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Heterologous expression of the mammalian nucleobase transporter rSNBT1 in the LEXSY *Leishmania tarentolae* protein expression system for functional and structural characterization

HELLENIC PASTEUR INSTITUTE

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Introduction

The parasites of the genus ***Leishmania*** are protozoan organisms of the *Trypanosomatidae* family. When transmitted to the mammal host by the bite of the insect host (sandflies of the genus *Phlebotomus* or *Lutzomyia*) cause a wide spectrum of diseases called Leishmaniases.

Leishmania tarentolae is a lizard-infecting species belonging to the genus Sauroleishmania (Lainson and Shaw, 1987, Rev Soc Bras Med Trop. Sep;22(3):125-30.), which has not been considered pathogenic to humans (***Fig.1***).

The ***L. tarentolae*** system (Reinhard Breitling et. al 2002, Protein Expr. Purif., 25(2):209-18) is cultured at biosafety S1 conditions. It requires simple nutrients for growth and can be grown in large volume cultures, it has a cell cycle of 4-6 hrs, allows efficient N-glycosylation of mammalian proteins (using a mammalian type glycan pattern, absent in yeast or *Drosophila)* and displays physical auxotrophy on several amino acids, which facilitates potential protein structural studies.

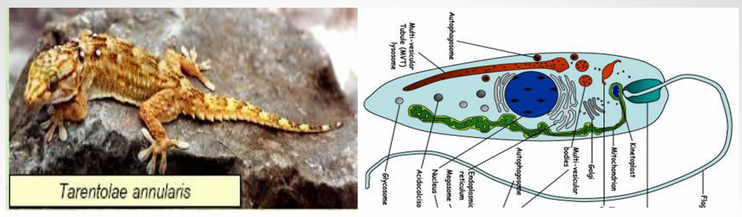

Fig.1 *Photo of the lizard Tarentolae annularis from which the parasite* ***L. tarentolae*** *was initialy isolated (Left). Schematic representation of a Leishmania parasite morphology (Right)*

We used the ***L. tarentolae*** organism to express the **rat rSNBT1 nucleobase transporter** (Yamamoto S. et al 2009. *J Biol Chem* 285(9):6522-31). This transporter belongs to the **NAT/NCS2 family of nucleobase transporters**, an evolutionarily conserved and phylogenetically widespread family with a single known x-ray structure from a distantly related bacterial homologue (UraA) and with a limited number of studied homologues.

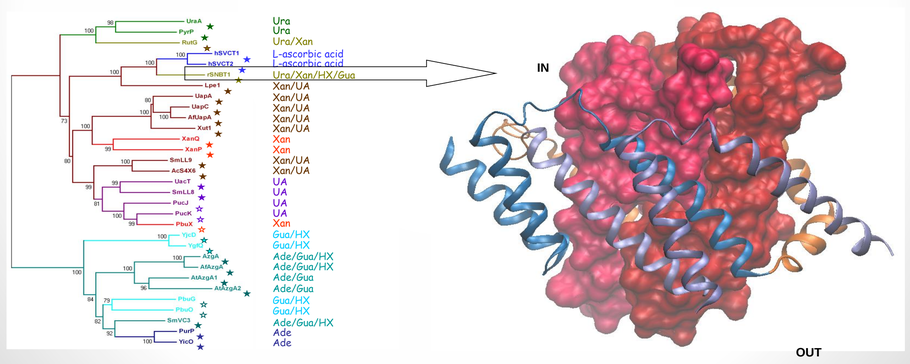

Fig. 2 *Phylogeny of the NAT/NCS2 family (constructed with program MEGA) and structure model of the rSNBT1 transporter based on the homology with UraA (3QE7) ; helices of the core domain are shown in spacefilling representation (TM 1-3 and 8-10) (red) and of the gate domain in ribbons (blue).*

Aim of this project is to examine the potential of the LEXSY *Leishamania tarentolae* parasites to heterologously express human NAT homologues for structural and functional studies.

For this, the rSNBT1 transporter gene was cloned into the *Leishmania* specific pLexsy-sat expression vector at the 5' end of the mRFP coding gene. *L. tarentolae* parasites were transfected with the pLexsy-rSNBT1-mRFP plasmid and transgenic parasites harboring the plasmid were selected by antibiotic treatment. The growth characteristics of the transgenic population were compared to the wild type strain and the plasma membrane localization of the rSNBT1-mRFP was evaluated in transgenic parasites by confocal microscopy. Finally the expression of the correct size hybrid proteins was confirmed by Western Blot using an anti-mRFP antibody. We currently assess the functional properties of the rSNBT1 as nucleobase transporter in live *L.tarentolae*-rSNBT1-mRFP parasites.

Methodology-Results

1 Cloning of *rSNBT1* from Rattus norvegicus (AB511909.1) in pLexsy-sat-mRFP plasmid

The rSNBT1 gene was amplified by PCR from *Rattus norvegicus* genomic *DNA* (***Fig. 3***) using primers specific for the beginning and end of the ORF.

Subsequently the rSNBT1 PCR product was inserted into the pLexsy-sat-mRFP vector which is specific for expression in protozoan parasites of *Trypanosomatidae* family. Insertion was confirmed after digestion of the PCR product and vectors with the suitable restriction enzymes and ligation of the digested DNA fragments. (***Fig. 4***)

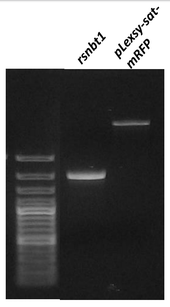

Fig. 3 *PCR-mobilized rsnbt1 and pLexsy-sat-mRFP digested with BglII. They have migrated at the expected molecular weights, 1845 bp for rsnbt1, ~ 7000bp for pLexsy-sat-mRFP.*

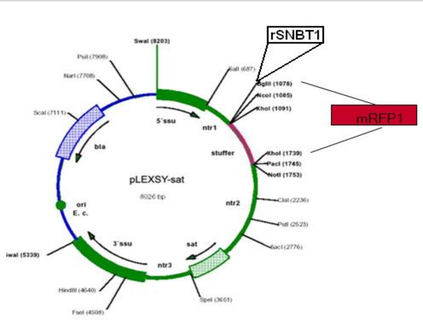

Fig. 4 *pLexsy-sat plasmid with the rSNBT1 and mRFP1 gene inserted in the BglII & BglII/XhoI sites*

2 Generation of *L. tarentolae* -rSNBT1- mRFP transgenic parasites

Transgenic parasites *L. tarentolae - rSNBT1 - mRFP* were generated by electroporation with the pLexsy-sat-rSNBT1-mRFP plasmid and incubation in selection medium consisting of BHI and the antibiotic nurseothricin (100mg/ml) at 25°C (***Fig. 5***).

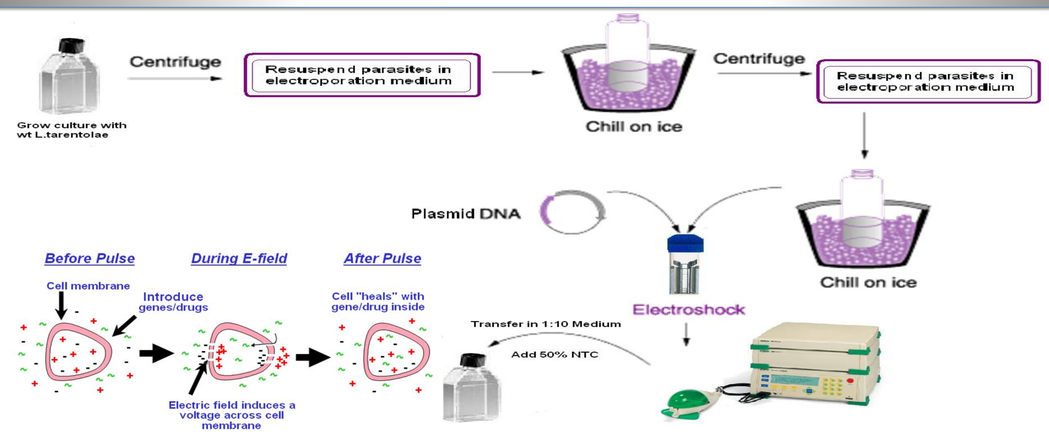

Fig. 5 *Schematic presentation of plasmid introduction in Lexsy L. tarentolae wt parasites* (http://www.biochem.arizona.edu)

3 Growth characteristics of the L. tarentolae -rSNBT1-mRFP transgenic parasites

Parasite cultures of both the transgenic and the wild type *L. tarentolae* strains were seeded at $1x10^6$ parasites/ml *and the number of parasites was counted using a Malassez hemocytometer and by measuring the* OD_{600} *every day for 8 days* (***Fig. 6***)

➔The *L.tarentolae-rSNBT1-mRFP* parasites seem to have similar growth characteristics to the wt Lexsy *L. tarentolae* strain.

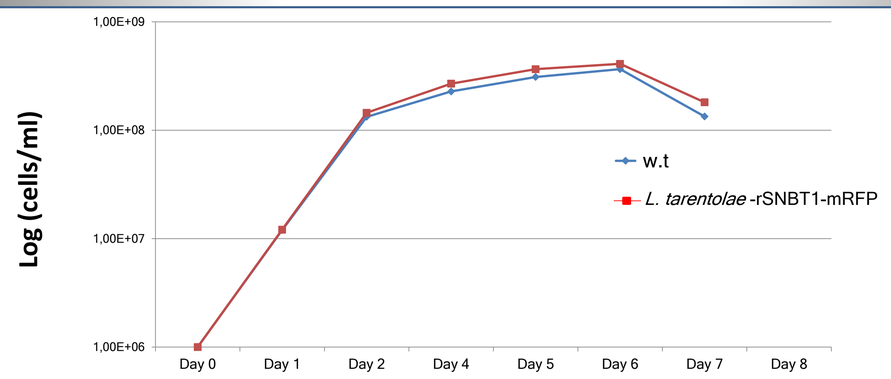

Fig. 6 *Growth curves of the L. tarentolae-rSNBT1-mRFP transgenics and the Lexsy L. tarentolae wt parasites*

4 Expression of the rSNBT1 transporter tagged with mRFP in *L. tarentolae* transgenic cells

Microscopy analysis

The expression of rSNBT1-mRFP in the *L. tarentolae* - rSNBT1-mRFP transgenic parasites was verified by observation of the red fluorescence (mRFP) under a confocal fluorescence microscope (Fig. 7)

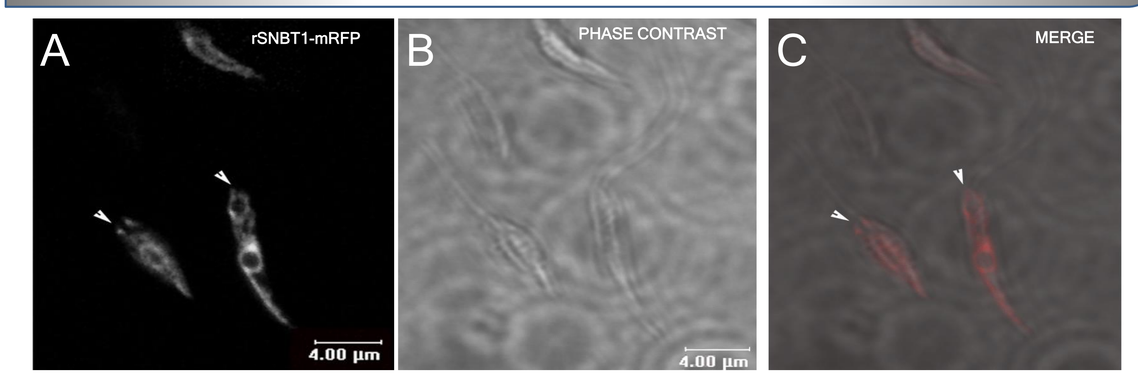

Fig. 7 *Transgenic parasites L. tarentolae-rSNBT1-mRFP. Images from a Leica TCS-SP confocal microscope. The arrows indicate the position of the flagelar pocket. The rSNBT1-mRFP shows perinuclear, ER-like and surface membrane localization. It also localises at the flagelar pocket, a deep invagination at the base of the flagellum that is responsible for uptake of larger nutrients via receptor-mediated endocytosis, for secretion of proteins into the extracellular medium, and for integration of membrane proteins into the cell surface.*

5 Expression of the rSNBT1 transporter tagged with mRFP in *L. tarentolae* transgenic cells Western Blot analysis

The rSNB1-mRFP was detected in Triton X-100 soluble and insoluble protein fractions from *L.tarentolae-rSNBT1-mRFP* parasites by Western Blot analysis. *The apparent MW of the specifically detected protein is close to the calculated MW of the hybrid* rSNB1-mRFP *(92,1KDa) (Fig. 8)*

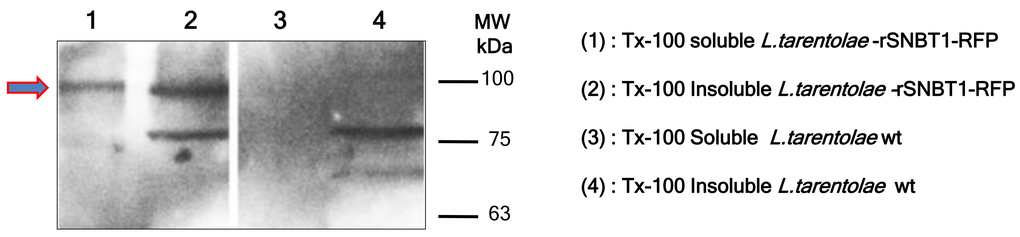

Fig. 8 *Western Blot of Triton X-100 soluble and insoluble parasite protein fractions separated side by side in a 10%(w/v) SDS-PAGE. The nitrocellulose membrane was probed with 0.5 µg/ml of the anti-mRFP Ab. The arrow indicates the position of the rSNB1-mRFP protein.*

CONCLUSIONS-FUTURE PERSPECTIVES

We have generated a transgenic *L. tarentolae-rSNBT1-mRFP* strain that expresses efficiently the rSNBT1 membrane transporter, tagged with mRFP1. The rSNBT1-mRFP is correctly targeted to the surface of the parasites proving that the *L. tarentolae* secretion system recognizes the surface membrane targeting signals of mammalian cells and seems to be subjected to post -translational modifications. *The L. tarentolae-rSNBT1-mRFP* strain has similar morphological and growth characteristics with the wt *L. tarentolae* strain and can be grown to large amounts. The transport characteristics of the transporter is in progress. [putative rSNBT1 substrates include: Uracil, Hypoxanthine, Xanthine, Guanine (see Fig. 2)]

Acknowledgment

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