Review on the Ph.D. Thesis of Ms. Deepika Uttam Kale, entritled "Studies to determine the structure and function of Trk - K^+ translocating system"

Presented at the University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic

The presented Thesis focuses on the analysis at the functional and structural level of the Trk potassium translocation proteins present at the plasma membrane in two different yeast, *S. cerevisiae* (Sc), an organism widely used as research model and with immense biotechnological applications, and *Pichia pastoris* (Pp, now renamed *Komagataella pastoris*), extensively exploited for heterologous protein expression.

The work appears organized in three parts. In the first part, the author investigates a characteristic structural feature of ScTrk1, the so call "Long Hydrophilic Loop" or LHL. On the basis of the comparison of cells expressing the Trk1 protein devoid of this LHL portion with cell expressing the entire protein, in terms of growth in K^+-deficient medium and uptake of K^+ and Na^+ cations, the authors extract the conclusion that, although it is dispensable for transport, influences activity and selectivity. These experiments have been already published in the BBA – Biomembranes journal.

In the second section, a comparative functional analysis of Trk1 from Sc and Trk from Pp (PpTrk, the single gene in the Pichia genome) is carried out. The author found that although PpTrk complements the growth defect of a Sc *trk1,2 tok*1Δ strain in low [K^+] medium this strain, in contrast to the equivalent strain expressing ScTrk1, was very sensitive to high [Na+]. From this, and from the fact that the PpTrk-expressing strain accumulates high amounts of Na+ (when NaCl is added to the medium), the author hypothesize that PpTrk has a higher selectivity for Na^+. It is postulated that a substitution of a Gly in SFA of ScTrk by Ala in PpTrk could lead to an increase in the diameter of the selectivity filter and to promotion of Na^+ influx into the cytosol.

In the third part, the production of full-length or part of Trk proteins is attempted in three different expression systems: one bacterial (*E. coli*) and two eukaryotic (*S. cerevisiae* and *P. pastoris*). Indeed, expression of such a long membrane protein in amounts suitable for X-ray crystallography is not a trivial issue. The attempts to express in the full-length protein in *E. coli* were unsuccessful. The protein could be expressed up to some extent in Sc and in Pp but was detected only by immunoblot (it was a GFP-fusion protein) and scaling-up was (very reasonably) not attempted. In contrast, the cytosolic amino- and carboxy-terminal tails (N and C-tail) was achieved in *E. coli* and the C-tail was even successfully crystallized. Attempt to solve the structure were made but details are not provided.

There are some points of criticism that have to be mentioned:

1.- In several parts the document is not concisely and carefully written. There are some sentences that are ill-constructed or obscure. An example in p. 4 "Membrane transport proteins are proteins that are involved in the movement of ions, molecules such as proteins across biological membrane." Please, also note that quite a few figures, mostly in the Introduction section, are unnecessarily small, in a way that precludes their understanding. To me, the most evident case is Fig. 2.

2.- Concerning the second section, I do believe that before publication of this part the experiment mutating the candidates Ala/Gly residues must be done. Additionally this yet part lacks a proper discussion, which has to be added before publication.

3.- The last part, (general) Discussion and Conclusions, is surprisingly short to be a Discussion and surprisingly unstructured to work as "Conclusions". There is little of discussion here, mostly repetition of results.

As a conclusion, this Thesis presents a considerable amount of work that significantly increases our knowledge about the structure and function of fungal Trk potassium transport systems. The introduction is complete and well-focused. In contrast, the discussion is abnormally short (see below). Indisputably, during the development of this work a large diversity of techniques has been used, thus resulting in excellent training of the candidate.

To me, this work meets the international standards for the obtention of the Ph.D. degree and I recommend its acceptance.

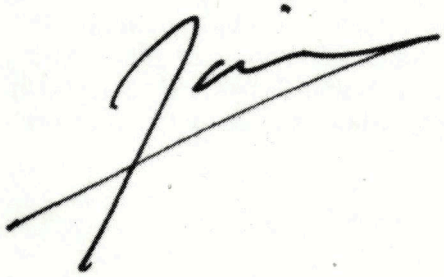
Questions for the discussion:

(i) In the manuscript draft in part 2 it is shown that PpTrk is less selective for K^+ over Na^+ than S.c. Trk1. Could the candidate speculate about possible physiological reasons for that?

(ii) Could K^+ uptake systems in fungi like Trks or Acu1 be possible targets in the treatment of diseases caused by pathogenic yeast?

(iii) Assuming that crystals of the C-tail become sufficient for X-ray diffraction and 3D-structure determination, up to what extend do the candidate considers this information will contribute to the understanding of Trk transport mechanism?

Campus de Bellaterra, 24-02-2020



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PhD thesis Report

This report is based on the Ph.D. of Ms. Deepika Uttam Kale, entitled "Studies to determine the structure and function of Trk - K+ translocating system".

This thesis resulted in 1 one publication in a Q1 Journal. In addition, one manuscript is in preparation.

The general aim of the Ph.D. Thesis was to advance understanding on the structure and function of the plasma membrane potassium-specific uptake systems Trk1 and Trk2, in yeast. This is a relevant area of research, considering that these systems are essential for the maintenance of cell homeostasis, in specific conditions.

The thesis is well organized, the methodology described is adequate for the proposed goals and the conclusions drawn from the results are, in general, well founded. The thesis reports interesting and novel observations.

The Introduction Section consists of a literature review and it contains the most recent advances in the field. The part regarding regulation of Trk proteins is vague and could have been further developed. Still, a good and useful work of literature analysis was carried out.

The experimental component of the thesis is presented in 3 parts, each addressing a specific scientific problem. Part 1 makes novel contributions to our understanding of the role of the "long hydrophilic loop" for the expression, activity and specificity of Sc Trk1. Part2 details the characterization of the *Pichia pastoris* PpTrk protein and identifies critical residues potentially involved in selectivity. Part 3 describes the efforts towards the production of single and truncated versions of Trk proteins, in different expression systems. This part represents a large body of work and it is a solid base for further studies aimed at determining the crystal structure of these relevant plasma membrane proteins.

In the final Chapter, the general conclusions of the work are summarized but the discussion and future perspectives are limited.

Overall, this thesis reports an interesting investigation and it meets the international standards for the obtention of the Ph.D. degree. I recommend its acceptance.

Questions for the discussion

General Questions:

a) Please integrate the latest advances in the understanding of potassium homeostasis and plasma membrane nutrients (sugars, aminoacids, carboxylates) transporters' function and regulation. For example, how do TrK transporters operate under distinct metabolic constraints (e.g. carbon or nitrogen limitation or starvation) that also impact the expression of other nutrients transporters dependent on the ion potential?

b) Part 3A: Although the fluorescence microscopy images in Fig 6 are blurry, they are consistent with correct localization of most of the fusion proteins. The gels shown on figures 7,8 and 9 show successful production of GFP/Trk1. Yet, the system was abandoned after the fusion protein failed to bind or elute from the Nickel column used in Fig 11. The problem seems more likely to have been related to the purification methodology rather than the expression system. Perhaps a more sustained effort here would have been more worthwhile than the investment in the Pichia system which ultimately produced truncated proteins (Fig 14). Please comment on this and discuss what other variables could have been tested or optimized. Include discussion on buffers, other tags and successful examples of plasma membrane proteins expression and purification.

c) Detail how your work can impact our understanding of the architecture of substrate binding sites and the gating mechanism underlying ion transport in higher eukaryotes.

Specific question:

The pYEX vector was engineered for a very high copy number (Fig. 5 page 114) that can lead to a traffic jam in the secretory pathway and ER retention due to too high overexpression. Please, comment and present the reasoning behind this choice of plasmid instead of a lower copy number vector.

Sincerely,

Sandra Paiva

Sandra Paiva
Assistant Professor
Vice-Director of the Centre for Molecular and Environmental Biology (CBMA), University of Minho