Protein Expression and Purification 114 (2015) 44–47

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Phytomonas: A non-pathogenic trypanosomatid model for functional expression of proteins

Mariana R. Miranda [a], Melisa Sayé [a], Chantal Reigada [a], Carolina Carrillo [b], Claudio A. Pereira [a,*]

[a] Laboratorio de Parasitología Molecular, Instituto de Investigaciones Médicas Alfredo Lanari, Universidad de Buenos Aires and CONICET, Buenos Aires, Argentina
[b] Instituto de Ciencia y Tecnología Dr. César Milstein (ICT Milstein), CONICET, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 18 September 2014
and in revised form 11 June 2015
Accepted 29 June 2015
Available online 30 June 2015

Keywords:
Phytomonas
Protein expression
Trypanosoma cruzi
Trypanosomatids

ABSTRACT

Phytomonas are protozoan parasites from the Trypanosomatidae family which infect a wide variety of plants. Herein, *Phytomonas* Jma was tested as a model for functional expression of heterologous proteins. Green fluorescent protein expression was evaluated in *Phytomonas* and compared with *Trypanosoma cruzi*, the etiological agent of Chagas' disease. *Phytomonas* was able to express GFP at levels similar to *T. cruzi* although the transgenic selection time was higher. It was possible to establish an efficient transfection and selection protocol for protein expression. These results demonstrate that *Phytomonas* can be a good model for functional expression of proteins from other trypanosomatids, presenting the advantage of being completely safe for humans.

1. Introduction

The *Phytomonas* genus comprises unicellular flagellates which infect a wide variety of plants. Although being phylogenetically close to trypanosomatids parasites that cause several human diseases, until 1970 these protozoan organisms were of little interest to researchers [1]. *Phytomonas* are non-pathogenic for humans, and can be cultivated in inexpensive undefined media. For example, in *Trypanosoma cruzi* media, such as LIT (Liver Infusion Tryptose) or BHT (Brain Heart Tryptose), *Phytomonas* grows with high replication rates [2]. The evolutionary proximity of these parasites to human protozoan pathogens makes it an ideal vehicle for the functional expression of genes from lower eukaryotes. Although no information about the regulation of gene expression in *Phytomonas* is available, in this work we tested the expression of the green fluorescent protein (GFP) in this organism using a well-known *T. cruzi* expression vector [3]. The performance of the *Phytomonas* Jma model was compared with *T. cruzi* in terms of selection time and protein expression levels.

2. Materials and methods

2.1. Cell cultures

Phytomonas Jma cells [4] isolated from the latex of *Jatropha macrantha* (Euphorbiaceae) were kindly provided by Prof. Israel Algranati (Fundación Instituto Leloir, Argentina), and originally obtained from the "Trypanosomatid Culture Collection", University of São Paulo (TCC-USP). *T. cruzi* epimastigotes are from the Y strain. *Phytomonas* and *T. cruzi* epimastigotes were cultured at 28 °C in plastic flasks (25 cm^2), containing 5 ml of Liver Infusion Tryptose (LIT) medium (started with 10^6 cells/ml) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin [5]. Cells were subcultured every 7 days, unless otherwise indicated, and counted using a hemocytometer.

2.2. Plasmids and parasites transfection

GFP gene was cloned into the pTREX expression vector [3] and the construction was confirmed by sequencing. *Phytomonas* Jma and *T. cruzi* cells were transfected with covalently closed circular plasmids using a gene pulser electroporator (BioRad Laboratories) under the following conditions: 10^8 cells grown in LIT medium at 28 °C were harvested by centrifugation, washed with PBS, and resuspended in 0.35 ml of electroporation buffer (PBS containing 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$). The cell suspension was mixed with 50 μg of plasmid DNA purified by passage through a Qiagen column (Qiagen) in 0.2 cm gap cuvettes (Bio-Rad Laboratories). The parasites were electroporated with a single discharge of 400 V, 500 μF with a time constant of about 5 ms [6]. Cells expressing the green fluorescent protein (GFP) were screened under a fluorescence microscope.

To analyze the fate of the plasmids into *Phytomonas*, two different techniques were carried out. For episome detection, a low-molecular weight DNA extraction was performed using

* Corresponding author at: IDIM, Combatientes de Malvinas 3150, 1427 Bs. As., Argentina.
E-mail address: cpereira@retina.ar (C.A. Pereira).

http://dx.doi.org/10.1016/j.pep.2015.06.019
1046-5928/

epimastigote samples and plasmid minipreps protocols [7]. After bacterial transformation and ampicillin selection, the plasmids were purified and analyzed by electrophoresis and DNA sequencing. The second technique was designed to evaluate the insertion of the plasmid and the recombination region by seminested PCRs performed using the forward primer of the GFP gene and random primers. Amplification products were then checked by a second PCR using the forward and reverse GFP primers. In the case of obtaining positive amplification, selected fragments are cloned in pGEM T-easy (Promega) and sequenced.

2.3. Western Blot analysis

Western Blots were performed using total parasite extracts obtained from 5×10^6 parasites (about 50 μg). Samples were fractioned by electrophoresis in polyacrylamide denaturing gels and transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were treated for 1 h with 5% non-fat dry milk in PBS and then incubated overnight with the anti-GFP antibody (Invitrogen) diluted 1:5000. Membranes were washed and incubated with the secondary antibody for two hours (anti-mouse HRP 1:2500, Vector Labs). Detection was done by chemiluminescence (Pierce). Total protein Ponceau S-staining was used for sample normalization.

3. Results and discussion

In order to test if a reporter gene could be expressed in *Phytomonas* Jma using a *T. cruzi* expression vector, the GFP gene was cloned into the pTREX plasmid [3]. This vector was designed for protein expression in *T. cruzi* and carries a mRNA processing signal (HX1) downstream of the RNA polymerase I ribosomal promoter that highly improves expression efficiency. The HX1 sequence was obtained from the upstream region of the *T. cruzi* TcP2β gene and cloned into the pRIBOTEX vector [8].

To compare the GFP expression in *Phytomonas* with the *T. cruzi* epimastigote control, both parasites were transfected with the pTREX-GFP plasmid using identical conditions, as described under Section 2. After transfection, parasites were left to recover for 24 h at 28 °C in LIT medium and the selection was performed in the same medium starting with 100 μg/ml G418. After 48 h parasites were diluted 1:10 and maintained with the same concentration of G418. From day 10 until the selection was completed the concentration of G418 was increased to 200 μg/ml. During this period the parasites were subcultured according the growth rate and monitored under a fluorescence microscope. The complete protocol is schematized in Fig. 1.

Although, in *T. cruzi* there are some fluorescent cells in the first 7 days, GFP expression begins to increase significantly from day 20. However, this early expression did not happen in *Phytomonas* which took about 30 days in starting to express GFP. In the case of *T. cruzi* the G418 selection took between 25 and 30 days while in *Phytomonas* complete selection took 45–50 days (Fig. 2).

In Trypanosomatids, the absence of DNA polymerase II promoters makes that regulation of gene expression rely on post-transcriptional processes. In consequence, the observed difference in the selection time could be due to the functionality of the regulatory elements such as the mRNA processing signal HX1. If this processing signal is not active in *Phytomonas*, the GFP expression should be lower than in *T. cruzi* until selection was completed. Once selected the levels of GFP expression were evaluated by Western Blot analysis using anti-GFP antibodies. Although it is a qualitative analysis the inset in Fig. 2 indicates that *T. cruzi* epimastigotes and *Phytomonas* appears to have similar levels of GFP, while the non-transfected *Phytomonas* lack the GFP signal. Images of *Phytomonas* expressing GFP are also showed in Fig. 3. As occurs in *T. cruzi*, GFP levels are heterogeneous in the *Phytomonas* population. The expression of GFP in transfected *Phytomonas* was followed in the presence or absence of G418. Under antibiotic selection GFP expression was observed for more than 3 years, and for at least 2 months without selection. To test if the pTREX-GFP vector was integrated in the *Phytomonas* genome or is maintained as an episome, plasmid purification was performed from *Phytomonas* cultures after selection. Four representative DNA samples from bacteria transformed with *Phytomonas* DNA corresponded to the pTREX-GFP plasmid with identical characteristics as the original source. Samples were partially digested with restriction enzymes to detect the presence of plasmid concatemers but the isolated episomes remain as plasmid monomers (Fig. 4). These data represent a difference with *T. cruzi*, which is capable of forming head to tail concatemers with episomal plasmids [9]. Moreover, successive attempts using a PCR approach failed to detect the plasmid integration in the *Phytomonas* genome. Identical results, in terms of plasmid stability, were obtained using samples from *Phytomonas* cultured for 2 months without G418 selection (Supplementary Fig. 1).

These results demonstrate that the pTREX-GFP vector, unlike what occurs in *T. cruzi*, was not integrated in *Phytomonas* genome but remains as stable episomes without forming concatemers. The presence of such structures is an evidence of the lack of integration by itself, because, in *T. cruzi* the integration of the pTREX plasmid produces a rapid degradation of extrachromosomal copies [10].

In order to establish if the *T. cruzi* regulatory elements present in the pTREX vector are in the *Phytomonas* genome, a bioinformatics approach was performed using the preliminary data of the *Phytomonas* genome project. A sequence similar (42% identity) to *T. cruzi* HX1 was found 105 bp upstream the putative ribosomal P2β homologue (60% identity). The reason of large lag time of GFP expression in *Phytomonas* as compared to *T. cruzi* could be due to the lack of functionality of HX1 as an mRNA processing signal in *Phytomonas*. The low nucleotide identity between both HX1 elements supports this hypothesis. Another possibility is that elements binding to the HX1 regulatory region in *Phytomonas* may

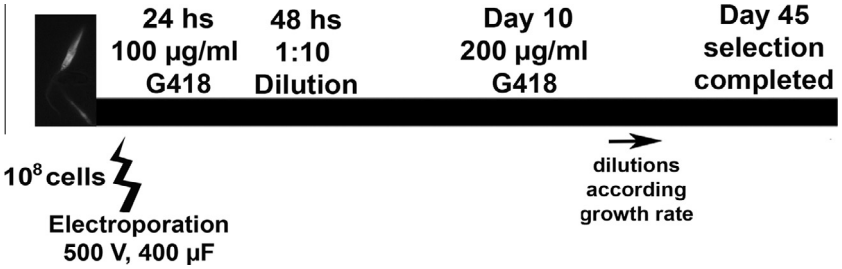

Fig. 1. Schematic representation of the selection protocol. A time-course of the treatments is represented by a black bar, starting from the parasites' electroporation and finishing on day 45 when the antibiotic selection was completed.