

Recombinant Protein Expression in *Leishmania tarentolae*

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Abstract A variety of recombinant protein expression systems have been developed for heterologous genes in both prokaryotic and eukaryotic systems such as bacteria, yeast, mammals, insects, transgenic animals, and plants. Recently *Leishmania tarentolae*, a trypanosomatid protozoan parasite of the white-spotted wall gecko (*Tarentola annularis*), has been suggested as candidate for heterologous genes expression. Trypanosomatidae are rich in glycoproteins, which can account for more than 10% of total protein; the oligosaccharide structures are similar to those of mammals with N-linked galactose, and fucose residues. To date several heterologous proteins have been expressed in *L. tarentolae* including both cytoplasmic enzymes and membrane receptors. Significant advances in the development of new strains and vectors, improved techniques, and the commercial availability of those tools coupled with a better understanding of the biology of *Leishmania* species will lead to value and power in commercial and research labs alike.

Keywords *Leishmania* · Heterologous protein production · Foreign gene expression

Introduction

The family Trypanosomatidae (*Euglenozoa*, *Kinetoplastida*), which includes genera *Leishmania*, is one of the

oldest groups of eukaryotes with a number of species that are extra- and intracellular parasites of humans and livestock causing a range of debilitating or fatal diseases [1]. They are notorious for finding unique solutions to general process of the eukaryotic cell as RNA editing, arrangement of genes in tandem arrays, polycistronic transcription followed by trans-splicing and regulation of gene expression exclusively at the post-transcriptional level [2]. Due to its public health significance, the Trypanosomatidae group is one of the best-studied eukaryotic groups after yeast. *Leishmania* are protozoan parasites with a complex life cycle, involving several developmental forms. These forms represent an adaptation to the changing environmental conditions encountered by the parasites within their two hosts: the mammalian host, to which they are pathogenic, and the sandfly insect vector. In the sandfly, *Leishmania* replicate as extracellular and actively motile flagellated cells known as promastigotes, which reside primarily in the insect's alimentary tract. Two main forms can be distinguished: multiplicative, but not mammalian-infective, procyclic promastigotes that are present in the insect's midgut; non-dividing, but mammalian-infective, metacyclic promastigotes in the thoracic midgut and proboscis of the sandfly. The metacyclic promastigotes, when inoculated into a mammalian host through a sandfly bite, differentiate (after being phagocytosed by a macrophage) into the intracellular aflagellate amastigote form. This form of the parasite resides within a vacuole with lysosomal features that is termed the parasitophorous vacuole [3]. The development of methods of trypanosomatid cultivation in vitro and genetic manipulation has allowed the dissection of the mechanism of non-conventional gene expression [4]. In this review, the basic aspects of the *Leishmania tarentolae* isolated from the Moorish gecko *Tarentola mauritanica* as expression system are highlighted. Further

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information on the *Leishmania* can be found in the numerous reviews describing the trypanosomatid's biology [5–7] and on Jena Bioscience web site (<http://www.jenabioscience.com>).

Secretion of Heterologous Proteins

With *L. tarentolae*, heterologous proteins can either be expressed intracellularly or secreted into the medium. Because *L. tarentolae* secretes only low levels of endogenous proteins and because its culture medium contains few added proteins, a secreted heterologous protein comprises the vast majority of the total proteins in the medium [8]. Thus, secretion serves as a major first step in purification, separating the foreign protein from the bulk of cellular proteins. Secretion requires the presence of a signal sequence fused to foreign protein, *L. mexicana* secreted acid phosphatase and *Staphylococcus aureus* phage 42D staphylokinase [4] have been utilized to allow recombinant protein export in the media. Moreover, wild type secretion signal sequence has been used successfully for human erythropoietin secretion [9].

Expression Vectors

Genes transcription in *Leishmania* host can be driven by endogeneous RNA polymerase I or II [3] or heterologous RNA polymerase such as T7 or T3 [10]. Moreover, inducible expression can be efficiently realized by tetracycline repressor [11–13]. Interestingly gene regulation, in Trypanosomatidae, occurs predominantly after transcription through intergenic untranslated regions (UTRs) [3] (Fig. 1). Therefore, choice of suitable UTRs is crucial for construction of an efficient expression cassette suitable for the large-scale recombinant proteins production. Breitling et al. [9] reported an UTRs that mediate green fluorescent protein high-level expression. These UTRs are mainly composed by *L. tarentolae* calmodulin cluster, containing three tandemly arranged regions and splice acceptor sites. The 5' UTR of the multi cloning site is a 0.4 kb fragment with the *camA* splices acceptor whereas its 3' UTR consisted of the entire 1.8 kb *camBA* intergenic region fused to an antibiotic resistance gene. The 3' terminus of the antibiotic resistance gene has been fused to the *L. major* DNA of the dihydrofolate reductase-thymidylate synthase locus (1.7 k-IR). Finally, the expression cassette is flanked by two fragments of the small subunit rRNA (5' ssu and 3' ssu) locus for double homologous recombination. In fact, small ribosomal subunit RNA gene is strongly transcribed by RNA polymerase I (Fig. 1) [14]. Integration of the expression cassette is achieved by homologous recombination coupled to drug

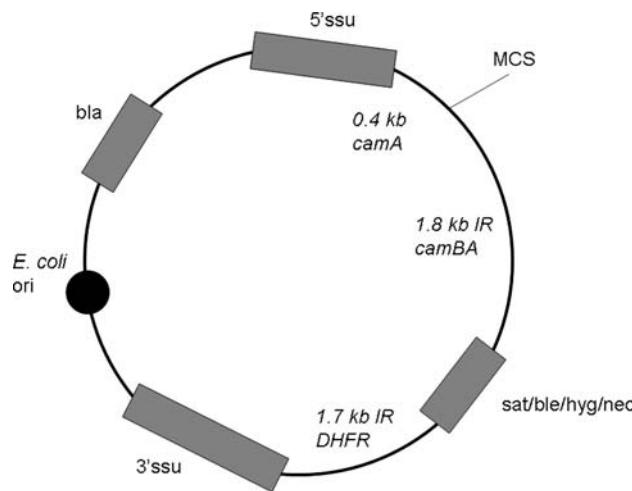


Fig. 1 *Leishmania* expression vector map. Abbreviations: *bla* β -lactamase, 5' and 3' ssu 5' and 3' of the small subunit of *L. tarentolae* rRNA gene, 0.4 kb *camA* fragment with the *L. tarentolae* calmodulin A gene splice acceptor, MCS multiple cloning site, 1.8 kb IR *camBA* a portion of the intergenic region between *L. tarentolae* calmodulin A and B genes, *sat/ble/hyg/neo* nourseothricine, bleomycin, hygromycin, or neomycin resistance genes, 1.7 kb IR *DHFR* 3' untranslated region of the *L. major* dihydrofolate reductase gene

selection [15]. Interestingly, reduction of homologous sequences length below 1 kb, linearly decreases the targeting frequency with no homologous recombination occurring when the targeting regions are below 180 bp [16]. As expected, recombination is strongly affected by base pair mismatches and chromosomal locations [17, 18]. Overall, gene targeting mediated by homologous recombination in *Leishmania* shows similarities to that of yeast and mammals [19, 20]. Trypanosomatids can be efficiently transformed by electroporation of in vitro cultivated promastigotes [21], moreover, transformants selection is easily performed using antibiotic resistance genes, such as streptothricin acetyltransferase (sat), phleomycin–bleomycin binding protein (ble), hygromycin phosphotransferase (hyg), or aminoglycoside phosphotransferase (neo), that confer resistance to nourseothricin, bleomycin, hygromycin, and neomycin, respectively.

Posttranslational Modifications

Trypanosomatidae are rich in glycoproteins which can account for more than 10% of total protein [22]. Probably due to their parasitic lifestyle, the oligosaccharide structures of their glycoproteins are often similar to those of mammals that include complex-type oligosaccharides with α -linked galactose and fucose residues (Fig. 2) [23]. N-glycosylation pathway of *L. tarentolae* is able to produce higher-eukaryote-like biantennary N-glycans, where only the sialylation of the glycans is missing. However, efficient

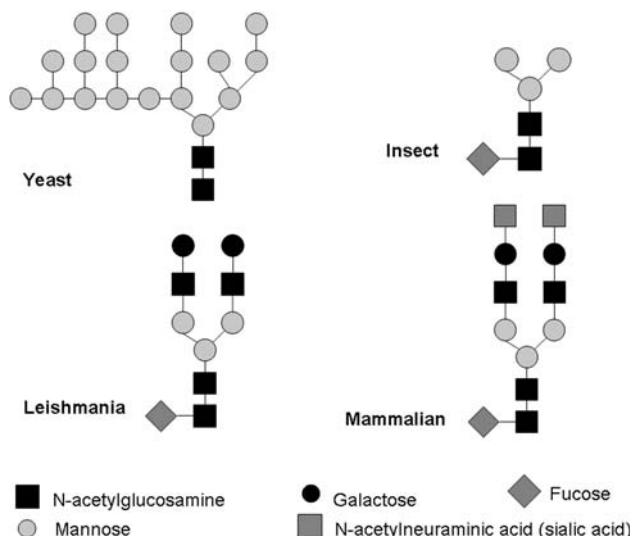


Fig. 2 Glycoproteins expressed in *L. tarentolae* are similar to those of mammalian with complex type oligosaccharides

in vitro sialylation procedures have been developed and are available; moreover *Trypanosoma cruzi* trans-sialidase can be expressed in *Leishmania* cells as active enzyme [24]. Another potentially beneficial feature of N-glycans produced by *L. tarentolae* is their homogeneity, this is particularly important when recombinant proteins are need for structural studies and for pharmaceutical purpose. Lack of tri- and tetrantennary glycans, probably due to absence of *Leishmania* N-acetylglucosaminyl transferase IV-activity, lead to absence of higher branched N-glycans on expressed proteins.

Expression in Fermentor Cultures

Promastigotes has been mainly cultivated in liquid media containing serum such as Brain Heart Infusion (BHI) or in animal-derived nutrient media [25]. Several media, suitable for different *Leishmania* species [26–29], in particular a Hemin-supplemented BHI has been successfully utilized for *L. tarentolae* growth [30]. Hemin (Fig. 3) is an essential growing element [31] and, even if the detailed function is currently unknown, it is important as prosthetic group of various proteins, a source of energy and an intracellular regulator for metabolic pathways involved in respiration and protein synthesis [32]. Other chemically defined media have been described for *L. tarentolae* cultivation [33, 34] but unfortunately, only complex (BHI-medium) allowed to obtain high-cell densities of *Leishmania* species. Interestingly, Fritzsche et al. [35, 36] reported *L. tarentolae* growth behavior in yeast extract-based medium (YE), containing buffer salts and hemin. Large-scale fermentation (2 l) resulted in a small doubling time (6.7 h) and high cell

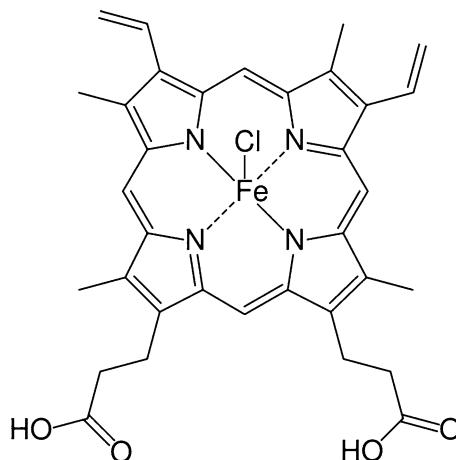


Fig. 3 Iron-containing molecule hemin: an essential intracellular regulator for metabolic pathways, involved in respiration and protein synthesis

density $0.65\text{--}1 \times 10^9$ cell/ml, about five times higher than reported by Meehan et al. with hemin supplemented Brain Heart Infusion media [30].

Expression of Proteins for Structural Studies

Nuclear magnetic resonance spectroscopy (NMR) is an extensively utilized spectroscopic technique that allows determination of protein structure at atomic resolution. A majority of NMR techniques in biology require recombinant proteins labeling with isotope containing amino acids (^2H , ^{13}C , and/or ^{15}N). Currently, isotopically labeled recombinant proteins have been expressed in *Escherichia coli*. Despite its obvious advantages such as rapid growth, developed methods of protein expression and cheapness of cultivation, *E. coli* has a range of shortcomings that limits its utility in protein studies. The most prominent problem relates to inefficiency of *E. coli* to assist folding of eukaryotic polypeptides producing only ca. 15% of eukaryotic proteins in their active form [37]. Moreover, the prokaryotic expression system lacks the posttranslational modifications often essential for functionality of eukaryotic proteins. To circumvent these problems several eukaryotic expression systems have been tested for their ability to produce significant amounts of isotopically labeled recombinant proteins. Several authors reported successful labeling of recombinant proteins in methylotrophic yeast [38, 39], instead a limited number of studies reported successful isotopic labeling in baculoviral expression system [40, 41]. Niculae et al. [42] and Foldynovà-Trantirkovà et al. [43] reported a NMR analysis of Enhanced Green Fluorescent Protein (EGFP) purified from recombinant *L. tarentolae* strain cultivated in a synthetic medium for selective ^{15}N -

Table 1 Heterologous proteins expressed in *Leishmania*

Protein	Mode	Signal peptide	Fusion	Amount (mg/l)	Reference
Amylase	Secreted	Erythropoietin	–	0.1–5	[8]
Bradykinin receptor B2	Secreted	Erythropoietin	–	0.1–5	[8]
Bradykinin receptor B2	Intracellular	–	GST	0.1–5	[8]
Checkpoint kinase Chk2	Intracellular	–	GST	0.1–5	[8]
CNAk	Intracellular	–	–	0.1–5	[8]
Chloramphenicol acetyl transferase	Intracellular	–	–	–	[45]
Cu/Zn Superoxide dismutase	Intracellular	–	–	30	[8, 9]
G-protein coupled receptor 56 (Ex segment)	Secreted	Native	–	0.1–5	[8]
Erythropoietin	Secreted	Native	–	30	[8, 9]
Erythropoietin	Secreted	Acid phosphatase	–	0.1–5	[8]
EpocI	Intracellular	–	GST	0.1–5	[8]
EpocII	Intracellular	–	GST	0.1–5	[8]
Green Fluorescent Protein	Intracellular	–	–	30	[9, 13, 42]
GTPase Rab7	Intracellular	–	GST	30	[8, 9]
GTPase Rab7	Intracellular	–	–	0.1–5	[8]
HIV-1 gag	Intracellular	–	–	–	[46]
Interferon γ	Secreted	Native	–	0.1–5	[8]
Intein-Myc	Intracellular	–	–	0.1–5	[8]
Laminin-322	Secreted	Acid phosphatase	–	0.55	[47]
LBH9	Secreted	Native	–	0.1–5	[8]
Luciferase	Intracellular	–	–	–	[48]
MAK-HC	Secreted	Acid phosphatase	–	0.1–5	[8]
MAK-LC	Secreted	Acid phosphatase	–	0.1–5	[8]
Max-2	Intracellular	–	–	0.1–5	[8]
MDP1	Secreted	Acid phosphatase	–	0.1–5	[8]
Miz-1	Intracellular	–	–	0.1–5	[8]
Mn Superoxide dismutase	Intracellular	–	–	0.1–5	[8]
MPP1	Intracellular	–	–	0.1–5	[8]
NNMT	Intracellular	–	–	0.1–5	[8]
P85alpha	Intracellular	–	–	0.1–5	[8]
PDM9	Intracellular	–	–	0.1–5	[8]
PFTa	Intracellular	–	–	0.1–5	[8]
PFTb	Intracellular	–	–	0.1–5	[8]
Pro BNP	Secreted	Acid phosphatase	–	0.1–5	[8]
Proto-oncogene Myc	Intracellular	–	GST	30	[8]
Proprotein convertase 4	Secreted	Native	–	4	[49]
Red fluorescent protein	Intracellular	–	–	–	[50]
Retinoic acid receptor γ	Secreted	Acid phosphatase	–	6	[51]
Rep	Intracellular	–	–	0.1–5	[8]
SCA	Secreted	Acid phosphatase	–	0.1–5	[8]
SCA	Secreted	Staphylokinase	–	0.1–5	[8]
smmyHC	Intracellular	–	–	0.1–5	[8]
SOD1	Intracellular	–	GST	0.1–5	[8]
T7 RNA polymerase	Intracellular	–	–	30	[8, 9]
Tissue plasminogen activator	Intracellular	–	–	0.17	[52]
WASP	Intracellular	–	–	0.1–5	[8]

labeling. The most important feature is *L. tarentolae* natural auxotrophy for several aminoacids (V, R, H, M, W, F, S, Y, T, L, and K) [44], therefore isotopic labeling is easily obtained supplementing expression media with required ¹⁵N-labeled aminoacid.

Summary

Leishmania tarentolae, a trypanosomatid protozoan parasite of the white-spotted wall gecko (*Tarentola annularis*), has been suggested as candidate for heterologous genes expression (Table 1 shows a comprehensive list of heterologous protein expressed in *L. tarentolae*). Its unique features are: ability to produce mammalian like complex type N-glycosylation, easy genetic manipulation, straightforward adaptation to large-scale production, and minimal nutrition requirement. Therefore, *Leishmania tarentolae* is a promising host for large-scale recombinant protein production for both pharmaceutical purpose and structural studies.

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