cerevisiae*. *PpTrk* complements the growth defect of a K^+ uptake deficient strain of *S. cerevisiae* (*trk1,2 tok1Δ*), in low [K^+] medium. Growth of the *PpTRK* expressing strain was highly sensitive to high [Na^+] under [K^+]-limiting conditions, in contrast to *S. cerevisiae* (*trk1,2 tok1Δ*) cells expressing *TRK1* under control of the same promoter. This indicated a decreased selectivity of PpTrk for K^+ over Na^+. Sequence analysis and homology modeling revealed a residue in the selectivity filter (Alanine 93), that might play an important role in the determination of ion-selectivity.

Part 3

Production of full length and parts of Trk proteins

Part 3A

Heterologous and homologous production of Trk proteins in E. coli, P. pastoris and S. cerevisiae

(Unpublished data)

and

Part 3B

Overproduction and functional studies of transmembrane parts (A and D), long hydrophilic loop (LHL) and cytosolic parts (C- and N-tail) of S. cerevisiae Trk1

(Unpublished data)

This part is comprised of 60 pages (92-152) unpublished data, which is present in the original thesis deposited at the Faculty of Science, University of South Bohemia

3A) Heterologous overexpression of TRK1 in E. coli and P. pastoris and its homologous overexpression.

Abstract

As the crystal structure of Trk1 is unknown an attempt was made to overexpression full length protein in *E. coli*. Because of failure to overexpress full length protein in *E. coli*, eukaryotic expression systems, *S. cerevisiae* and *Pichia pastoris* were used. Different constructs where TRK1 fused, to the C-terminal and N -terminal of GFP were made and used to transform *S.c.* strain BY4741-3M and functional/expression studies were carried out with these strains. When observed microscopically, the fusion proteins were targeted correctly. By western blot and immuno-staining with anti-GFP antibodies, it was shown that the produced fusion proteins were full length. Furthermore, crosslinking experiments showed that Trk proteins can form oligomers. The expression of both, *TRK1* and *TRK1ΔLHL* was attempted. Optimization studies for determining suitable buffer, methods of cell disruption and solubilisation were performed. Using the identified optimal conditions for purification, an attempt was made to purify the Trk1 (pYEX-*6xHis/GFP/TRK1*) by metal affinity chromatography. The amount of protein produced in *S.c.* was low, and protein purification was not successful. Constructs suitable for expression of *TRK1* and *TRK1(ΔLHL)/GFP*-fusion genes under control of the MeOH activated *AOX1* promoter were generated and used to transform different *P. pastoris* strains. Production of Trk proteins was checked after induction with methanol. Fluorescence microscopy of GFP/Trk fusion proteins showed plasma membrane localization in *P. pastoris*. Also, protein analysis by SDS-PAGE and western blot/immunostaining using anti-GFP and anti-His antibodies showed the presence of Trk1- and Trk1(ΔLHL). Thus, expression of *TRK* using both yeast expression systems *S. cerevisiae* and *P. pastoris* was successful.

3B) Overexpression and functional studies of transmembrane parts (A and D), regulatory domain (LHL) and cytosolic parts (C- and N-tail) of S. cerevisiae TRK1.

Abstract

A Trk1 monomer resembles a fully functional K^+ channel. Hence the idea was to overexpress and purify single MPM domains of TRK1, each corresponding to a K^+ channel alpha subunit. The production of A and D domains in *E. coli* was attempted. However, only low protein production was observed. Thus, further experiments focused on the targeting and possible function of these domains when the genes were expressed *S. cerevisiae*. Furthermore, the heterologous expression of (GFP-fusion) genes encoding LHL, C-terminal cytosolic "tail" (C-tail) and N-terminal cytosolic part (N-tail) in *E. coli* and their purification was carried out. Only *E. coli* was used as host for the production of cytosolic parts of Trk1 (cytosolic amino-terminal part, "N-tail", carboxy-terminal part "C-tail" and LHL) and transmembrane domains (A and D). Production of the cytosolic amino- and carboxy-terminal tails (N- and C-tail) was achieved in *E. coli* and the C-tail was successfully crystallized. However, its structure could not be solved, most likely because it is too flexible. Trk1(LHL) could not be produced in 4 different *E. coli* expression strains due to its toxicity for the bacterial cells. Also, the attempts to produce single MPM domains in *E. coli* failed. However, single domains A and D were successfully produced in *S. cerevisiae*, though also not yet in amounts allowing purification and crystallization. Taken together, the results obtained confirmed that the solution of the structure of membrane proteins is a challenging task. The whole procedure is laborious and usually results are obtained based on numerous trial and error experiments. As first step towards a crystal structure of a yeast Trk, production of ScTrk and PpTrk in *P. pastoris* and *S. cerevisiae* has been achieved.

Discussion and Conclusions

This thesis work was focused on the functional and structural analysis of potassium translocation proteins (Trk) in the plasma membrane of yeast (*S. cerevisiae* and *Pichia pastoris*). In yeast, Trk's are the potassium uptake systems and required for growth in potassium limiting conditions. Trk proteins possess at the cytosolic side a "long hydrophilic loop" (LHL), that comprises more than 50% of the total residues. However, there was no information available about structure and function of LHL. In order to investigate the role of LHL, comparative analyses between full length Trk1 and strain lacking LHL (Trk1ΔLHL) were performed (part 1).

Results described in part 1, showed that Trk1 is functional without LHL. However, growth experiments with low $[K^+]$ and 3000-fold surplus $[Na^+]$, Trk1[ΔLHL] expressing cells revealed increased NaCl sensitivity, compared to full-length Trk1. Non-invasive ion flux measurements using FLISE showed that K^+-influx through Trk1 and Trk1[ΔLHL] is decreased in the presence of surplus Na^+, due to permeability of the proteins for both cations and competition between them. Surprisingly, the deletion of LHL increased the selectivity of Na^+ over K^+.

Thus, the observed higher sensitivity of Trk1[ΔLHL] to NaCl is not due to toxic effects of Na^+ but caused by a too low intracellular concentration of monovalent cations. LHL might also accelerate/enhance a switch from low to high affinity mode that has been proposed for Trk1. In this respect, LHL resembles the role of RCK domains (in K-cannels) or subunits of SKT proteins (KtrA and TrkA) that modulate activity of these related transport systems. An explanation for the action of LHL could be a mechanic influence of LHL on a transmembrane helix which might modify the size of the pore and thus selectivity.

The *P. pastoris* Trk protein (PpTrk), was not functionally analysed before. Hence, a comparative functional analysis of Trk1 and Trk2 (*ScTRK1, -2*) from *S. cerevisiae* and Trk from *P. pastoris* was performed (part 2). Growth tests, revealed differences in the properties of PpTrk and ScTrk proteins. The growth of *S.c.* (Δ trk1,1, tok1) cells expressing *PpTRK*

was completely inhibited under K^+-limiting conditions and 3000-fold surplus Na^+. AAS measurements revealed that *PpTRK* expressing cells had under these conditions a 3.5 times higher intracellular Na concentration than cells expressing *ScTRK1* or *ScTRK2*, indicating decreased selectivity for K^+ over Na^+. Sequence analysis and homology modeling indicated that due to differences between the selectivity filter sequences, the pore of PpTrk might possess a larger diameter, letting pass Na^+ more easily. Furthermore, these analyses showed that charges and charge distribution in LHLs differ between PpTrk and ScTrk1 and -2, possibly causing differences in the release of K^+ and Na^+ to the cytosol.

In Part 3, as a first step towards structure elucidation by X-ray diffraction crystallography, production of Trk proteins from *S. cerevisiae* and parts of these proteins was attempted using different expression systems (*E. coli*, *S. cerevisiae* and *P. pastoris*). Attempts to overexpress genes encoding full-length Trks were carried out using all three systems. Only *E. coli* was used as host for the production of cytosolic parts of Trk1 (cytosolic amino-terminal part, "N-tail", carboxy-terminal part "C-tail" and LHL) and transmembrane domains (A and D). After optimization of expression conditions, membrane purification and solubilisation of membrane proteins, the isolation of full-length Trk1 in *S. cerevisiae* and *P. pastoris* was accomplished. However, amounts were too small for crystallization experiments.

Production of the cytosolic amino- and carboxy-terminal tails (N- and C-tail) was achieved in *E. coli* and the C-tail was successfully crystallized. However, its structure could not be solved, most likely because it is too flexible. Trk1(LHL) could not be produced in 4 different *E. coli* expression strains due to its toxicity for the bacterial cells. Also, the attempts to produce single MPM domains in *E. coli* failed. However, single domains A and D were successfully produced in *S. cerevisiae*, though also not yet in amounts allowing purification and crystallization.

Taken together, the results obtained confirmed that the solution of the structure of membrane proteins is a challenging task. The whole

procedure is laborious and usually results are obtained based on numerous trial and error experiments. As first step towards a crystal structure of a yeast Trk, production of ScTrk and PpTrk in *P. pastoris* and *S. cerevisiae* has been achieved. Future studies should focus on improving protein yield and purification from *P. pastoris*. To conclude, the structure and function of the K^+ channels are well known. Nevertheless, for fungal Trk proteins, due to the lack of crystal structure information, the exact mechanism for the ion selectivity remains unknown. The results obtained in this study, showed that LHL might play a regulatory role in the functioning of Trk1.

Bibliography

Agre P. The Aquaporin Water Channels. *Proc Am Thorac Soc* 2006;**3**:5–13.

Ahmed A, Sesti F, Ilan N *et al.* A molecular target for viral killer toxin: TOK1 potassium channels. *Cell*1999;**99**:283–91.

Alberts B, Johnson A, & Lewis J. Molecular biology of the cell; **4th** ed., Garland Science, New York 2002; pp712

Ambesi A, Miranda M, Petrov VV. *et al.* Biogenesis and function of the yeast plasma-membrane H^+-ATPase. *J Exp Biol* 2000;**203**:155–60.

Anderson JA, Best LA, Gaber RF. Structural and functional conservation between the high affinity K^+ transporters of *S. uvarum* and *S. cerevisiae*. *Gene* 1991;**99**:39–46.

Arechaga I, Miroux B, Karrasch S *et al.* Characterisation of new intracellular membranes in *Escherichia coli* accompanying large scale over-production of the b subunit of F(1)F(o) ATP synthase. *FEBS Lett* 2000;**482**:215–9.

Ariño J, Ramos J, Sychrova H. Alkali metal cation transport and homeostasis in yeasts. *Microbiol Mol Biol Rev* 2010;**74**:95–120.

Ariño J, Ramos J, Sychrova H. Monovalent cation transporters at the plasma membrane in yeasts. *Yeast* 2019;**36**:177–93.

Armstrong CM. Voltage-gated K channels. *Sci Signal* 2003;**2003**:re10–re10.

Baev D, Rivetta A, Li XS *et al.* Killing of *Candida albicans* by human salivary histatin 5 is modulated, but not determined, by the K^+ channel TOK1. *Inf Immun* 2003;**71**: 3251–60.

Barreto L, Canadell D, Valverde-Saubí D *et al.* The short-term response of yeast to potassium starvation. *Environ Microbiol* 2012;**14**:3026–42.

Benito B, Garciadeblás B, Schreier P *et al.* Novel P-Type ATPases mediate high-affinity potassium or sodium uptake in fungi. *Eukaryot Cell* 2004;**3**:359–68.

Berg JM, Tymoczko JL *et al*. *Biochemistry*; **5th** ed., WH Freeman, NewYork 2002; pp2396

Bertani G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli. J Bacteriol* 1951;62:293–300.

Bertl A, Ramos J, Ludwig J *et al.* Characterization of potassium transport in wild-type and isogenic yeast strains carrying all combinations of trk1, trk2 and tok1 null mutations. *Mol Microbiol* 2003;**47**:767–80.

Bertl A, Slayman C, Gradmann D. Gating and conductance in an outward-rectifying K^+ channel from the plasma membrane of *S. cerevisiae*. *J Membr Biol* 1993;**132**:183–99.

Bihler H, Slayman CL, Bertl A. NSC1: a novel high-current inward rectifier for cations in the plasma membrane of Saccharomyces cerevisiae. *FEBS Lett* 1998;**432**:59–64.

Bossemeyer D, Borchard A, Dosch DC *et al.* K^+-transport protein TrkA of *Escherichia coli* is a peripheral membrane protein that requires other trk gene products for attachment to the cytoplasmic membrane. *J Biol Chem* 1989;**264**:16403–10.

Breinig F, Tipper DJ, Schmitt MJ. Kre1p, the plasma membrane receptor for the yeast K1 viral toxin. *Cell* 2002;**108**:395–405.

Cagnac O, Leterrier M, Yeager M *et al.* Identification and Characterization of Vnx1p, a Novel Type of Vacuolar Monovalent Cation/H^+ Antiporter of *Saccharomyces cerevisiae*. *J Biol Chem* 2007;**282**:24284–93.

Cao Y, Jin X, Huang H *et al.* Crystal structure of a potassium ion transporter, TrkH. *Nature* 2011;**471**:336–40.

Crosslinking Technical Handbook. 2012:1–53.

Cubillos-Rojas M, Amair-Pinedo F, Tato I *et al.* Simultaneous electrophoretic analysis of proteins of very high and low molecular mass using Tris-acetate polyacrylamide gels. *Electrophoresis* 2010;**31**:1318–21.

Cubillos-Rojas M, Schneider T, Sánchez-Tena S *et al.* Tris-acetate polyacrylamide gradient gel electrophoresis for the analysis of protein oligomerization. *Anal Bioanal Chem* 2016;**408**:1715–9.

Cyert MS, Philpott CC. Regulation of Cation Balance in *Saccharomyces cerevisiae*. *Genetics* 2013;**193**:677–713.

Doyle DA, Morais-Cabral J, Pfuetzner RA *et al.* The Structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science* 1998;**280**:69–77.

Drew D, Newstead S, Sonoda Y *et al.* GFP-based optimization scheme for the overexpression and purification of eukaryotic membrane proteins in *Saccharomyces cerevisiae*. *Nat Protoc* 2008;**3**:784–98.

Dulbecco R, Vogt M. Plaque formation and isolation of pure ines with Poliomyelitis virus. *J Exp Med* 1954;**99**:167–82.

Durell SR, Guy HR. Structural Models of the KtrB, TrkH, and Trk1,2 symporters based on the structure of the KcsA K^+ channel. *Biophys J* 1999;**77**:789–807.

Durell SR, Hao Y, Nakamura T *et al.* Evolutionary relationship between K^+ channels and symporters. *Biophys J* 1999;**77**:775–88.

Evans, H.J. and Wildes, R.A. Potassium and its role in enzyme activation: Potassium in Biochemistry and Physiology, International Potash Institute, Berne 1971;13-19.

Gaber RF, Styles CA, Fink GR. TRK1 encodes a plasma membrane protein required for high-affinity potassium transport in *S. cerevisiae*. *Mol Cell Biol* 1988;**8**:2848–59.

Gasteiger E, Hoogland C, Gattiker A *et al.* Protein Identification and Analysis Tools on the ExPASy Server. *The Proteomics Protocols Handbook*. Vol 112. Totowa, NJ: Humana Press, 2005, 571–607.

Gietz RD, Schiestl RH. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat Protoc* 2007a;**2**:31–4.

Gietz RD, Schiestl RH. Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat Protoc* 2007b;**2**:35–7.

Goffeau A, Barrell BG, Bussey H *et al.* Life with 6000 Genes. *Science* (1996);**274**:546–67.

Gouaux E. Principles of selective ion transport in channels and pumps. *Science* 2005;**310**:1461–5.

Hames BD. *Gel Electrophoresis of Proteins: A Practical Approach*. **3rd** ed. Hames B. Oxford University Press, 1998; pp373.

Haro R, Garciadeblas B, Rodriguez-Navarro A. A novel P-type ATPase from yeast

involved in sodium transport. *FEBS Lett* 1991;**291**:189–91.
Haro R, Sainz L, Rubio F *et al.* Cloning of two genes encoding potassium transporters in Neurospora crassa and expression of the corresponding cDNAs in *Saccharomyces cerevisiae*. *Mol Microbiol* 1999;**31**:511–20.
Hille B. Ion Channels of excitable membranes, **3rd** ed. Sinauer Associates 2001; pp37
Ho SN, Hunt HD, Horton RM *et al.* Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 1989;**77**:51–9.
Inoue H, Nojima H, Okayama H. High efficiency transformation of *Escherichia coli* with plasmids. *Gene* 1990;**96**:23–8.
Jiang Y, Lee A, Chen J *et al.* Crystal structure and mechanism of a calcium-gated potassium channel. *Nature* 2002;**417**:515–22.
Junge F, Schneider B, Reckel S *et al.* Large-scale production of functional membrane proteins. *Cell Mol Life Sci* 2008;**65**:1729–55.
Kabsch W. XDS. *Acta Crystallogr Sect D Biol Crystallogr* 2010;**66**:125–32.
Kale D, Spurny P, Shamayeva K *et al.* The *S. cerevisiae* cation translocation protein Trk1 is functional without its "long hydrophilic loop" but LHL regulates cation translocation activity and selectivity. *Biochim Biophys acta Biomembr* 2019;**1861**:1476–88.
Karginov A, Agaphonov M. A simple enrichment procedure improves detection of membrane proteins by immunoblotting. *Biotechniques* 2016;**61**:260–1.
Ketchum KA, Joiner WJ, Sellers AJ *et al.* A new family of outwardly rectifying potassium channel proteins with two pore domains in tandem. *Nature* 1995;**376**:690–5.
Knaus H-G, Eberhart A, Koch ROA *et al.* Characterization of tissue-expressed α subunits of the high conductance Ca^{2+} -activated K^+ channel. *J Biol Chem* 1995;**270**:22434–9.
Ko CH, Buckley AM, Gaber RF. TRK2 is required for low affinity K^+ transport in *Saccharomyces cerevisiae*. *Genetics* 1990;**125**:305–12.
Ko CH, Gaber RF. TRK1 and TRK2 encode structurally related K^+ transporters in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1991;**11**:4266–73.
Kuroda T, Bihler H, Bashi E *et al.* Chloride channel function in the yeast TRK-potassium transporters. *J Membr Biol* 2004;**198**:177–92.
Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–685.
Leigh RA, Wyn Jones RG. A Hypothesis relating critical potassium concentrations for growth to the distribution and functions of this ion in the plant cell. *New Phytol* 1984;**97**:1–13.
Lockless SW, Zhou M, MacKinnon R. Structural and thermodynamic properties of selective iion binding in a K^+ channel. Aldrich RW (ed.). *PLoS Biol* 2007;**5**:e121.
Lodish H *et al*. Molecular Cell Biology **5th** Edition, Harvey; Berk, Arnold; Matsudaira, Paul; Kaiser, Chri published by W. H. Freeman ISBN-13: 978-0716743668 2003; pp961.
Loukin SH, Lin J, Athar U *et al.* The carboxyl tail forms a discrete functional domain that blocks closure of the yeast K^+ channel. *Proc Natl Acad Sci* 2002;**99**:1926–30.

Lubin M, Ennis HL. On the role of intracellular potassium in protein synthesis. *Biochim Biophys Acta - Spec Sect Nucleic Acids Relat Subj* 1964;**80**:614–31.
MacKinnon R. Potassium channels. *FEBS Lett* 2003;**555**:62–5.
Madrid R, Gómez MJ, Ramos J *et al.* Ectopic potassium uptake in trk1 trk2 mutants of *S. cerevisiae* correlates with a highly hyperpolarized membrane potential. *J Biol Chem* 1998;**273**:14838–44.
Maresova L, Urbankova E, Gaskova D *et al.* Measurements of plasma membrane potential changes in *Saccharomyces cerevisiae* cells reveal the importance of the Tok1 channel in membrane potential maintenance. *FEMS Yeast Res* 2006;**6**:1039–46.
Mathieu K, Javed W, Vallet S *et al.* Functionality of membrane proteins overexpressed and purified from *E. coli* is highly dependent upon the strain. *Sci Rep* 2019;**9**:2654.
Mendoza I, Rubio F*et al.* The protein phosphatase calcineurin is essential for NaCl tolerance of Saccharomyces cerevisiae. *J Biol Chem* 1994;**269**:8792–6.
Mohanty AK, Wiener MC. Membrane protein expression and production: effects of polyhistidine tag length and position. *Protein Expr Purif* 2004;**33**:311–25.
Murray RK, Granner DK, Mayes PA & Rodwell VW. Harper's biochemistry- **24th** edition. Appleton & Lange, Stamford 1996, pp 868.
Nakamura T, Yuda R, Unemoto T *et al.* KtrAB, a New Type of Bacterial K(+) - uptake system from *Vibrio alginolyticus*. *J Bacteriol* 1998;**180**:3491–4.
Nass R, Cunningham KW, Rao R. Intracellular sequestration of sodium by a novel Na^+/H^+ exchanger in yeast is enhanced by mutations in the plasma membrane H+-ATPase. Insights into mechanisms of sodium tolerance. *J Biol Chem* 1997;**272**:26145–52.
Novy R, Drott D, Yaeger K *et al.* Overcoming the codon bais of *E. coli* for enhanced protein expression. *Innovations* 2001:1–3.
Nowikovsky K, Froschauer EM, Zsurka G *et al.* The LETM1 / YOL027 gene family encodes a factor of the mitochondrial K^+ homeostasis with a potential role in the Wolf-Hirschhorn Syndrome. *J Biol Chem* 2004;**279**:30307–15.
Page MJ, Di Cera E. Role of Na^+ and K^+ in enzyme function. *Physiol Rev* 2006;**86**:1049–92.
Petrezsélyová S, Ramos J, Sychrová H. Trk2 transporter is a relevant player in K^+ supply and plasma-membrane potential control in *S. cerevisiae*. *Folia Microbiol* 2011;**56**: 23–8.
PichiaPink™ Expression System. 2014:86.
Prior C, Potier S, Souciet J-L *et al.* Characterization of the NHA1 gene encoding a Na^+ /H^+ -antiporter of the yeast *Saccharomyces cerevisiae*. *FEBS Lett* 1996;**387**:89–93.
Ramírez J, Ramírez O, Saldaña C *et al.* A *Saccharomyces cerevisiae* mutant lacking a K^+/H^+ exchanger. *J Bacteriol* 1998;**180**:5860–5.
Ramos J, Alijo R, Haro R *et al.* TRK2 is not a low-affinity potassium transporter in Saccharomyces cerevisiae. *J Bacteriol* 1994;**176**:249–52.
Ramos J, Ariño J, Sychrová H. Alkali-metal-cation influx and efflux systems in nonconventional yeast species. *FEMS Microbiol Lett* 2011;**317**:1–8.
Reid JD, Lukas W, Shafaatian R *et al.* The *S. cerevisiae* outwardly-rectifying

potassium channel (DUK1) identifies a new family of channels with duplicated pore domains. *Receptors Channels* 1996;**4**:51–62.

Rivetta A, Kuroda T, Slayman C. Anion currents in yeast K^+ transporters (TRK) characterize a structural homologue of ligand-gated ion channels. *Pflügers Arch - Eur J Physiol* 2011;**462**:315–30.

Rivetta A, Slayman C, Kuroda T. Quantitative modeling of chloride conductance in yeast TRK potassium transporters. *Biophys J* 2005;**89**:2412–26.

Rodríguez-Navarro A. Potassium transport in fungi and plants. *Biochim Biophys Acta - Rev Biomembr* 2000;**1469**:1–30.

Rosano GL, Ceccarelli EA. Recombinant protein expression in *Escherichia coli*: advances and challenges. *Front Microbiol* 2014;**5**:1–17.

Schägger H. Tricine–SDS-PAGE. *Nat Protoc* 2006;**1**:16–22.

Schlesser A, Ulaszewski S, Ghislain M *et al.* A second transport ATPase gene in *Saccharomyces cerevisiae*. *J Biol Chem* 1988;**263**:19480–7.

Smith TF, Waterman MS. Identification of common molecular subsequences. *J Mol Biol* 1981;**147**:195–7.

Su H, Golldack D, Zhao C *et al.* The Expression of HAK-Type K^+ transporters is regulated in esponse to salinity stress in common ice plant. *Plant Physiol* 2002;**129**:1482–93.

Swulius MT, Jensen GJ. The Helical MreB cytoskeleton in *E. coli* MC1000/pLE7 is an artifact of the N-terminal yellow fluorescent protein tag. *J Bacteriol* 2012;**194**:6382–6.

Tholema N, Brüggen MV der, Mäser P *et al.* All four putative selectivity filter glycine residues in KtrB are essential for high affinity and selective K^+ uptake by the KtrAB system from *Vibrio alginolyticus*. *J Biol Chem* 2005;**280**:41146–54.

Vieira-Pires R, Szollosi A, Morais-Cabral J. The structure of the KtrAB potassium transporter. *Biophys J* 2013;**104**:23a.

Villalobo A, Boutry M, Goffeau A. Electrogenic proton translocation coupled to ATP hydrolysis by the plasma membrane Mg^{2+}-dependent ATPase of yeast in reconstituted proteoliposomes. *J Biol Chem* 1981;**256**:12081–7.

Winn MD, Ballard CC, Cowtan KD *et al.* Overview of the CCP4 suite and current developments. *Acta Crystallogr Sect D Biol Crystallogr* 2011;**67**:235–42.

Yaffe MP, Schatz G. Two nuclear mutations that block mitochondrial protein import in yeast. *Proc Natl Acad Sci* 1984;**81**:4819–23.

Zahrádka J, Sychrová H. Plasma-membrane hyperpolarization diminishes the cation efflux via Nha1 antiporter and Ena ATPase under potassium-limiting conditions. *FEMS Yeast Res* 2012;**12**:439–46.

Zayats V, Stockner T, Pandey SK *et al.* A refined atomic scale model of the *Saccharomyces cerevisiae* K^+-translocation protein Trk1p combined with experimental evidence confirms the role of selectivity filter glycines and other key residues. *Biochim Biophys Acta - Biomembr* 2015;**1848**:1183–95.

e-mail address: kale@nh.cas.cz, deepikakale19@gmail.com

Studies to determine the structure and function of Trk - K^+ translocating system
Ph.D. Thesis Series, 2020, No. 6

Printed in the Czech Republic by Typodesign
Edition of 20 copies

University of South Bohemia in České Budějovice
Faculty of Science
Branišovská 1760
CZ-37005 České Budějovice, Czech Republic

Phone: +420 387 776 201
www.prf.jcu.cz, e-mail: sekret-fpr@prf.jcu.cz