MSc Thesis

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Construction and characterization of transgenic διαγονιδιακών *Leishmania tarentolae* strains for heterologous expression of the red fluorescent protein mCherry and of the nucleobase transporter rSNBT1 (from *Rattus norvegicus*)

SUBJECT AREA: Biotechnology, Protozoan parasite biology

KEYWORDS: *Leishmania tarentolae* LEXSY, recombinant protein expression, Fluorescent proteins, mCherry, Transmembrane proteins, Nucleobase transporter rSNBT1.

ABSTRACT

Aim of the present MSc thesis was to construct transgenic *Leishmania tarentolae* (*L. tarentolae*) strains expressing the red fluorescent monomeric protein mCherry from the organism *Discosoma sp*. or the nucleobase transporter rSNBT1 from *Rattus norvegicus* tagged with the red fluorescent protein mRFP1.

The *Leishmania tarentolae* organism (*L. tarentolae*) is a species of the protozoan parasite *Leishmania* from the Trypanosomatide family belonging to the *Sauroleishmania* genus. It was initially isolated from the lizard *Tarentolae annularis*. *L. tarentolae* is considered non- pathogenic to humans, unlike other types of *Leishmania* which when they are transferred to the mammalian host by the bite of the insect vector (sandflies of the genus *Phlebotomus* and *Lutzomyia*) are causing a wide range of diseases called leishmaniasis.

As *L. tarentolae* is non-pathogenic to humans, it can be cultured in S1 biosafety conditions and in large volumes at 25 °C. Furthermore, the protozoan *L. tarentolae* has a short cell cycle (~ 10 hours), allows efficient N-glycosylation of mammalian proteins and exhibits natural auxotrophy in several amino acids which allows incorporation into proteins of labeled amino acids with heavy metals, essential step for NMR structural studies. For all these reasons, *L. tarentolae* has been proposed as an alternative system for heterologous expression of proteins from lower and higher

eukaryotes in order to perform structural and functional studies and for biotechnological applications.

We used the *L. tarentolae* organism to express 1) the red fluorescent protein mCherry in order to study its utility as a biosensor in studies for pathogenic *Leishmania* species (e.g. *Leishmania donovani*, *Leishmania major* etc.) also use the transgenic parasites *L. tarentolae*-mCherry for antileismanial drug screening and 2) the nucleobase transmembrane transporter rSNBT1 which belongs to the NAT / NCS2 transporters' family in order to study its functional characteristics in an eukaryotic organism dividing faster and growing in less expensive medium than mammalian cell lines with the aim to isolate from these cells a sufficient amount of the transporter for crystallization and structural studies.

Initially, we perfromed cloning of the *mcherry* and *rsnbt1* genes in plasmid vectors specific for protein expression in protozoan cells from the *Trypanosomatidea* family (e.g. pLexsy-sat). These genes were amplified by PCR from plasmid DNA containing them and the PCR products obtained were inserted into digested with the appropriate restriction enzymes pLexsy-sat vector. Generation of the *L. tarentolae parrot II* transgenic strains was performed by electroporating the corresponding plasmids into the protozoan cells followed by selection with antibiotic pressure (Nourseothricin). Expression of the mCherry from transgenic cells was confirmed by fluorescence microscopy. The plasmid vector pLexsy-sat-*mcherry* constructed in this work will be further used as a cloning vector for expression of chimeric proteins with mCherry in cells of other *Leishmania* species in order to study their subcellular localization and functional properties and also use the transgenic parasites *L. tarentolae*-mCherry for antileismanial drug screening.

The gene coding for the rSNBT1 transporter was cloned into the pLesxy-sat-*mrfp1* expression vector at the 5' end and in frame with the gene coding for the monomeric mRFP1 fluorescent protein. Following electroporation, the *L. tarentolae* cells carrying the pLesxy-sat-*rsnbt1-mrfp1* plasmid, covalent closed circular for episomal expression or a 7800bp fragment of the linearized plasmid for chromosomal integration, were selected by culturing in the presence of the antibiotic Nourseothricin as mentioned above for the mCherry expressing transgenic populations. The growth characteristics of all the *L. tarentolae* transgenic populations were studied in comparison to wild type cells. Confirmation of the rSNBT1-mRFP1 expression and correct localization to the protozoan plasma membrane was followed by confocal

fluorescence microscopy. The correct size of the expressed rSNBT1-mRFP1 hybrid protein was confirmed by Western blotting using a specific anti-mRFP1 antibody in protein cell extracts enriched in membrane proteins.

Finally, the function of rSNBT1-mRFP1 as nucleobase transporter in the transgenic *L. tarentolae*-rSNBT1-mRFP1 cells was confirmed by an uptake assay using [^{3}H]-hypoxanthine as transport substrate and in competition of [^{3}H]-hypoxanthine uptake experiments with other nucleobases.