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Research internship

report

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Expression in*E.coli*and purification of a *Leishmaniadonovani*phosphoinositide binding protein from the nexin family(*Ld*PIBPnex)

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1. Introduction:

My 3 months internship was performed in the "Intracellular Parasitism" research group at the Hellenic Pasteur Institute in Athens, Greece. The aim of my work, under the supervision of DrHaralabia BOLETI, was to contribute to the study of ***L****eishmania****d****onovani*****P***hosphoinositide***B***inding ***P***rotein ***nex***in (*Ld*PIBPnex) in order to further analyze in the future its value as a potential virulence factor for visceral Leishmaniasis.

Leishmania are dimorphic protozoan parasites from the *Trypanosomatidae* family *(kinetoplastid class*) living as extracellular flagellated forms (promastigotes) in their sandfly vector gut and as intracellular aflagellated forms (amastigotes) inside modified phagolysomes of the mammalian host phagocytes. As other intracellular pathogens, *Leishmania* species have evolved strategies supporting invasion and persistence within the mammalian phagocytes. In some cases the underlying mechanisms involve export of virulence factors into the host cell cytosol. Proteomic analysis of the secretome of *Leishmaniadonovani/L. donovani* (causative agent of the fatal disease visceral Leishmaniasis/VL) revealed several proteins secreted in the extracellular medium of cultured promastigotes, possibly through an exosome-like route (Silverman, Chan et al. 2008) [1].

One of these was the product of the *Ld*BPK_352470.1 gene, highly conserved in *Leishmania* species, which encodes for a secreted proteinof 417 a.a. (predicted MW 46,6 kDa) with a PX phosphoinositide binding domain and structural features classifying it in the Sorting Nexin protein family. This is a large group of cytoplasmic proteins that bind to membranes either through their lipid-binding PX domain or through protein–protein interactions with membrane-associated protein complexes. Members of the Nexin family are shown to participate in an increasing array of endosomal sorting and intracellular membrane traffic events (Cullen 2008) [2], (Carlton, Bujny et al. 2005) [3], (van Weering, Verkade et al. 2010)[4].

The ***Ld*BPK_352470.1 gene** product was named***Ld*PIBPnex**, for ***L****eishmania****d****onovani*****P***hosphoinositide ***B***inding ***P***rotein ***nex***inand the gene *Ldpibp*$_{nex}$on the basis of its predicted structural features (the PX, PI binding domain and the BAR binding domain) (Figure 1) and its classification to the nexin family of proteins.Recombinant plasmids were constructed from the plasmid pGEX4T-1 (Figure 2), in which this gene was inserted in the EcoR1 restriction site and next to a GST gene (GST will be used as a

molecular tag to purify the protein) and an ampicillin resistance gene (to select transformed bacteria containing this plasmid). Three types of plasmids were used in this study. The pGEX-*Ldpibp*$_{nex}$with the full-length gene coding for the protein of interest. The pGEX-*Ldpibp*$_{nex}$-C-Term with the part of the gene encoding only the half C-Terminal part of the protein and the vector pGEX-4T1, which contains only the gene for the GST tag. The two latter plasmids will be used to express proteins that will be used as controls in the *in vitro* biochemical assays planned for the analysis of the Phosphoinositide binding properties of *Ld*PIBPnex.

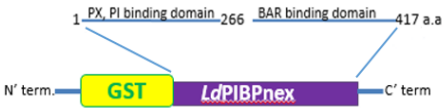


Figure 1- Schematic representation of the recombinant GST-*LdPIBP*$_{nex}$ hybrid protein produced by the pGEX-4T1*Ldpibp*$_{nex}$plasmid. The domains of the protein and their position in the primary structure are indicated.

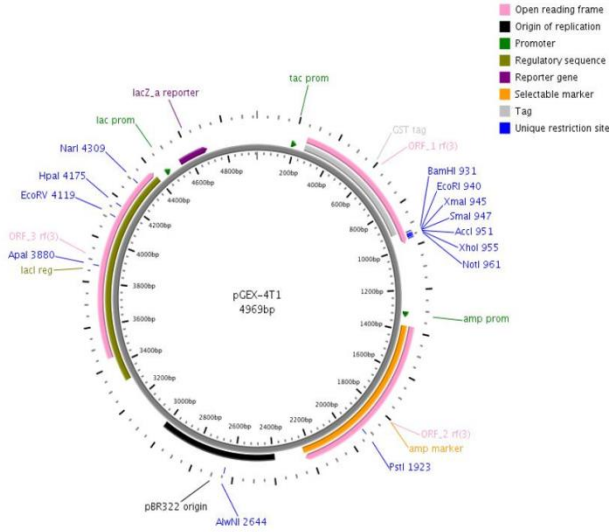


Figure 2- pGEX-4T1 map, including restriction sites and genes.The plasmid length is 4969pb and contains an EcoRI restriction site at 940 pb in which the *Ldpibp*$_{nex}$gene was inserted.

The aim of my work presented in this report has been to use these three recombinant plasmids, prepared in the lab by the MScstudent DrososKourounis who has just defended his thesis, to define the optimal conditions for expression of the 3 recombinant proteins (i.e. GST-*Ld*PIBPnex, GST-*Ld*PIBPnex-C-term and GST). As a first step I had to confirm that the plasmids given to me contained the correct insert at the correct orientation before proceeding to the analysis of the expression levels of the recombinant proteins as soluble under different induction conditions (i.e. length of induction, IPTG concentration and Temperature).

2. Materials and methods:

2.1. Preparation of media for bacteria cultures:

2.1.1 LB (Luria Broth):

LB (Luria Broth):media for bacteria culture was prepared by dissolvingNaCl,(1% w/v final concentration),Tryptone(2% w/v final concentration) and yeast extract (0,5 % w/v final concentration) into deionized pure water. The medium was sterilized by autoclaving and when cooled ampicillin was added to a concentration of 100 mg/ml.

2.1.2 LB agar

Liquid LB agar + Amp was added on petri dishes and let to cool and solidify.

2.2. Bacteria cultures

2.2.1 Solid bacteria cultures:

On cold LB-agar platesTOP 10 F *E.coli*bacteria from glycerol stocks stored at -80°C were inoculated by spreading on the agar surface with a sterile tooth pick.four different bacterial strains: clone Cl7 and Cl6 (two clones carrying pGEX 4T1-*Ldpibp*$_{nex}$plasmid), clone C-Term (carrying thepGEX-*Ldpibp*$_{nex}$-C-Term plasmid) and clone 4T-1 (carrying the pGEX-4T1 vector) were plated. Thesolid bacteria cultures with bacteria colonieswere used for up to one month to start all the liquid cultures. They were stored at 4°C wrapped withparafilm.

2.2.2Liquidbacteria cultures:

Bacteriaculture for plasmid isolation:

Pre-cultures were prepared in 3ml or 100 ml LB + Amp media and incubated overnight at 37°C, 120 rpm shaking.

Bacteria culture for recombinant protein expression:

Pre-cultures were prepared in 3ml LB + Amp media and incubated overnight at 37°C, 120 rpm shaking. One tenth dilutions (final volume 10-20 ml in 50 ml falcon tubes) were prepared the following morning and the cultures were incubated further at 37°C for the time required to reach an OD_{600} between 0,4 -0,6. Protein expression was induced by the addition of IPTG (Isopropyl β-D-1-thiogalactopyranoside) from 0,5 -1 mM concentration for 3hrs- 4hrs at 37°C or for 20hrs at 22°C. The $OD_{600}$was measured before induction and at the end of the induction period by removing 1ml from the culture. The bacteria in this 1 ml samples after measuring the OD_{600} for determining the number of bacteria were centrifuged at 14000 rpm for 5 min and the pellet was stored in 1,5 ml eppendorf tubes at -20°C. The other 8ml from the cultures

were also centrifuged at the end of the induction period and stored at -20°C in 50 ml falcon tubes.

2.3. Isolation of plasmids DNA (mini prep) from bacteria:

Plasmid DNA was isolatedfrom 3 ml cultures (for plasmid analysis by Restriction Enzyme digestion) or from 100ml culture (for sequencing and use for bacteria transformation and other uses). The cultures from different bacteria strains were started from single colonies taken from Petri dishes, were grown overnight at 37°C, 160 rpm. Plasmid was isolated using the kits NucleoSpin® miniprep and NucleoSpin® maxiprep from Macherrey-Nagel®.The DNA concentrations in the plasmid solutions were estimated by using a ThermoFisher® nanodrop 2000 spectrophotometer, at 260 nm.

2.4. Restriction Enzyme Digestion of plasmid DNA:

Plasmid DNA solutions were analyzed by electrophoresis in a 1% w/v agarose gel (prepared as in "Preparation of Agaroses gels for DNA analysis"). Positive bacteria colonies were analyzed for the correct size of the plasmid and insert by performing a digestion with the restriction enzyme EcoRI(Takara Bio Inc., Shiga, Japan)(1 h, 37°C in a heated bath).RE digestion reactions were performed in a 15µl reaction mix containing 1,5µl of 10X M buffer, the volume corresponding to 1µg plasmid DNA, the necessary volume of EcoR1 [15U/µl] restriction enzyme to achieve a complete digestion and the *QS* of dH_2O to obtain the 15µl final reaction final volume. Finally, 1,5µl of 10X sample buffer was addedbefore loading the samples to the gels.Non-digested plasmid DNA (0,1µg) was analyzed in parallel.The correct orientation of the insert inserted into the vector was analyzed by digesting (1 hrs at 37°C in a heated bath) the plasmids with Sal1 RE (Takara Bio Inc., Shiga, Japan) in a 15µl reaction mix containing 1,5µl of 10X H buffer(Takara Bio Inc., Shiga, Japan), the volume corresponding to 1µg plasmid DNA, the necessary volume of Sal1 [15U/µl] restriction enzyme and the *QS* of dH_2O to obtain the 15µl final reaction volume.As above 1,5µl of 10X sample buffer was added in each sample before loading to the agarose gels.

2.5. Preparation of Agaroses gels for DNA analysis:

Agarose gels were prepared by dissolving 1g of agarose, in 100 ml TBE Buffer, by heating in a microwave until boiling. After cooling (no more steam from evaporating water was produced) EtBrsolution (5*10^-5 mg/ml) was added (for the revelation) in the agarose TBE

solution that was then transferred into a casting apparatus. Nitrile gloves were used throughout this procedure, as EtBr is carcinogenic. The gel was then transferred to a running apparatus, filled with TBE buffer. The DNA samples were loaded in the wells and the DNA was subjected to migration into the gel by applying voltage 90V for 45min. After migration, gels were illuminated by transilluminationUV light in an Alphaimager® deviceandfluorescent DNA bands (due to EtBr binding) were photographed by the digital camera of the device.

2.6. SDS-PAGE gels for protein analysis:

10%SDS-PAGE gels were prepared in Laemli buffer system.

Resolving gel solution : 6,3 ml dH_20, 3,75 ml 4X Tris-HCl 1,5M buffer pH 8,8, 4,95 ml of 30%w/v Acrylamide solution, 75 µl 10% w/v APS. Stacking gel solution: 3,05 ml dH_20, 1,8 ml 4X Tris-HCl 0,5M buffer pH 6,8, 1 ml of 30% w/v Acrylamide solution, 37,5 µl 10% w/v APS. Gel polymerization wasstarted by the addition of7,5 µl TEMED(Tetramethylethylenediamine, Omnipur® Calbiochem®)and then it was castedbetween two glass plates (mini gel BioRad apparatus) with a 1,5 mm thickness spacer. Two gels were ran at the same time for 45 min at 200V in electrode buffer 1X prepared with 370 ml dH_20, 125 ml 4x Reservoir buffer (25 mMTris, 192 mM glycine, 0.1% w/v SDS).
The gels were stained overnight using a Coomasie stain solution (400 ml Methanol, 100 ml Acetic Acid (glacial), 500 ml dH_2O, 1 g Bromophenol blue) anddestained for minimum 4 hrswith the same solution prepared without the Bromophenol blue dye.After destaining, gels were illuminated with white light in an Alphaimager® deviceand photographed.

2.7. Storage and Preparation of protein samples

All samples,atall stages of protein analysiswere stored in ice throughout protocols to avoid protein degradation by proteolysis.

2.7.1. Samples used to compare theexpression of the protein of interest:

Sampleswith or without IPTG induction were prepared by resuspending the bacteria pellet, resulting from a centrifugation of 1 ml culture) in 100 µl of protein 1X sample buffer (SDS-PAGE laemli system). They were then boiled for 5 min at 95°C and stored in ice until analysis.

2.7.3 Samples used to study the solubility of the protein of interest:

Samplesafter induction were prepared by resuspendingthe bacteria pelletresultingfrom centrifugation of an 8ml culture in 1ml cold (4°C) PBS 1X + proteolytic inhibitors (Sigma, P 2714)(washing step). Then the bacteria were centrifuged for 5 min at 10,000 rpm, 4°C, resuspendedin 320 µl PBS 1X + proteolytic inhibitors + 1 mg/ml lysozyme and incubated at 4°C for 1hrs. The cells were then lysed by sonication (three cycles of 30s sonication interrupted by 30s breaks).Triton-X 100 at 1%v/v final concentration was addedto the samples for membrane solubilization. A volume of30 µl of the total lysate was kept for analysis. The soluble fraction of the total lysate (290 µl) was separated from the insoluble by a centrifugation(5 min, 10000 rpm, 4°C).The insoluble pellet was resuspended in the same volume (290 µl) as the soluble supernatant. Samples for analysis by SDS-PAGE were prepared by the addition of 5X Sample buffer in order to obtain 1X concentration of sample buffer and they were then boiled 5 min at 95°C and storedin ice until analyzed.

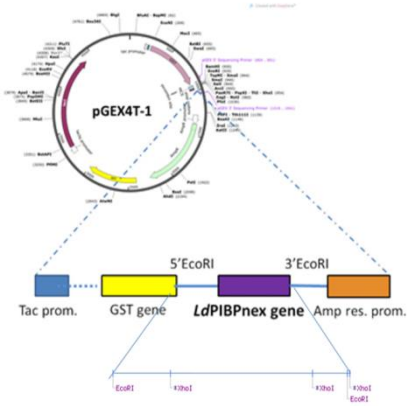
2.8. Buffer

TBE 1X (Tris-borate , EDTA): Tris 89 mM, boric acid 89 mM, 2 mM, EDTA, 1000 ml dH_2O.

3. Results:

3.1. Verification of the plasmids used in the study

As a first step of the project, I verified that the plasmids I was given to work with have the correct size insertin the correct orientation for maintaining the open reading frame of the GST coding gene that is upstream of the EcoRI site of insertion (Figures 3,4).



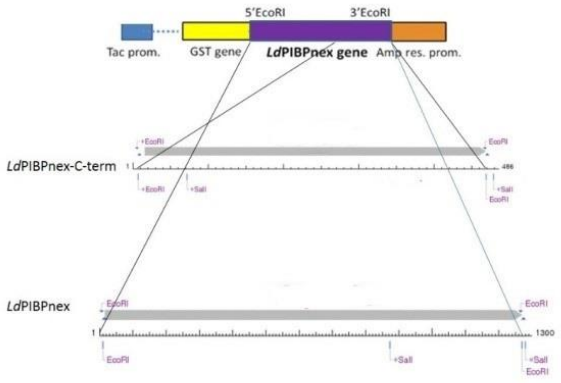


Figure 3–Schematicrepresentation of the *Ldpibp$_{nex}$*gene insertion site in the pGEX-4T1 plasmidmap

Figure 4- Schematic representation of the *Ld*PIBP$_{nex}$ and *Ld*PIBP-C-Term encoding gene features. The base pairs of the two genes and the important Restriction enzyme sites are indicated. Two different insertion, the full length*Ldpibp$_{nex}$* gene (1251 bps coding for a 417 aa protein) and *Ldpibp$_{nex}$*-C-Term (407 bps coding for a proteins150 aa that contains only the C-Term part of*Ld*PIBPnex) are shown. The SalI internal restriction site in the two genes was used to determine the orientation of the insert.

3.1.1 Verification of the correct insert size:

The plasmidspGEX-*Ldpibp$_{nex}$*from clone 6 (PIBPnex 6) and from clone 7 (PIBPnex 7) have the *Ldpibp$_{nex}$*full-length gene inserted in the EcoRI restriction site (expected size 1251bps). The plasmid C-Term contains a shortened version of *Ldpibp$_{nex}$*, also inserted in the EcoRI site (expected size 407 bps), coding for the half C-terminal part of *Ld*PIBPnex (a.a.-417)

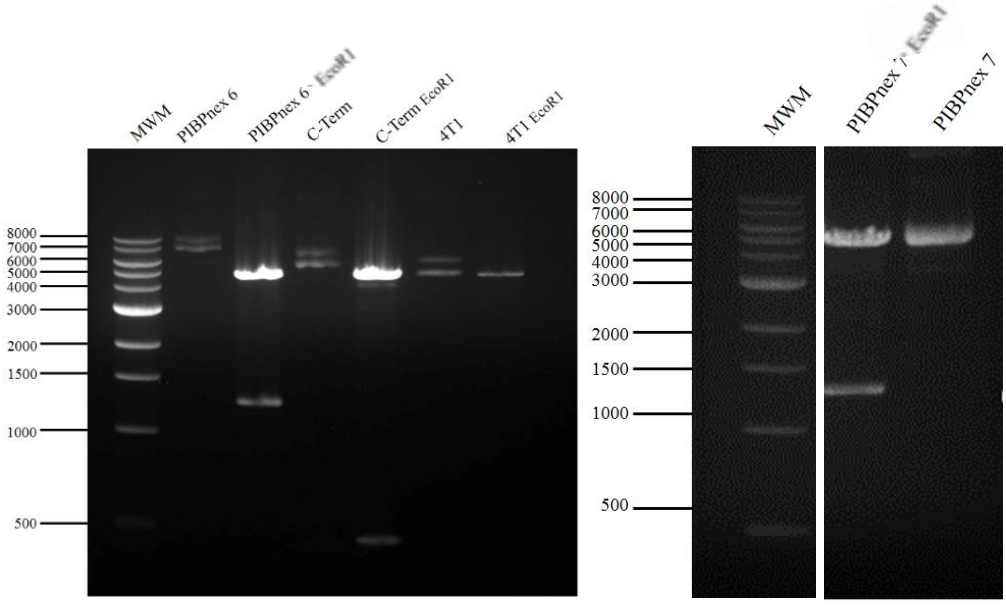


Figure 5– Electrophoretic migration of plasmids in agarose gels after digestion with the EcoRI RE. Plasmids pGEX 4T-1-*Ldpibp$_{nex}$* from clone 6, pGEX 4T-1-*Ldpibp$_{nex}$*C-Term, and pGEX 4T-1-digested or not with EcoRI (according to protocol in Methods) were analyzed on 1% w/v agarose gel at 90V, for 45 min in TBE buffer. Bands were revealed with EtBr binding as described in methods (paragraph 2.4).

Two bandsof the digested plasmids were expected. One representing the opened plasmid and the otherthe insert that has been excised. The lower molecular sizeband for the C-Term plasmid, representing the insert, is smaller than that of the, pGEX-*Ldpibp$_{nex}$* plasmid as expected indicated by its further migration in the gel than the full length gene fragments.

Plasmids from clones 6 and 7were prepared to be sent for sequencing for further verification of the sequence of the inserted fragment.

3.1.2. Verification of the correct orientation of the insert:

The *Ldpibp*$_{nex}$ gene was inserted in the EcoRI site. To confirm thecorrect orientation we digested the plasmids with the SalI enzyme that cuts in a single site inside the insert and a single site outside (inside the vector). The expected size of the generated fragments after RE digestion with this enzyme of the two plasmids is expected to be as shown in Figure 6.

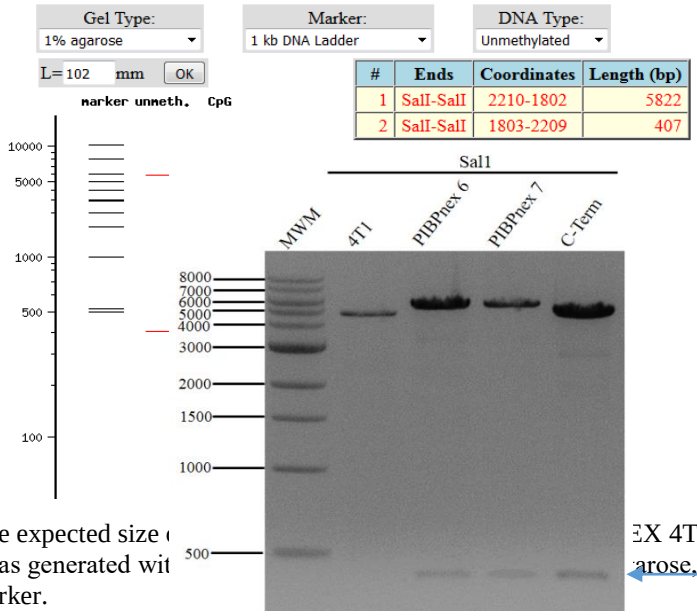


Figure 6-The expected size (EX 4T-1-*Ldpibp*$_{nex}$ with SalI. This virtual gel was generated wit arose, SalI digestion and 1 kb DNA ladder as marker.

Figure7-Migration of plasmid DNA digested with the SalI restriction enzyme.pGEX-

Precisely two bandsare expected for clones with inserts and one for the vector

*Ldpibp$_{nex}$*plasmid DNA from clones 6 and 7, pGEX-*Ldpibp$_{nex}$*-C-Term and pGEX-4T1were digested with SalI enzyme (Methods paragraph 2.4). MWM DNA size markers were analyzed in parallel on the far left lane. Colors were inverted. The insert is indicated with an arrow on the right

As expected, migration of plasmids with insert digested with SalI (Figure 7) show that we obtained two bands, one under 500bp corresponding to the insert fragment generated and one around 5500pb corresponding tothe rest of the plasmid. The pGEX-*Ldpibp$_{nex}$*-C-Term plasmid fragment appear smaller than the one from pGEX-*Ldpibp$_{nex}$*as expected (844 bp shorter), and pGEX-4T1 is just linearized.

We can conclude comparing the fragments generated by digestion with the SalIrestriction enzyme (Fig.4, Fig. 6), that the *Ldpibp$_{nex}$* and pGEX-*Ldpibp$_{nex}$*-C-Termgenes are inserted in the expected orientation. Otherwise, we would have observed bands with different sizes.

3.2. Optimization of the conditions for recombinant protein expression in *E.coli*:

3.2.1. Induction at 37°C for3 or 4 hrs with 0,5mM IPTG:

Protein expression was induced initially in small bacteria cultures (Top10F *E. coli*) grown at 37^{o}C. The induction was performed for 3 or 4 hrs with 5mM IPTG. At the end of the induction period a sample from the bacteria cultures before and after induction corresponding to the same number of cells was centrifuged and the protein content from clones 7 (CL7), 6 (CL6), C-Term and 4T-1 was analyzed by SDS-PAGE (Figure 8).

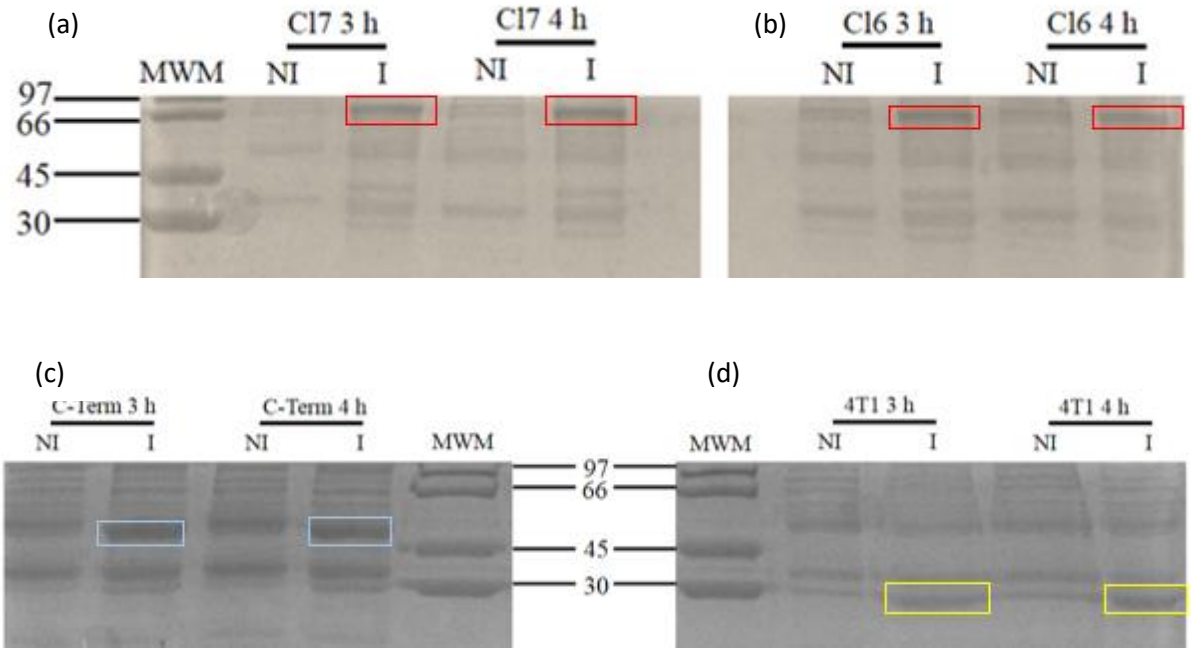


Figure 8 - Comparison of the protein migration profiles from bacteria total cell lysates from all four bacteria

strains after 3hrs and 4hrs induction at 37°C with 0,5mM IPTG. Samples from non-induced corresponding cultures were analyzed on the side. Proteins were analyzed on a 10% w/v acrylamide SDS-PAGE at 200 V for 45 min.Bands at the expected MW of the GST-*Ld*PIBPnex (~72 kDa), GST-*Ld*PIBPnex-C-Term (~45 kDa) and GST (~29kDa) that seem to be more abundant in the samples from induced bacteria cultures are framed in red, blue or yellow. NI=non induced, I= induced.

From the gels presented in Figure 8 we observethat the recombinant proteins GST-*Ld*PIBPnex, GST-*Ld*PIBPnex-C-Term and GST are well produced at 37°C. We observed no significant difference in the levels of the proteins induced after 3hrs with respect to4hrs induction. For clones 6 and 7 expressing GST-*Ld*PIBPnex we also observed overexpression of a protein species of approximate 35 kDa. This could be a proteolytic product of the full length protein.

We then tested the induction of expression at lower temperature which allows a slower production of the protein allowing for slower folding and increased chances for the protein to be more soluble.

3.2.2. Induction at 22°C, 20 hrswith 0,5mM IPTG:

The levels of expression of the 3 recombinant proteins of interest were examined under temperature of 22ºC for 20 hrs. Analysis as above of the migration profiles of the proteins from bacteria lysates showed that two of the three recombinant proteins i.eGST-*Ld*PIBPnex and GST-*Ld*PIBPnex-C-Termseem to be better expressed in these conditions (Figure 9, lanes 1-4 and 7-8). For GST we cannot conclude the same. It seems that the protein was not induced well in this experiment (Figure 9, lanes 5, 6).

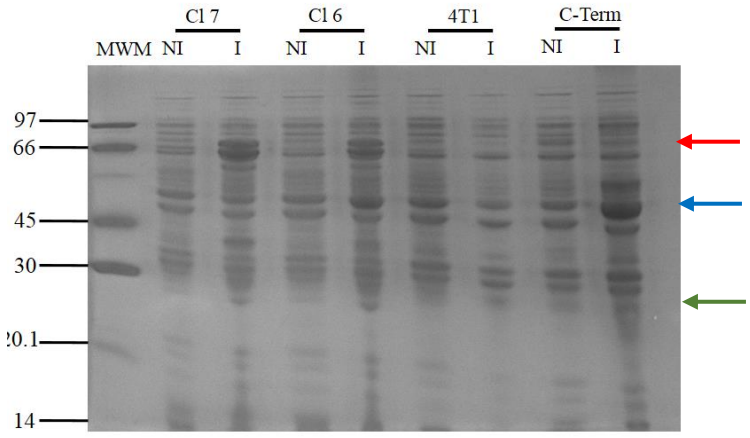
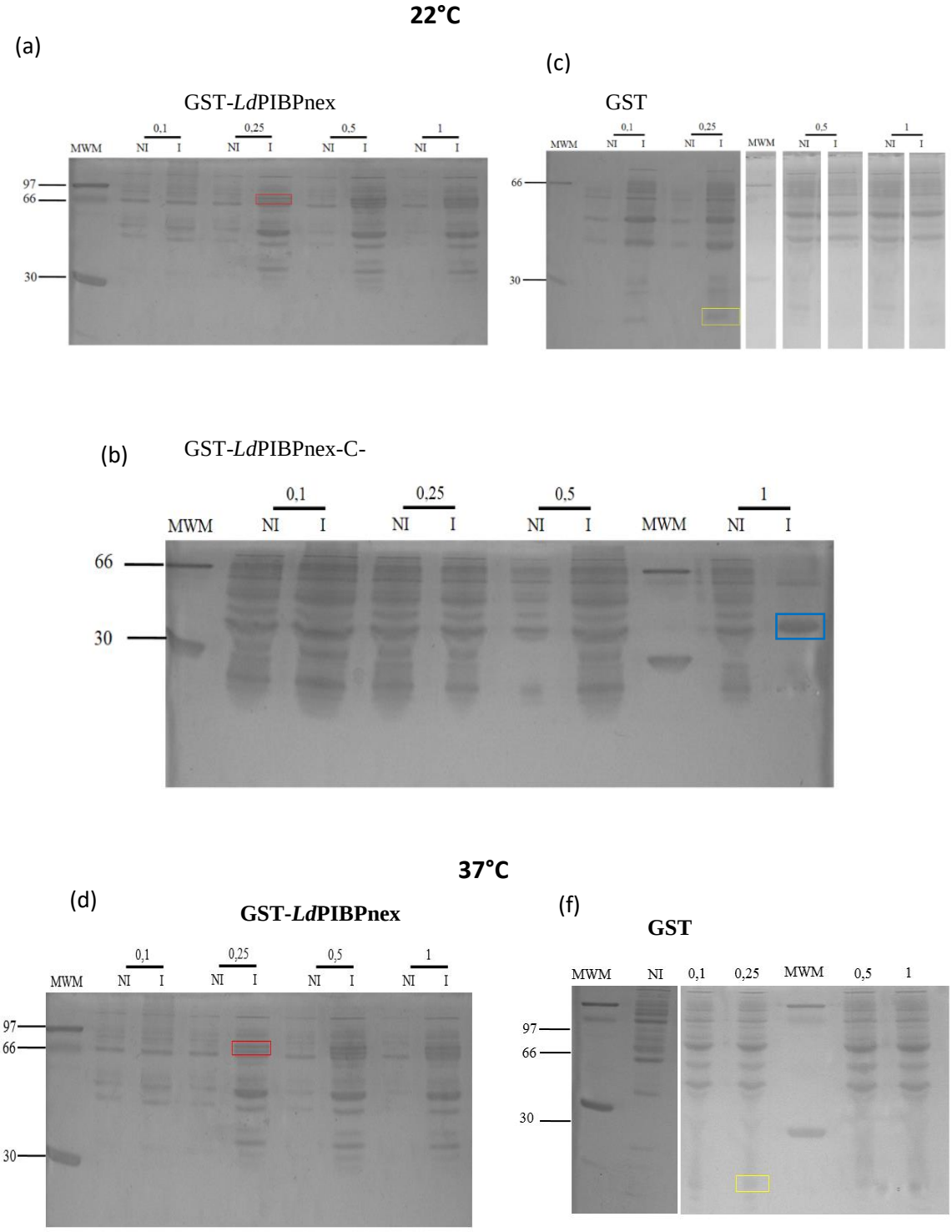


Figure 9- Induction of expression of GST-*Ld*PIBPnex, GST-*Ld*PIBPnex-C-Term and GST proteins for 20h at 22°C with 0,5mM IPTG. Proteins from bacteria pellets were analyzed on a 10% w/v SDS-PAGE at 200 V during 45 min. The migration positions of.GST-*Ld*PIBPnex, GST-*Ld*PIBPnex-C-Term and GST are indicated by the red, blue and green arrows on the right side respectively.

3.2.4 Influence of the IPTG concentration:

As a next step in defining better the optimal conditions of expression, we tested the effect of IPTG concentration in the induction of expression of the 3 proteins of interest. Four different IPTG concentrations (0,1, 0,25, 0,5, and 1 mM) were evaluated at 22°C and 37°C for 20 hrs (Figure 10) or 3 hrs (Figure 11) induction respectively.



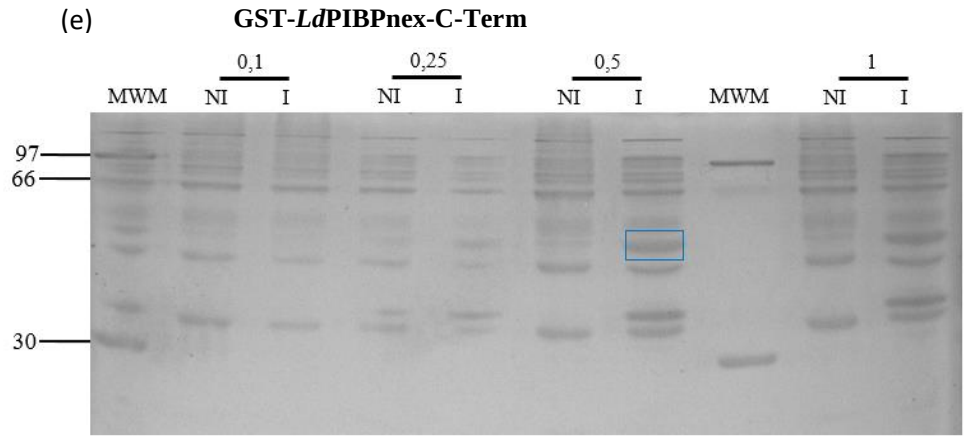


Figure 11- Effect of four different concentrations IPTG in the expression of the GST-*Ld*PIBPnex, GST-*Ld*PIBPnex-C-Term and GST proteins. Proteins from bacteria pellets from clones 6 (a), C-Term (b) and 4T1 (c) were analyzed on a 10% w/v SDS-PAGE at 200 V during 45 minafter 3h induction with 0,1, 0,25, 0,5, 1 mM IPTG at 37°C. Proteins from bacteria pellets from clones 6 (d), C-Term (e) and 4T1 (f) were analyzed on a 10% w/v SDS-PAGE at 200 V during 45 min after 3h induction with 0,1, 0,25, 0,5, 1 mM IPTG at 37°C. The migration position of GST-*Ld*PIBPnex, GST-*Ld*PIBPnex-C-Term and GST are indicated at the side of the gels by a red, blue or yellow frame respectively.

At 22ºC for clone 6, we can observe the induction of GST-*Ld*PIBPnex full length from 0,25 mM up to 1 mM IPTG. Better induction was observed at 0,5 mM concentration of IPTG (Figure 10 a). Similar results were obtained for induction of the protein at 37ºC (Figure 11a). Induction of GST-*Ld*PIBPnex-C-Term seems to be better at 1 mM (Figure 10 b) at 22ºC while it seems to be equally well expressed with 0,5mM and 1mM IPTG at 37ºC(Figure 11 b). Induction of GST is visible from 0,1-1 mM, however the amount of proteins observed at MWs lower than 30 kDa was very low and the bands very faint.

When a large amount of protein is produced during induction this may affect its solubility. This aspect must be considered in the selection of the optimal conditions for expression.

3.2.3 Evaluation of Solubility of the induced recombinant proteins:

As a next step we wanted to confirm that the recombinant proteins produced in our experiments are soluble, high concentration of IPTG can produce insolubility. We have decided to test the protein solubility as described in Methods, paragraph **2.7.3** on the recombinant proteins expressed at 22ºC after induction with 0,5 mM IPTG for 20hrs (Figure **12 (a)**): and at 37°C after induction for 3hrs with 0,5 mM IPTG (Figure **12 (b)**):

The GST-*Ld*PIBPnex recombinant protein from Clones 6 and 7 seem to have very low solubility at 22°C. However, this may result from inefficient lysis of the bacteria cells as most of the proteins of the bacteria lysates were recoverd in the insoluble fraction. Cells from the C-Term clone seem to be well lysed and the GST-*Ld*PIBPnex-C-Term protein seems to have

good solubility. Most of it is recovered in the soluble fraction of the lysates. The GST protein from the 4T-1 sample cannot be detected as in this experiment the proteins smaller than 30 kDa run out of the gel due to a wrong estimation of the running time of the gel. Shorter length of gel migration or a higher acrylamide concentration could solve this problem.

At 37ºC we also had a problem in the cell lysis and therefore we cannot conclude for the solubility of the GST-*Ld*PIBPnexin Clones 7 and 6 and for the GST-*Ld*PIBPnex-C-Term protein. In all three cases, it seems that these proteins are mostly recovered in the insoluble fraction of the cells as well but the experiment has to be repeated to achieve better bacteria lysis. The GST from the 4T-1was detected in the soluble fraction but it was present in a small amount.

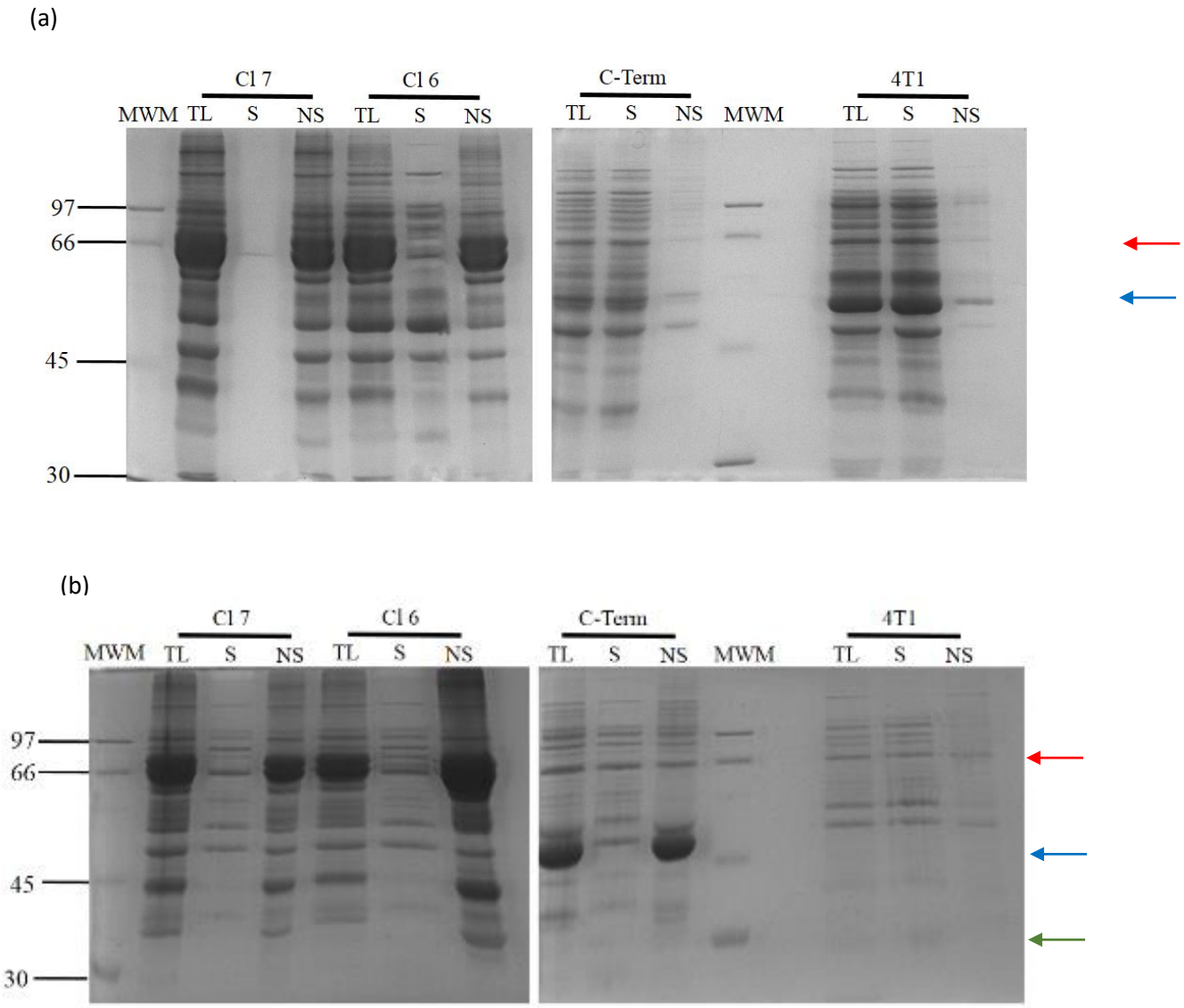


Figure 12-Comparison of the proteins expression and solubility of recombinant proteins GST-*Ld*PIBPnex, GST-*Ld*PIBPnex-C-Term and GST in clones 7, 6, C-Term and 4T1 after 20hrs induction at 22°C with 0,5mM IPTG (a) or at 37°C for 3hrs with 0,5 mM IPTG (b). Protein samples were analyzed on a 10% acrylamide sds page at

200 V during 45 min.TL=total bacteria lysate; S: soluble; NS: non-soluble. The suspected migration position of the GST-*Ld*PIBPnex, GST-*Ld*PIBPnex-C-Term and the GST bandsare indicated with arrows on the right side of the gels (red, blue and green respectively).

4. Conclusions

To summarize, from my two months' work describedabove I achieved

1) to verify the plasmids given to me for the presence of the correct inserts at the correct orientations.
2) to define some conditions that allow a good expression of the proteins of interest.

Analysis of the solubility of the induced recombinant proteinshas not been conclusive thus far due mainly to technical problems. Experiments are in progress to verify the original results and overcome the technical problems that I faced.

Certainly more experiments have to be performed to optimize the conditions of induction of expression of the three proteins.

For the one month left of my internship, I will try to assess the levels of expression of the endogenous *Ld*PIBPnex protein in the *Leishmaniadonovani* parasites at different stages of growth and development (i.e. log phase and stationary phase promastigotes and amastigotes). This will be performed by SDS-PAGE and Western blot analysis of *L. donovani* total protein lysates obtained from parasites in culture (axenic). This analysis will be completed by observation of parasites stained by Immunofluorescence with a confocal microscope. These experiments are planned to enable me to gain some experience with the culture of protozoan parasites *Leishmaniadonovani* and the techniques of immunofluorescence and confocal microscopy.

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