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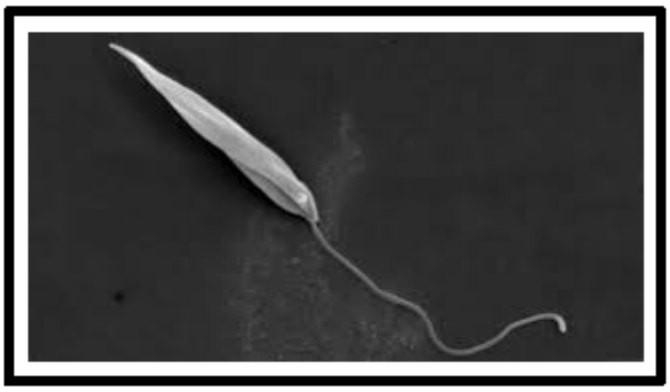
2nd year of MSc, BioPMBB – IMH

Molecular Biology of the protozoan parasites *Leishmania donovani* : Cloning of an *L. infantum* putative phosphatase into plasmid vectors for expression of His-tagged or *mcherry*-tagged protein in bacteria, mammalian and *Leishmania* cells.



Internship realized from the 2nd of February 2015 to the 31st of July 2015

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Under the supervision of Dr. Haralabia Boleti

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I) Introduction

Leishmaniasis is mostly known in southern France and other north Mediterranean countries as a disease which mainly strikes dogs [17]. It is caused by microorganisms belonging to the *Leishmania spp*. These are protozoan parasites from the Trypanosomatidae family and are transmitted to the mammalian host by the bite of infected sandflies [21]. The *Leishmania* parasites are also responsible for a global public health issue. Many *Leishmania* species are the etiologic agents of various forms of human leishmaniasis, ranging from self-healing cutaneous lesions to visceral complication which can lead to death [21]. According to the World Health Organisation (WHO), 350 million people throughout more than 90 countries worldwide are considered at risk in regards to leishmaniasis [1] (Figure 1). No efficient vaccine against human leishmaniasis is available and additionally, diagnostic methods and treatments are problematic [15]. Diagnostic methods are nowadays based on Immunofluorescence or PCR assays on blood and tissue samples, which make them hard to realize in rural areas. Currently, the most efficient treatment is chemotherapy. This term regroups a few drugs which are not completely specific to *Leishmania*, with important side effects for the patient and high cost. The last two problems are aggravated by the fact that leishmaniasis mainly strikes poor populations in developing countries. To crown all that, *Leishmania* parasites show a great ability to develop quickly drug resistance against most of these treatments and raise new issues with co-infections with other pathogens such as HIV [18]. It is now urgent to find solutions against leishmaniasis and the proliferation of its invertebrate vector sandflies, considering the quick emergence of this group of diseases in an increasing number of areas in Europe [5]. It is within this framework that many Institutes belonging to the Institut Pasteur International Network (IPIN) and other research and development laboratories have been acting in concert since several years.

My 6 months internship of the second year of my master (MSc) degree was performed in one of the IPIN institutes, the Hellenic Pasteur Institute in Athens, Greece, and more specifically in the "Intracellular Parasitism" research group. The aim of my work, under the supervision of Dr Haralabia BOLETI, was to contribute to the study of a newly discovered atypical Dual-Specificity Phosphatase (aDSP) from *Leishmania infantum* parasites, highly conserved in most *Leishmania* species, a potential virulence factor, in order to further analyze in the future its value as a potential drug target.

1) Generalities on Leishmaniasis

a) Clinical Manifestations

There are three main clinical manifestations of human leishmaniasis. These diseases patterns are due to the *Leishmania* parasite species involved in the infection.

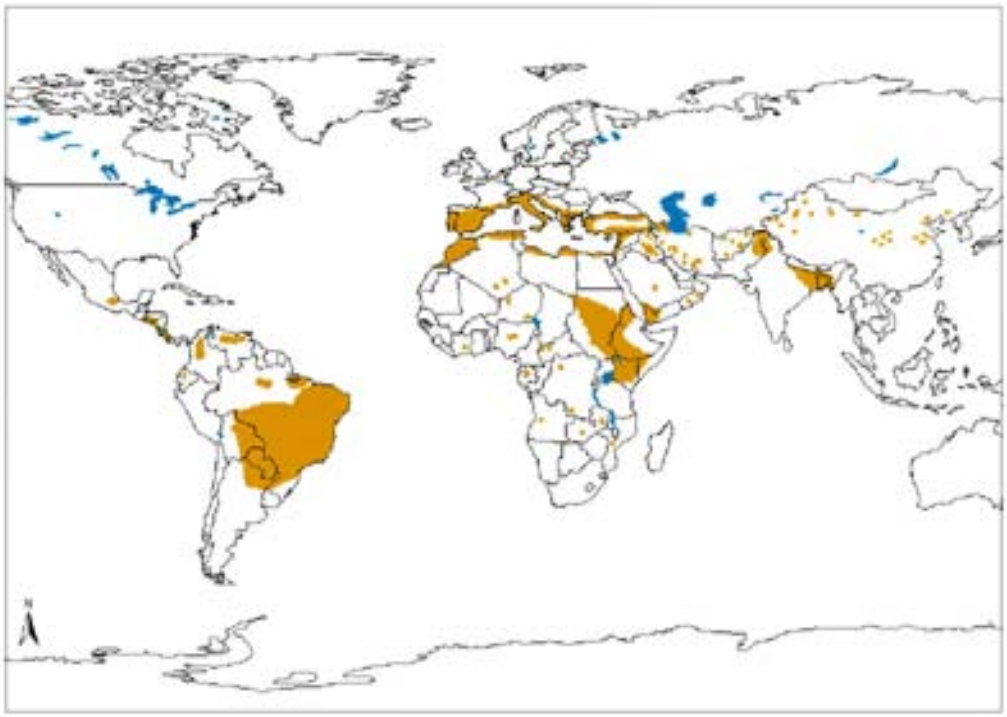


Figure 1 – **Geographical distribution of leishmaniasis in the Old World (Europe, Asia, and Africa) and in the New World (America).** (http://www.who.int/leishmaniasis/leishmaniasis_maps/en/)

Cutaneous leishmaniasis (Figure 2a) – this term describes the mildest form of leishmaniasis. It is characterized by sores appearing within a few weeks at the infected sandfly bite location. Sores can evolve into ulcers. These lesions disappear by themselves within a few months but leave permanent scars.

Mucocutaneous leishmaniasis (Figure 2c) – this manifestation of leishmaniasis is also known as "Espundia". Mucocutaneous disease is due to extension of local skin disease, similar to the cutaneous form, into the mucosal tissue via direct extension, blood stream or lymphatics. Lesions appear mainly in the oral and nasal mucosa and occasionally in the laryngeal and pharyngeal mucosa. This form is the less common one. The symptoms of mucocutaneous leishmaniasis appear within months to years after the first cutaneous lesion. If non-treated in a short delay, this clinical form can be really disfiguring and life-threatening.

Visceral leishmaniasis (Figure 2b) – a form also called Kala-Azar. In this case, parasites infect and multiply in cells of various organs such as spleen, liver, bone narrow and lymph nodes. This tropism is responsible for the following symptoms: fever, weight loss, hepatosplenomegaly, lymphadenopathy and pancytopenia. Kala-Azar leads to death if untreated because of massive hemorrhages or secondary infections. The incubation period usually varies from 3 to 8 months.

b) Epidemiology

At least 20 species of *Leishmania* are human pathogens. However, these species have typical geographic repartitions and provoke most of the time only one type of leishmaniasis.

Cutaneous leishmaniasis – This is the form of leishmaniasis the most widely spread with approximately twelve million individuals' suffering worldwide and 1 - 1.5 million new cases are estimated each year [15]. However, the great majority of cutaneous leishmaniasis is found in Afghanistan, Algeria, Brazil and Syria [1]. Two parasites' species are mainly responsible for it, *Leishmania major* and *Leishmania tropica* even if some other species have already been demonstrated as the etiologic agent of the disease such as *Leishmania aethiopica* and several subspecies of *Leishmania mexicana*. *L. tropica* is responsible for epidemics in large cities of the Middle-Orient and Central Asia due to its anthroponotic characteristic. *L. major* is rather involved in zoonotic outbreaks in rural areas [15].

Mucocutaneous leishmaniasis – There is no accurate number of related cases of this form of the disease. However, this kind of leishmaniasis is almost only observable in the South American continent where it is caused by *Leishmania braziliensis braziliensis* [21].

Visceral leishmaniasis – From 300 000 to 500 000 new cases of Kala-Azar are estimated each year which provoke the death of 70 000 persons mainly in India, Bangladesh, Sudan and Brazil [15]. Visceral leishmaniasis is also observable around the Mediterranean Sea. Two species are responsible

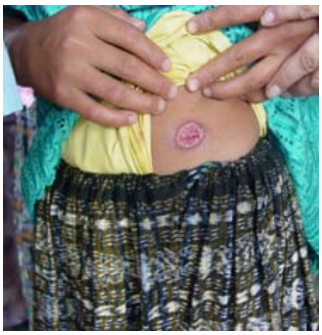


Figure 2a

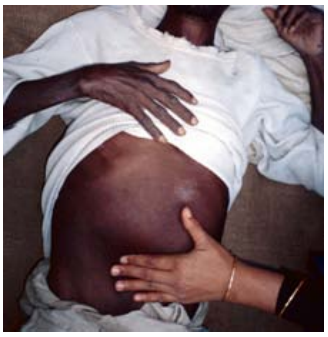


Figure 2b

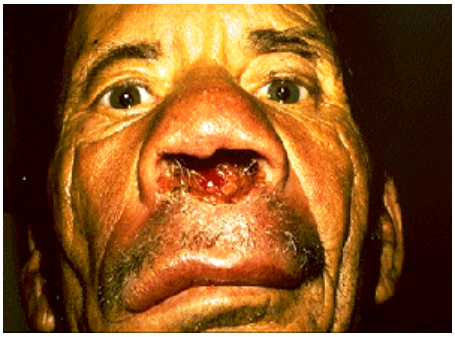


Figure 2c

Figure 2: **a** - Typical ulcerative lesion resulting from cutaneous infection. (Credit: B. Arana, MERTU, Guatemala). **b** - Splenomegaly occurring after a visceral leishmaniasis/Kala-azar infection. (Credit: C, Bern, CDC).(http://www.cdc.gov/parasites/leishmaniasis/disease.html),
c - Nasal mucosa destruction provoked by a mucocutaneous leishmaniasis/Espundia disease. (http://web.stanford.edu/group/parasites/ParaSites2003/Leishmania/leish%20web.html)

for this disease, *Leishmania donovani* and *Leishmania infantum*. *L. donovani* is strongly present in East Africa and India whereas *L. infantum* is mainly found in Middle-Orient, South America and in countries around the Mediterranean Sea [15]. As its name suggests, *L. infantum* is responsible for infections in children or immunodeficient persons whereas *L. donovani* infects people of all categories. Infections with *L. infantum* are the result of a zoonotic transmission whereas *L. donovani* is anthroponotic [15].

2) *Leishmania*'s Characteristics

a) Life cycle

Leishmania parasites are dimorphic microorganisms (Figure 3a). They realize a metamorphosis when they pass from the invertebrate vector to the mammalian host and the opposite [8]. Inside its insect vectors, which are sandflies from *Phlebotomus* genus in the Ancient World (i.e. Europe, Asia and Africa) and sandflies from *Lutzomyia* genus in the New World (i.e. America), the parasite is under the promastigote form (procyclic and metacyclic). Promastigotes are motile with a single anterior flagellum and have a spindle-shaped body. Their size is from 10 to 15 micrometer (μm) in length and from 1.5 to 3.5 μm at their widest part (Figure 3b) [8]. *Leishmania* parasites use mammalian species as host or reservoir such as canines, rodents, marsupials and the human being as well. Thus, inside the mammalian host, *Leishmania* is under the amastigote form. Amastigotes are non-flagellated spherical cells with a diameter ranging from 2 to 3 μm (Figure 3c) [8].

Insect Vector – Only female sandflies realize the bloodmeal and can thus accommodate the parasite. Insects get parasites into their amastigote forms from the infected blood of the mammalian host during a bloodmeal. The amastigote migrates to the sandfly midgut, where it transforms into the promastigote in a process that takes about 3 days [8]. After a period of days to weeks, as a result of replication by means of binary fission and subsequent migration to the foregut of the insect, the promastigotes partially obstruct the digestive tract of the insect [8]. When the infected sandfly takes a second bloodmeal, it regurgitates infectious promastigotes into the bloodstream of the future mammalian host.

Human host – *Leishmania* parasites establish a safe niche of differentiation and multiplication within phagocytic cells, mainly the macrophages. Infectious metacyclic promastigotes are quickly phagocytosed by macrophages. Parasites are able to resist to antimicrobial activity and modify to its favor the macrophage's metabolism. Indeed, these parasites, as other intracellular pathogenic microbes lead to the formation of a non-functional or partially functional phagolysosome which is permissive for *Leishmania*'s development. Different forms of leishmaniasis are due to the different cellular tropisms of the parasite species.

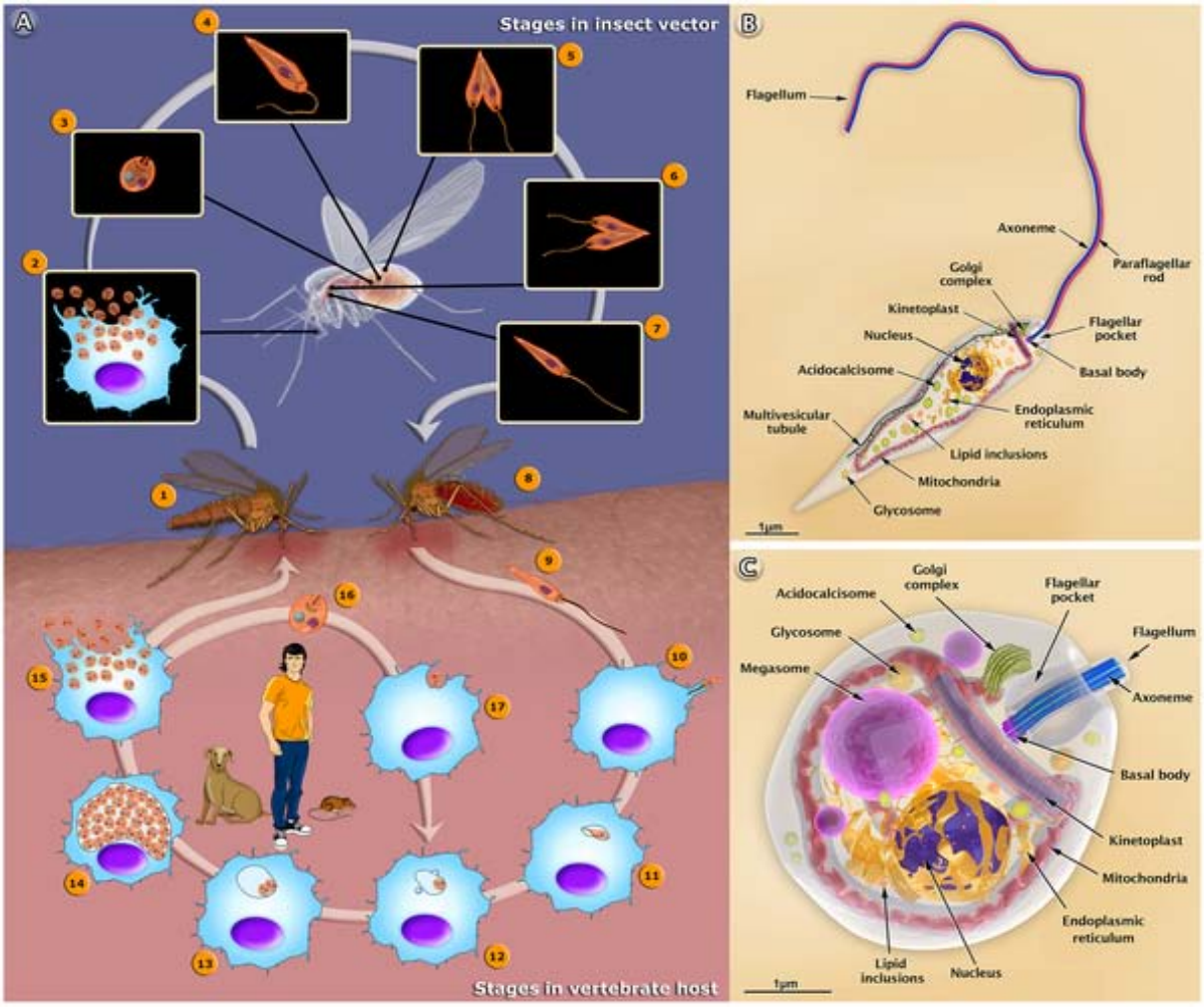


Figure 3 – **a.** Different life-cycle stages of a *Leishmania spp.* inside the insect vector and a mammalian host. **b.** Metacyclic promastigote stage of a *Leishmania spp.* parasite. **c.** Amastigote stage of a *Leishmania spp.* parasite. (http://www.scoop.it/t/tropical-diseases/p/4009089568/2013/10/11/plos-pathogens-the-cell-biology-of-leishmania-how-to-teach-using-animations)

b) Most studied virulence factors

Because of the two different forms of the parasites, they aren't harboring the same molecules at their cellular surface [16]. However, both parasitic forms must resist to innate immunity effectors before entering macrophages. One of the main innate immunity effectors that *Leishmania* has to resist to is the complement. It is composed of more than 30 serum proteins recognizing carbohydrates' motifs responsible for the phagocytosis, cell lysis and inflammatory signaling [7]. *Leishmania* disposes of various molecules that help it to avoid the formation of the Membrane Attack Complex (MAC) in order to be internalized safely by phagocytic cells.

Metacyclic promastigotes – Major molecules present on this form of the parasite are the LipoPhosphoGlycane (LPG) and the Leishmanolysin (GP63 – an extracellular protease) [12] [13]. Promastigotes are entirely covered by LPG, with around 5 million copies per cell which form a dense glycocalyx [16]. GP63 is also abundant on the promastigote membrane. Both contribute to the resistance of the parasite to the complement mediated lysis. They also permit a complement mediated phagocytosis which is devoid of the usual oxidative burst occurring after a direct phagocytosis. Promastigotes are also directly phagocytosed, and they achieve to block the assembly of the Nicotinamide Adenine Dinucleotide PHosphate complex (NADPH) at the leishmaniophorous phagosome membrane [16]. NADPH is the enzymatic complex responsible for the reactive oxygen compounds' production. Another important role of the LPG molecules is the delay of dissociation of the periphagosomal F-actin which acts as a physical barrier to prevent the phagolysosomal fusion and the inclusion of the acidification pump V-ATPase at the phagosomal membrane [16]. Therefore, promastigotes employ molecular mechanisms, still under active investigation, which ensure the modification of the phagolysosome microbicidal properties so that they can survive, differentiate and replicate in an otherwise destructive biochemical environment.

Amastigote – Once the promastigotes differentiate to the amastigote form, the parasites still possess the GP63 at their surface but they possess another type of phosphoglycan instead of LPG [16]. They are phagocytosed like the promastigotes but they can also be opsonized by immunoglobulin G (IgG). Physical characteristics are not the only differences between metacyclic promastigote and amastigote, metabolic properties also differ. Indeed, there is no need for the amastigote to prevent neither the phagolysosomal fusion nor the V-ATPase fusion because this parasitic form develops in an acidic environment comprised from a pH from 4.5 to 5.5 and is resistant to lysosomal enzymes [8]. However, the amastigotes also block the NADPH formation in order to avoid the oxidative burst [14]. Thus, the amastigote form is perfectly adapted to macrophages' environment and can then replicates, lyses the host cell and carries on the infection to a neighboring cell.

3) Phosphatases as newly discovered Virulence Factors

As elaborated above, the GP63 and molecules from the phosphoglycan family seem to be very

important for *Leishmania* to establish infection. Recent work has also highlighted the role of the flagellum in the infection by metacyclic promastigotes and sandfly saliva [6] [10]. However many other virulence factors exist. Recent studies, based on the comparison of pathogenic bacteria' phosphatomes (i.e. analysis of the all phosphatase genes) and some protozoan parasites' phosphatomes revealed sequence homologies as well as similarities in metabolic activities [4]. A group of these microorganisms, shared phosphatase sequences belonging to the atypical Dual-Specificity Phosphatase family sharing a P-loop motif (characteristic for tyrosine phosphatases) in their catalytic site [4]. Interestingly, *Leishmania major*, a *Leishmania* specie that causes cutaneous leishmaniasis, has in its genome two phosphatases with sequence homologies with the *Mycobacterium tuberculosis* protein tyrosine phosphatase (MptpB), a very important virulence factor for the *M. tuberculosis* survival in the macrophage remodeled phagolysosome [2]. MptpB is an excreted virulence factor which exhibits both Serine/Threonine phopshatase activity together with the Tyrosine phosphatase activity plus a phosphoinositide phosphatase activity [3]. *Mycobacterium* profits from these abilities to disrupt phosphoinositide metabolism in order to block phagolysosome biogenesis and also disrupt several normal host signaling pathways involving mitogen-activated protein kinases (MAPKs), Interferon-γ (IFN-γ), calcium signaling and apoptosis [11]. Because of the sequence similarities shared between these two phosphatases in the two organisms and the lack of knowledge about how *Leishmania* is responsible for the same cellular effects, we hypothesize that *Leishamnia* may use these enzymes for the establishment of infection in the macrophages. In the *Leishmania donovani* complex (i.e. *L. donovani* and *L. infantum*), that causes visceral leishmaniasis, the genes coding for these enzymes were identified during the *Leishmania* genome sequencing projects in the chromosomes 22 and 33 and were registered with the accession numbers LDBPK_220120 and LDBPK_332990. In the work of the intracellular parasitism group at the Hellenic Pasteur Institute, the proteins coded by these genes were called *Ld*PIPh22 and *Ld*PIPh33 for *Leishmania donovani* Phosphoinositide Phosphatase at chromosome 22 or 33.

The purpose of my internship was to begin the structural and functional characterization of the LDBPK_332990 gene product. We chose to clone this phosphatase from *L.infantum*, the most closely *Leishmania spp.* related to *L.donovani,* because previous efforts to clone the *Ld*PIPh33 coding gene from the *L. donovani* strain used in the lab (LG13 Ethiopian strain) gave a product with a stop codon before the predicted stop codon of the LDBPK_332990 gene, probably due to strain variations. The *L. infantum* PIPh33 protein is predicted to have only four amino acid differences with the *L. donovani* PIPh33 (See annex 1). For this, I started by amplifying the *piph33* gene by PCR from genomic DNA of *L. infantum* GH12 parasites and cloning of the PCR product into 2 different expression vectors (pTriex1.1 & pF4X1 – 4sat – *mcherry*) (Figures 4a&b respectively) for expression of the enzyme in bacteria, mammalian and *Leishmania* cells with different molecular tags.

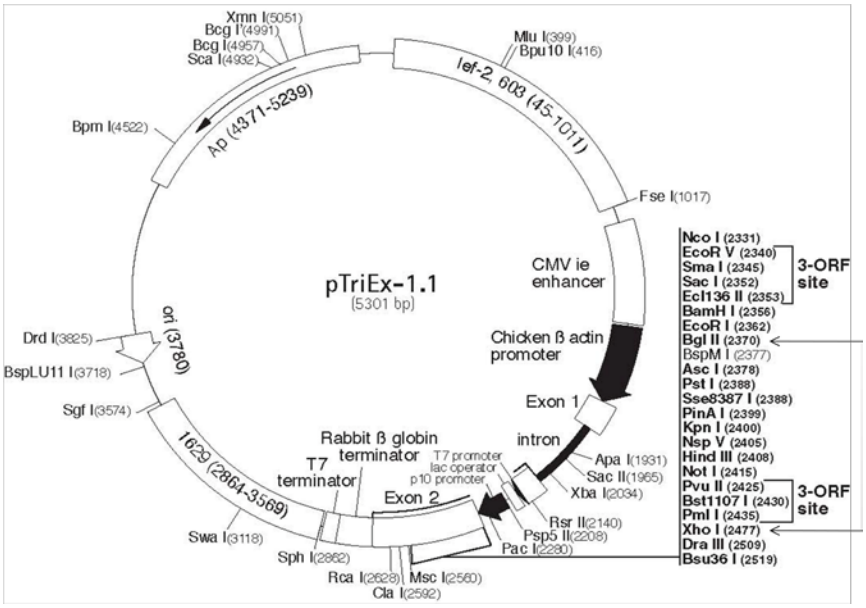


Figure 4a – **Sequence and map of the pTriex1.1 plasmid.** The *piph33* gene will be inserted between the BglII and the XhOI RE sites (see arrows). This vector is appropriate for expression in bacteria and mammalian cells.

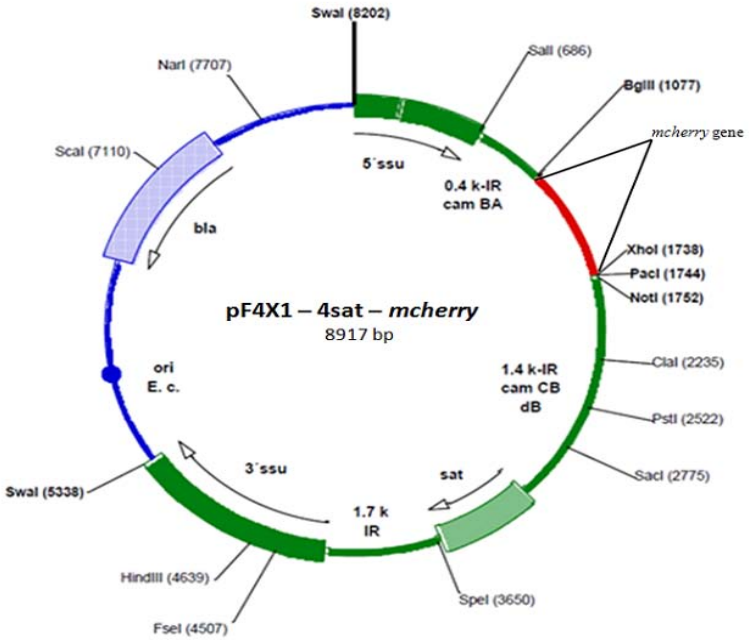


Figure 4b – **Sequence and map of the pF4X1 – 4 sat – *mcherry* plasmid.** The *mcherry* gene was inserted between the BglII and the XhOI RE sites. The *piph33* gene will be inserted upstream to the *mcherry* gene at the BglII RE site only. The pF4X1 – 4 sat plasmid is appropriate for expression in *Leishmania* cells.

II) Materials & Methods

1) PCR amplification of the LinJ.33.2990 (*piph33* gene) from *Leishmania infantum* GH12 genomic DNA

PCR reactions were realized in a MJ Research PTC-200 Thermal Cycler (GMI, Minnesota, USA) using the KAPA HiFi PCR kit (Kapa Biosystems, Massachusetts, USA). Two different amplification reactions were performed using one forward and two different reverse primers containing specific restriction enzyme sites that were added at the 5' and the 3' ends of the PCR products (Table 1). Amplification of the *piph33* gene was realized in two steps. Genomic DNA was first used as template and at a second step purified PCR products of the *piph33* gene were used as template to realize a final amplification. The PCR conditions (i.e. Temperatures and concentrations of Mg^{2+} or DMSO) were established in 50µl reactions (Tables 3a and 3b). Preparative PCRs were performed in 100µl reaction volumes. The PCR protocols for the two different products prepared in this work are shown in Tables 2a and 2b.

2) Restriction enzyme (RE) digestions

a) RE digestions of PCR products

PCR products of the *piph33* sequence containing the RE sites at the 5' and 3' ends were digested in 60µl final reaction mix containing DNA solution (8µg-16µg of DNA) and 6µl of (10X) H Buffer (Takara Bio Inc., Shiga, Japan). The 5' BglII/3' BglII and 5' BglII/3' XhOI fragments were digested with the BglII RE (Takara Bio Inc., Shiga, Japan) or the BglII and XhOI (Takara Bio Inc., Shiga, Japan) enzymes together respectively (Table 4a). The enzyme 10X buffer volume should not exceed the 1/10th of the reaction volume (final glycerol concentration <10%v/v). For each reaction, 3-5U of enzyme ($10U.\mu l^{-1}$) per µg of DNA was added. Digestions were performed at 37°C for 20 hours.

b) RE digestions of expression plasmids

The pTriex-PIPh22 plasmid was digested with the BglII and XhOI REs (Takara Bio Inc., Shiga, Japan) to remove the *piph22* insert. The restriction enzyme digestion was performed in 40µl final reaction volume according to the protocol described in table 4b. Digestion was performed at 37°C for 20 hours.

The pF4X1 – 4sat – *mcherry* plasmid was digested with the BglII RE (Takara Bio Inc., Shiga, Japan) to open the plasmid at this restriction site. Digestion was set up in a 30µl final reaction as described in table 4b. Digestion was also performed at 37°C for 20 hours.

Table 1 – **Primers used for PCR amplifications**

Primers	Sequences
5’ BglII Lin*piph33*A (Forward)	5’- GAAGATCTACCATGACCTCCGTCATTCAGCGCC -3’
3’ BglII Lin*piph33*B (Reverse)	5’- GAAGATCTTCCTGATCCCTCTAGCATCAGCTCTCGC -3’
3’ XhOI Lin*piph*33 (Reverse)	5’- CCGCTCGAGCTCTAGCATCAGCTCTCGC -3’

Table 2a – **Genomic DNA based PCR protocol**

Steps	Temperatures (C°)	Times	Number of cycles
Initial denaturation	95	3 minutes	-
Denaturation	98	20 seconds	
Primer annealing	52	30 seconds	5
Extension	72	1 minute	
Denaturation	98	20 seconds	
Primer annealing	63	30 seconds	30
Extension	72	1 minute	
Final extension	72	5 minutes	-
Cooling	4	∞	

Table 2b – **PCR product based PCR protocol**

Steps	Temperatures (C°)	Times	Number of cycles
Initial denaturation	95	3 minutes	-
Denaturation	98	20 seconds	
Primer annealing	60	30 seconds	35
Extension	72	1 minute	
Final extension	72	5 minutes	-
Cooling	4	∞	

3) Purification of DNA fragments

a) Purification of digested PCR products and digested plasmids

Both digested PCR products were first analyzed by electrophoresis on a 1%w/v agarose gel in 1X TBE buffer (89mM Tris base, 89mM Boric acid, 2 mM EDTA). Digested pTriex1.1 plasmid and digested pF4X1 – 4sat – *mcherry* were analyzed by electrophoresis in the same 1X TBE buffer in a 1% w/v or a 0.8%w/v agarose gel respectively. The entire amount of digested PCR product and plasmid were loaded in one big size well. The band corresponding to the fragment of interest was then excised and the DNA in the gel slice was purified either with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) according to the manufacturers' recommendations or by using the Costar® Spin-X® centrifuge tube filters kit (Sigma – Aldrich Corporation, Saint-Louis, USA). The DNA solution obtained after centrifugation of the Spin-X column (10,000 rpm, 10 min at 4°C) was mixed with an equal volume of a phenol/chloroform solution (1:1 ratio) and centrifuged at 10,000 rpm for 1 min. The upper aqueous phase was recovered and mixed with an equal volume of chloroform. The solution was then centrifuged at 10,000 rpm for 1 min and a standard ethanol precipitation was realized with the upper phase by adding 1/10 of the volume of the upper phase a solution of sodium acetate (CH_3COONa , 3M, ph 5,2) and by adding 2,5 of the volume of the upper phase of ethanol 100% as well. The samples were left at -20°C overnight, then centrifuged for 15 min at 14,000 rpm at 4°C, the pellets were washed with 70% ethanol and the same centrifugation step was performed again. Supernatants were once again discarded, pellets were dried in a Thermo Scientific™ SpeedVac™ (Thermo Fisher Scientific Inc., Waltham, USA) and the DNA was resuspended in 20µl of deionised super clean water (dH_2O)

4) Ligation reactions

a) Ligation of pTriex1.1 plasmid with the 5'BglII – *piph33* – 3'XhOI fragment

Ligation reaction of the purified digested with BglII and XhOI pTriex1.1 plasmid and the 5'BglII – *piph33* – 3'XhOI PCR fragment was realized in a 15µl final volume reaction mix using the 10X Ligase Buffer (Kapa Biosystems, Massachusetts, USA) and the KAPA Ligase (Kapa Biosystems, Massachusetts, USA). Two different types of ligations were performed with the same quantity of plasmid. The first one had a vector to insert molar ratio (Equation 1) of 1:3 (Transformation n°1) and the second of 1:4 (Transformation n°2) (Table 5a). These ligations were performed for 4 hrs at room temperature on the bench (~ 25°C) (Table 5b). Two controls were realized as well. The first control consisted of a ligation reaction with vector and insert but without the ligase and the second one was a ligation reaction with just the vector without the insert.

Table 3 – **PCR reaction mix (Genomic DNA used as template)**

Reaction mix	Volume (µl)	Volume (µl)	Volume (µl)	Final concentration
dH_2O	33.0	30.0	30.5	
Buffer KAPA Hifi 5X	10.0	10.0	10.0	1X
dNTPs (10 mM)	1.0	1.0	1.0	200µM
Oligo 5' **BglI 10µM**	2.5	2.5	2.5	0.5µM
Oligo 3' **XhOI 10µM**	2.5	2.5	2.5	0.5µM
Template	0.5	0.5	0.5	3.25ng/reaction
DMSO	-	3	-	
Mg2+	-	-	2.5	
Enzyme KAPA Hifi	0.5	0.5	0.5	50U/reaction
Final Volume	**50.0**	**50.0**	**50.0**	

Table 3b – **PCR reaction mix (PCR product used as template)**

Reaction mix	Volume (µl)	Volume (µl)	Volume (µl)	Final concentration
dH_2O	33.0	30.0	30.5	
Buffer KAPA Hifi 5X	10.0	10.0	10.0	1X
dNTPs (10 mM)	1.0	1.0	1.0	200µM
Oligo 5' **BglI 10µM**	2.5	2.5	2.5	0.5µM
Oligo 3' **XhOI 10µM**	2.5	2.5	2.5	0.5µM
Template	0.5	0.5	0.5	15ng/reaction
DMSO	-	3	-	
Mg2+	-	-	2.5	
Enzyme KAPA Hifi	0.5	0.5	0.5	50U/reaction
Final volume	**50.0**	**50.0**	**50.0**	

b) Ligation of pF4X1 – 4sat – *mcherry* plasmid and 5'BglII – *piph33* – 3'BglII fragment

Ligation reaction of purified BglII digested pF4X1 – 4sat – *mcherry* plasmid and 5'BglII – *piph33* – 3'BglII fragment was realized as described in the previous section (Method II-4a) in a 12µl reaction volume. Two different ligations were performed with the same quantity of plasmid. The first one was realized using a vector to insert molar ratio 1:5 and the second with a ration of 1:6 (Table 5a).

5) Transformation of *E. coli* chemically competent cells with ligation reactions or with plasmid DNA

The entire quantity (15µl) of the ligation reaction products were added to a 100µl *E. coli* XL1 Blue chemically competent cells' suspension. These samples were incubated on ice for 30 min. The mixtures were then transferred to 42 °C for exactly 45 sec to increase the membrane fluidity and were immediately chilled on ice for 10 min. One ml of Lysogeny Broth (LB) was added to each sample and the cells were incubated at 37 °C for 1 hr to allow the expression of the antibiotic resistance marker (β-lactamase) encoded by the plasmid. The cells plus the 1 ml of LB were centrifuged for 1 min at 14,000 rpm. The supernatants were removed and the pellets were resuspended in 100 µl of LB. The entire volume of the samples was spread on plates of agar-LB medium with ampicillin (50µg.ml^{-1}). To finish, the plates were incubated at 37°C for 20 hrs.

6) Isolation of plasmid DNA (mini prep and midi prep) from bacteria

Plasmid DNA was isolated from a 3 ml overnight bacteria culture started from a single colony. A volume of 1.5 ml was transferred to an eppendorf tube and bacteria were pelleted by centrifugation (14,000 rpm, 15 min, at 4°C). The bacteria pellet was resuspended with 100µl resuspension buffer (P1 =50mM Tris-Cl, pH 8.0, 10mM EDTA) (© QIAGEN, Hilden, Germany) containing RNase A (Thermo Fisher Scientific Inc., Waltham, USA). Then, 200µl of P2 lysis buffer (200mM NaOH, 1% SDS) (© QIAGEN, Hilden, Germany) were added and the tubes were inverted 3-5 times and left at room temperature for 4 min. Then 150µl of P3 neutralization buffer (3.0 M potassium acetate, pH 5.5) (© QIAGEN, Hilden, Germany) was added; the tubes were inverted once and left on ice for 10 min. A centrifugation step was then performed at 14,000 rpm at 4°C for 10 min and the supernatants were collected in new tubes. Plasmid DNA was precipitated by the addition of 2 volumes absolute chilled (-20°C) Ethanol and collected by a centrifugation (14,000 rpm, 10 min, 4°C). The supernatant was removed and the pellet was resuspended in 20 µl dH_2O. A volume of 5µl from the plasmid DNA solution was analyzed by electrophoresis in a 1% w/v agarose gel (prepared as in 3a). Clones which seem to contain the plasmid construction in regard to their size on the agarose gel were then digested for 2 hrs at 37°C with BglII and XhOI RE. Each sample was added to a 15µl reaction mix containing 2µl of 10X H buffer, 0.3µl of BglII and XhOI RE respectively and 12.4µl of dH_2O were added to obtain the 20µl final reaction volume. Once more, after the 2 hrs digestion, the entire volume of the

Table 4a – **Restriction enzyme digestion of PCR products**

RE digestion of *piph33* – 5'BgllII/3'XhOI fragment		**RE digestion of *piph33* – 5'BglII/3'BglII fragment**	
Reaction Mix	**Volume (µl)**	**Reaction Mix**	**Volume (µl)**
Template	29.0	Template	29.0
10X H Buffer	6.0	10X H Buffer	6.0
BglII RE ($10U.\mu l^{-1}$)	3.0 – 5.0	BglII RE ($10U.\mu l^{-1}$)	3.0 – 5.0
XhOI RE ($10U.\mu l^{-1}$)	3.0 – 5.0	-	-
dH_2O	15.0 – 19.0	dH_2O	20.0 – 22.0
Final Volume	**60.0**	**Final Volume**	**60.0**

Table 4b – **Restriction enzyme digestion of expression plasmids**

RE digestion of pTriex plasmid		**RE digestion of pL – 4sat – *mcherry* plasmid**	
Reaction Mix	**Volume (µl)**	**Reaction Mix**	**Volume (µl)**
Template	2.0	Template	2.0
10X H Buffer	4.0	10X H Buffer	3.0
BglII RE ($10U.\mu l^{-1}$)	1.0	BglII RE ($10U.\mu l^{-1}$)	2.0
XhOI RE ($10U.\mu l^{-1}$)	1.0	-	-
dH_2O	32.0	dH_2O	23.0
Final Volume	**40.0**	**Final Volume**	**30.0**

Equation 1: **Calculation of the vector to insert molar ratio**

$$Amount\ of\ insert\ (ng) = \frac{Quantity\ of\ vector\ (ng)\ x\ Size\ of\ the\ insert\ (kb)}{Size\ of\ the\ vector\ (kb)\ x\ insert{:}\ vector\ ratio}$$

positive clones were analyzed by electrophoresis in 1X TBE buffer prepared as in 3.a. in a 1% w/v agarose gel. Positive clones containing a good quantity of DNA according to the electrophoresis analysis were selected to make another starter culture. In order to realize it, 100µl of the previous starter culture of the selected clones was added to 4ml of LB plus ampicillin (50µg.ml^{-1}) and the new liquid cultures were left at 37°C for 20 hrs.

7) Verification of the *piph33* sequence in the positive clones

To verify the nucleotide sequence of the selected positive clones, 3µg of the purified pTriex1.1-*piph33* plasmid and 30µl of both the 5'BglII and 3'XhOI oligos (10µM) were sent to a professional DNA sequencing company (VBC-Biotech Service GmbH, Vienna, Austria). Sequencing results were analyzed with the Clustal W2 (UCD, Dublin, Ireland) and BioEdit (Ibis Biosciences, Carlsbad, USA) softwares.

8) Expression of the *Lin*PIPh33 – 8His recombinant protein in *E.coli* BL21 cells.

pTriex1.1 – *piph33* plasmid isolated from confirmed positive XL1 Blue clones was used to transform *E. coli* BL21 competent cells. An aliquot of 100µl competent cells was transformed (section II-5a) with 300 ng of pTriex1.1-*piph33* plasmid DNA. One single colony per plate was picked as described in section II-6 and used to inoculate starter cultures of 4ml LB plus ampicillin (50µg.ml^{-1}) in 15ml flacon tubes which were incubated at 37°C overnight. The following day, a volume of 1.5ml of these cultures was transferred to 50ml fresh LB plus ampicillin (50µg.ml^{-1}) and incubated at 37°C until the optical density at 600nm (OD_{600}) reached a value 0.4 -0.5 corresponding to a bacterial density of 3.2 to 4.0 x 10^8 cells per ml. Then, 75.0µl of Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce recombinant protein expression and the samples were incubated at 37°C for 2 hrs. The bacteria were then pelleted by centrifugation (4,000 rpm, 20 min). The pellets were resuspended in 1ml 1X SDS-PAGE sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue). The sample was boiled (95^{o}C, 3 min) and the proteins were separated on a 12% w/v SDS-PAGE gel (2.9 ml dH_2O, 4ml Tris – 1M/pH 8.8, 3ml 20% acrylamide, 54µl 20% w/v SDS, 54µl 10% w/v Amonium persulfate and 10 µl TEMED) and electroblotted onto a nitrocellulose membrane (Porablot NCP, Macherey-Nagel, Düren, Germany). The blot was then blocked (1 hr, at RT) with 5% w/v milk in Tris-Buffered Saline with 0.05% v/v Tween-20 (TBST) and further incubated overnight at 4 ^{o}C with a primary mouse anti-histidine antibody diluted in TBST and washed three times with TBST (0.1% v/v Tween-20, 10 min/wash). Subsequently, the membrane was incubated for 2 hrs at RT with a secondary anti-mouse antibody conjugated to Horse Radish Peroxidase (HRP), washed three times with TBST and finally the enzyme activity was detected by a chromogenic reaction using 3,3- diaminobenzidine tetrahydrochloride (C-8890 Sigma) and H_2O_2 as substrates.

Table 5b – **Ligation reactions of pTriex1.1 plasmid and 5'BglII- *piph33* -3'XhOI fragment**

pTriex1.1/5'BglII- *piph33* -3'XhOI Vector to insert molar ratio of 1:3		**pTriex1.1/5'BglII- *piph33* -3'XhOI Vector to insert molar ratio of 1:4**	
Reaction Mix	**Volume (µl)**	**Reaction Mix**	**Volume (µl)**
Vector (350ng)	9.1	Vector (350ng)	9.1
Insert (160ng)	0.9	Insert (210ng)	1.2
10X Ligase Buffer	1.5	10X Ligase Buffer	1.5
KAPA Ligase ($5U.\mu l^{-1}$)	1.0	KAPA Ligase ($5U.\mu l^{-1}$)	1.0
dH_2O	2.5	dH_2O	2.2
Final Volume	**15.0**	**Final Volume**	**15.0**

Table 5c – **Ligation reactions of pF4X1 – 4sat – *mcherry* plasmid and 5'BglII-*piph33*–3'BglII fragment**

pF4X1 – 4sat – *mcherry*/5'BglII-*piph33*–3'BglII Vector to insert molar ratio of 1:5		**pF4X1 – 4sat – *mcherry*/5'BglII-*piph33*–3'BglII Vector to insert molar ratio of 1:6**	
Reaction Mix	**Volume (µl)**	**Reaction Mix**	**Volume (µl)**
Vector (400ng)	7.35	Vector (400ng)	7.35
Insert (175ng)	1.85	Insert (210ng)	2.3
10X Ligase Buffer	1.2	10X Ligase Buffer	1.2
KAPA Ligase ($5U.\mu l^{-1}$)	1.0	KAPA Ligase ($5U.\mu l^{-1}$)	1.0
dH_2O	0.6	dH_2O	0.15
Final Volume	**12.0**	**Final Volume**	**12.0**

III) Results

1) Cloning of the the LinJ.33.2990 (*piph33*) gene into bacteria and *Leishmania* expression vectors (pTriex1.1 and pF4X1 – 4sat – *mcherry*)

The open reading frame of the gene encoding the *Lin*PIPh33 protein (GenBank access number: CAM71428.1), a putative phosphoinositide and tyrosine phosphatase (dual specificity phosphatase), was amplified from *Leishmania infantum* GH12 genomic DNA according to the protocol described in tables 2a, 2b and 3.

a) For cloning into the pTriex1.1 vector

PCR amplification of the *piph33* gene using the 5'BglII and 3'XhOI primers (Table 1) was performed in order to insert the product into the pTriex1.1 vector (Figure 4a) at the 5' end site of a sequence coding for 8 Histidine moieties. The anticipated size of the PCR product (789 base pairs) was verified by electrophoresis of the PCR reaction on a 1%w/v agarose gel (Figure 5a). We detected a main abundant PCR product at ~ 790 bp and a second weak with an approximate size of 1600 base pairs (bp).

b) For cloning into the pF4X1 – 4sat – *mcherry* vector

PCR amplification of the *piph33* gene with the 5'BglII and 3'BglII primers was performed in order to insert it into the 5' end of the *mcherry* gene into the pF4X1 – 4sat – *mcherry* vector (Figure 4b). The anticipated size of the PCR product (795 base pairs) was detected by electrophoresis analysis on a 1%w/v agarose gel (Figure 5b). As in the case of the 5'BglII- *piph33* -3'XhOI fragment, we observed a main abundant band at ~800 bp and a less abundant at ~ 1400 bp.

These two PCR reactions seemed to need extra magnesium to be efficient (Figure 5a – lane 1). In both cases the PCR products of ~800 bps from 300µl amplification reactions were purified as described in Methods II-3 and gave a yield of 9.5 µg of 5'BglII – *piph33* – 3'XhOI product and 16.74 µg of 5'BglII – *piph33* – 3' BglII product.

2) Restriction enzyme (RE) digestion of inserts and vectors and purification of the digested PCR DNA fragments

a) 5'BglII – *piph33* – 3'XhOI and 5'BglII – *piph33* – 3'BglII PCR products

The 5'BglII – *piph33* – 3'XhOI and 5'BglII – *piph33* – 3' BglII PCR products (9.5 µg and 16.74 µg respectively) were subjected to digestion by the BglII and XhOI or BglII alone restriction enzymes as described in Methods II-2a. The entire amount of the digested 5'BglII – *piph33* – 3'XhOI and 5'BglII – *piph33* – 3' BglII fragments were purified on a 1% agarose gel as described in the

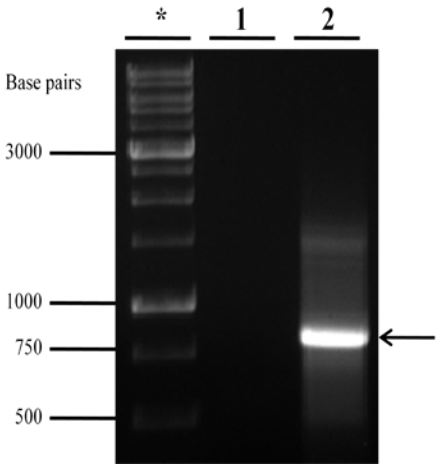


Figure 5a

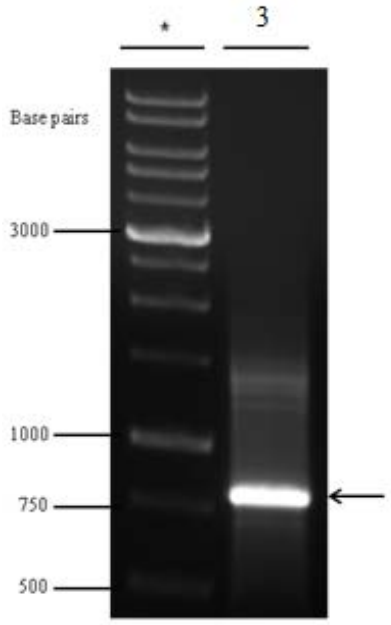


Figure 5b

Figure 5 – **Amplification of the *piph33* gene by PCR.** Analysis of the preparative PCR reaction products by agarose gel electrophoresis (1% w/v agarose in TBE buffer, 1 h at 120V). The *piph33* gene was amplified from *L. infantum* GH12 genomic DNA using the 5'BglII and 3'XhOI oligonucleotides (Figure 5a) and the 5'BgllI and 3'BglII oligonucleotides (Figure 5b) according to the protocol described in the methods' section II – 1. The main PCR product (arrow) had the expected size for the *piph33* (i.e. 780 bp). (*) 1 kb DNA Ladder RTU (5.0 µl). (1) 15.0 µl of the PCR reaction mix without extra Mg^{2+} + 3µl of 6X Loading Buffer. (2) (3) 15.0 µl of PCR reaction mix with extra Mg^{2} plus 3µl of 6X Loading Buffer.

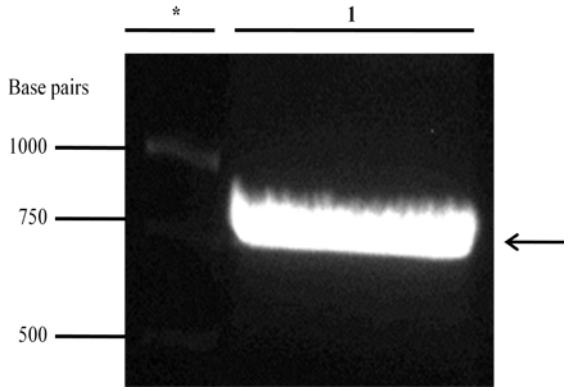


Figure 6 – **Purification on agarose gel of the digested BglII – *piph33* – XhOI PCR product.** The entire volume of the digested PCR product was loaded in a big size well in an agarose gel electrophoresis (1% w/v agarose in TBE buffer) and analyzed for 1:30 hrs at 120V. The *piph33* gene was amplified from the PCR product using the 5'BglII and 3'BglII oligonucleotides according to the protocol described in the methods' section II – 3a. The band corresponding to the digested PCR product (arrow) was excised for further purification of the DNA fragment. (*) 1 kb DNA Ladder RTU (5.0 µl). (1) 60 µl of the digested BglII – *piph33* – XhOI PCR product plus 12µl of 6X Loading Buffer.

methods sections II-2a and II-3a (Figure 6); and yielded 2.54μg and 2.69μg of 5'BglII – *pip33* – 3'XhOI and of the 5'BglII – *pip33* – 3' BglII inserts respectively for further ligation with the pTriex1.1 vector or pF4X1 – 4sat – *mcherry* vector respectively digested with the same restriction enzymes as the corresponding inserts.

b) pTriex1.1 and pF4X1 – 4sat – *mcherry* plasmids

The amounts of 10μg of the pTriex1.1 and 20μg of pF4X1 – 4sat – *mcherry* plasmids were digested with the restriction enzymes BglII/XhOI or BglII alone respectively and purified according to the protocol described in section II-3b (Figures 7 a&b). The purification provided 2.30 μg and 5.09 μg of purified digested pTriex1.1 and pF4X1 – 4sat – *mcherry* plasmids respectively for further ligations with the 5'BglII – *pip33* – 3'XhOI or the 5'BglII – *pip33* – 3' BglII digested insert.

3) Ligation reactions

a) Ligation of pTriex1.1 plasmid and 5'BglII- *piph33* -3'XhOI

Two different ligation reactions were realized at a vector to insert molar ratio of 1:3 and 1:4 according to the protocols described in section II-4a. A volume of 5μl of each ligation reaction, 250 ng of vector alone (lane 1) and of ligation reaction mix without ligase (lane 4) as controls were analyzed by electrophoresis on a 1% w/v agarose gel (Figure 8). The multiple bands for both vector to insert molar ratios (lanes 2 and 3) suggest successful ligation. However, the yield of the ligated vector insert seems to be low.

4) Transformation of *E. coli* with ligation reactions and selection of positive bacterial colonies

a) Transformation of *E. coli* XL1 Blue cells with the pTriex1.1-*piph33* (product of ligation 4a)

Transformation of chemically competent *E. coli* XL1 Blue cells was performed according to the protocol described in Methods paragraph II-5a. Bacterial colonies grown on Amp LB-agar plates after overnight incubation at 37°C were detected for both ligation reactions performed with the two different vector to insert molar ratios. The transformation realized with the 1:3 molar ratio ligation mix (Transformation n°1), yielded 61 colonies while the ligation with 1:4 molar ratio (Transformation n°2) yielded 34 colonies (Figure 9).

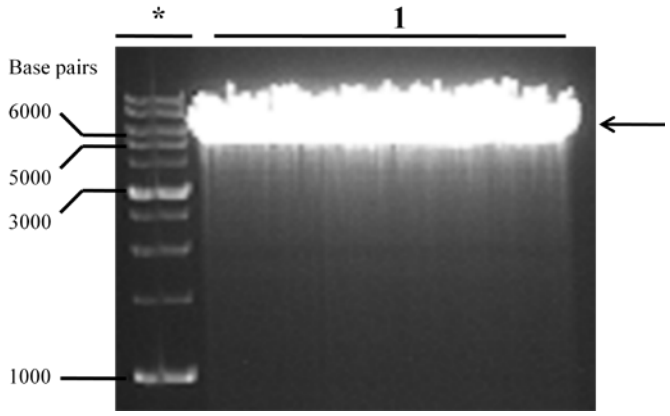


Figure 7a – **Purification on agarose gel of the digested pTriex1.1 plasmid**. The entire volume of the digested plasmid was loaded in a big size well in an agarose gel (1% w/v agarose in TBE buffer) and analyzed by electrophoresis for 1:30 h at 120V. The pTriex1.1 plasmid was digested using the BglII and XhOI restriction enzymes according to the protocol described in the Methods' section II – 3b. The band corresponding to the digestion product (arrow) was excised for further purification of the DNA fragment. (*) 1 kb DNA Ladder RTU (5.0 µl). (1) 40 µl of the digested pTriex1.1 plasmid plus 8 µl of the 6X Loading Buffer.

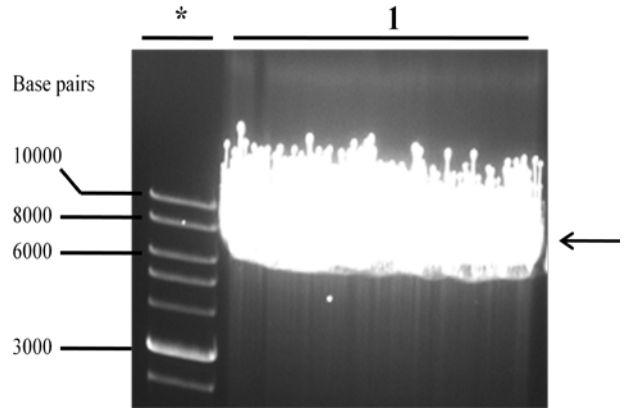


Figure 7b – **Purification on agarose gel of the digested pF4X1 – 4sat – *mcherry* plasmid.** The entire volume of the digested plasmid was loaded in a big size well in an agarose gel (0.8% w/v agarose in TAE buffer) and analyzed by electrophoresis for 1:30 hr at 120V. The pF4X1 – 4sat – *mcherry* was digested with the BglII restriction enzyme according to the protocol described in the methods' section (pg II – 3b). The band corresponding to the digestion product (arrow) was excised for further purification of the DNA fragment. (*) 1 kb DNA Ladder RTU (5.0 µl). (1) 50 µl of the digested pF4X1 – 4sat – *mcherry* plasmid plus 10 µl of 6X Loading Buffer **.**

5) Selection of *E. coli* colonies carrying the pTriex1.1-*piph33* plasmid. Isolation of positive clones.

A number of 22 colonies were picked with pipette tips from both LB agar plates (Figure 9). Eleven colonies were taken from the Transformation n°1 plate and 11 from the Transformation n°2 plate. A small (3ml) liquid bacteria culture (i.e Luria Broth + Ampicilin) was inoculated with each of these colonies and was incubated for 20 hrs at 37°C (protocol described in section II-6). High turbidity of the medium indicated a bacterial growth in all the starter cultures except for clone 21 from the Transformation n°2. Subsequently plasmid DNA was isolated from each of these colonies as described in Methods paragraph II-6. Electrophoresis analysis in a 1% w/v agarose gels of the plasmid DNA isolated from each of the putative positive colonies revealed that only clones 3, 4, 7, 9, 10, 11, 13, 14, 15, 16, 17, 20 and 22 seemed to contain the pTriex1.1 – *piph33* plasmid (Figure 10). RE digestions with the BglII and XhOI enzymes were then performed on the plasmid DNA from these clones and these reactions were analyzed by another electrophoresis in order to reveal the presence of the pTriex1.1 plasmid and the 5'BglII – *piph33* – 3'XhOI insert. According to the size of the DNA fragments detected, the pTriex1.1 plasmid and the *piph33* gene were present in clones 3, 7, 8, 9, 11, 14, 15 and 20 (Figure 11). The clones 9, 11, 14 and 15 seemed to have yielded the largest amount of plasmid DNA.

6) Preparation of pTriex1.1 – *piph33* plasmid DNA for sequencing and bacteria glycerol stocks from the positive clones for storage.

A volume of 4 ml liquid *E. coli* XL1 Blue cultures were inoculated with 100µl from 9, 11, 14 and 15 starter cultures (mentioned in the previous paragraph) and grown overnight at 37°C. These cultures were used to prepare pTriex1.1 – *pip33* plasmids with the NucleoSpin Plasmid MiniPrep kit (Macherey-Nagel, Düren, Germany) as described in section II-6. The purified plasmids were once more analyzed by electrophoresis on agarose gels (Figure 12). The plasmid preparations yielded 11.63 µg, 13.91 µg, 14.7 µg and 9.2 µg for the clones 9, 11, 14 and 15 respectively. Two glycerol stocks of 1 ml (30% glycerol) were prepared from bacteria cultures of each clone and stored at -80°C.

Plasmid DNA from clones 11 and 14 were sent for sequencing to the VBC Biotech (VBC-Biotech Service GmbH, Vienna, Austria), a professional company offering DNA sequencing services. The sequencing results were analyzed with the Clustalw2 algorithm and proved that, for the clone 11, the *pip33* sequence is 100% identical to the LinJ.33.2990 sequence and that the histidine tag coding sequence is in frame with the *piph33* sequence at its 3' end (Figure 13). For the clone 14, a mutation seemed to have occurred at position 687 of the *piph33* gene. This mutation consisted on a nucleotide substitution of a C by a T base (Figure 14). However, translation of the clone 14 sequence in amino acids and comparison with the *Lin*PIPh33 protein sequence revealed a 100% identity in the amino acid sequence which supports the fact that this substitution will not change the amino acid composition of the recombinant protein (Figure 16).

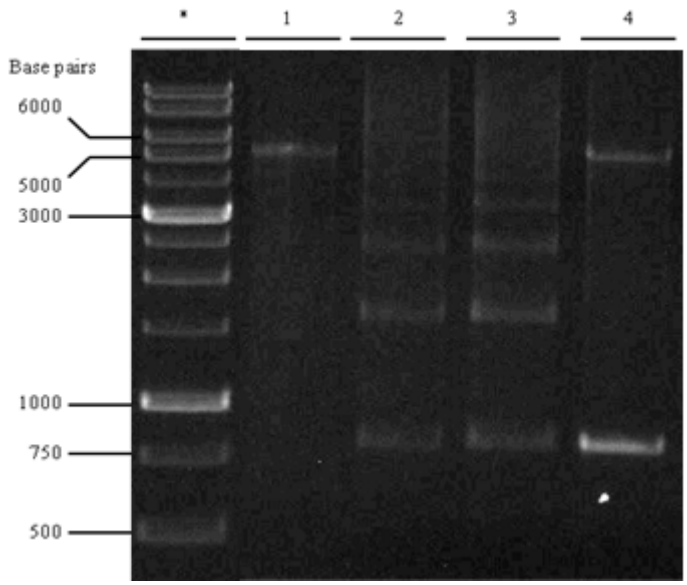


Figure 8 – **Ligation reaction of pTriex plasmid and 5'BglII – *pip33* – 3'XhOI fragment.** Analysis of the ligation reactions' products by agarose gel electrophoresis (1% w/v agarose in T**B**E buffer,1 h, 120V). (*) 1 kb DNA Ladder RTU (5.0 µl). (1) 5.0 µl of pTriex plamsid plus 1.0 µl of 6X Loading Buffer. (2) 5.0 µl of the 1:3 ligation reaction product. (3) 5.0 µl of the 1:4 ligation reaction product. (4) 5.0 µl of ligation reaction mix without enzyme.

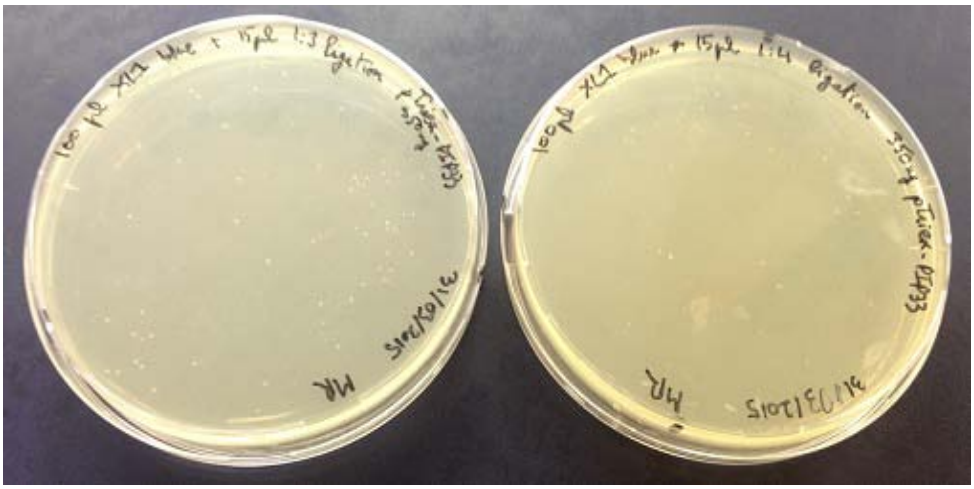


Figure 9 – **Transformation of *E. coli* with the products obtained from the ligatin of the pTriex1.1 plasmid with the 5'BglII – *piph 33* – 3'XhOI fragment.** Chemically competent XL1blue cells were transformed with 15 µl from the two different vector to insert molar ratio ligations of the pTriex1.1 – *piph33* plasmid. Photography of the LB ampicillin plates on which were plated transformed XL1 Blue cells after incubation for 20 hrs at 37°C. On the left, XL1 Blue cells transformed with the ligation with 1:3 vector to insert molar ratio. Thirty four colonies were counted. On the right XL1 Blue cells transformed with the ligation with 1:4 vector to insert molar ratio, 61 colonies were counted.

7) Expression of the *LinPIPh33*-His protein in BL21 *E. coli* cells.

We then proceeded to examine the expression of the *LinPIPh33*-His protein from clones 11 and 14 of the pTriex1.1 – *piph33* plasmid in the BL21 *E. coli* recombinant strain that expresses the T7 polymerase. BL21 chemically competent cells were transformed with the pTriex1.1 – *piph33* clone11 and 14 plasmids (Methods II-8) and plated on an LB-agar ampicilin plate. A positive bacteria colony was selected from each plate and used to inoculate 5 ml cultures that were grown overnight at 37^{o}C. The following day, the cultures were diluted with 1:10 with LB-Ampicilin medium to 50 ml cultures and when the OD_{600} reached 0.6, IPTG (0.5 mM final concentration) was added and the BL21 bacteria from clones 11 and 14 were incubated for 2hrs at 37°C to express the recombinant protein. At the end of this, 1ml of cells was withdrawn centrifuged, and the bacteria pellets were resuspended in 100 µl 1XSB and prepared for SDS-PAGE electrophoresis (Methods II-8). The rest of the bacteria in the 50 ml culture were pelleted and the pellets were frozen at -20^{o}C for further analysis. The bacteria proteins present in the small samples of the cultures were further transferred from the gel to a nitrocellulose membrane which was probed with an anti-His antibody to reveal the recombinant His-tagged proteins. Two protein bands were revealed by the antibody. One corresponds to the expected molecular weight of ~30 kDa (calculated molecular weight is 30.753 kDa) and the other band with a smaller size, ~ 29 kDa (Figure 15).

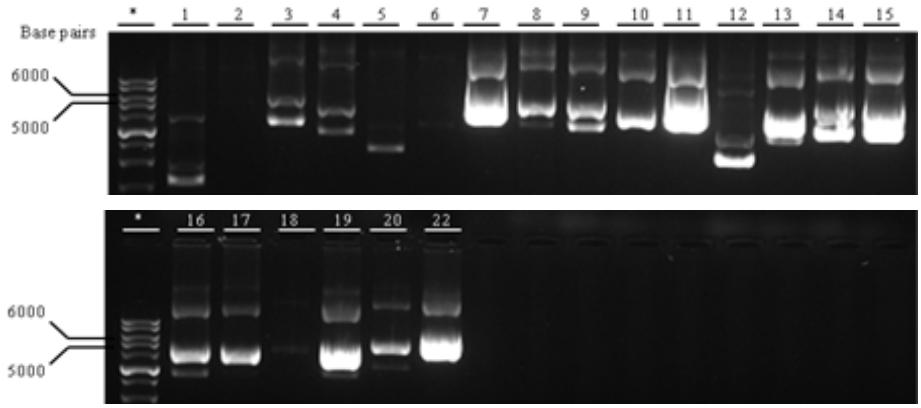


Figure 10 – **Analysis of the pTriex1.1 – *pip33* plasmid DNA from positive bacteria colonies.** Analysis of the prepared pTriex1.1 – *pip33* plasmid from mini cultures of the selected positive colonies by agarose gel electrophoresis (1% w/v agarose in TAE buffer) for 1:30 h at 120V. (*) 1 kb DNA Ladder RTU (5.0 µl). Numbers correspond exactly to the name of each clone. The volume of 5.0µl plus 1.0 µl of 6X Loading Buffer pTriex1.1 plasmid and *piph33* gene seem to be present only in clone 3, 7, 8, 9, 11, 14, 15 and 20.

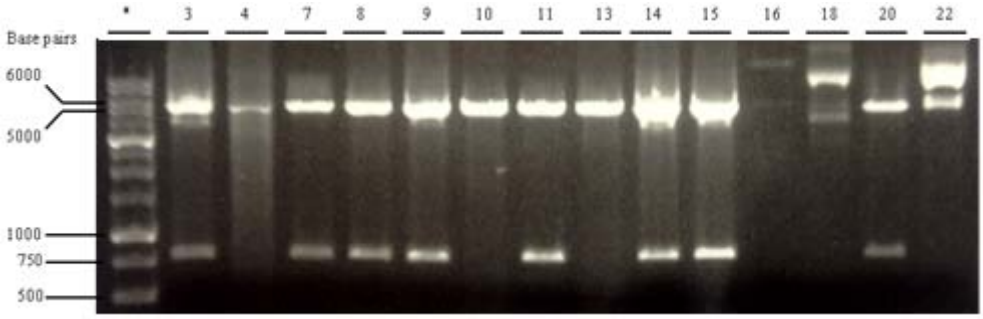


Figure 11 – **RE digestions of the potential pTriex1.1-*pip33* positive clones with BglII and XhOI REs.** Analysis of the prepared pTriex1.1 – *pip33* plasmid of the previously selected clones digested for 2 hours at 37°C with BglII and XhOI REs by agarose gel electrophoresis (1% w/v agarose in TAE buffer) for 1:30 h at 120V. (*) 1 kb DNA Ladder RTU (5.0 µl). Numbers correspond exactly to the name of each clone. The entire volume of each digested sample were loaded (20.0ml) plus 4.0 µl of 6X Loading Buffer pTriex1.1 plasmid and *piph33* gene seem to be present only in clone 3, 7, 8, 9, 11, 14, 15 and 20.

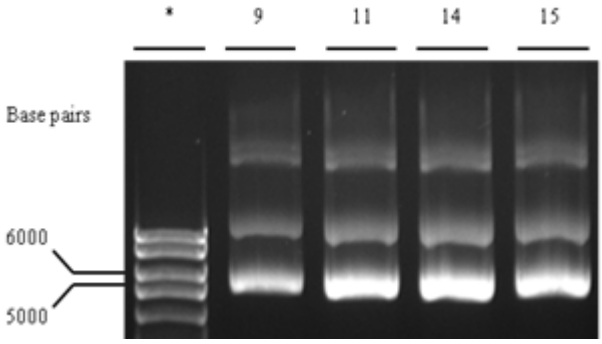


Figure 12 – **Miniprep/Plasmid preparation of positive clones 9, 11, 14 and 15 containing the pTriex1.1 – *pip33* construction.** Analysis of the prepared pTriex1.1-*pip33* plasmid from positive clones n° 9, 11, 14, 15 by agarose gel electrophoresis (1% w/v agarose in TAE buffer) for 1:00 h at 120V. (*) 1 kb DNA Ladder RTU (5.0 µl). Numbers correspond exactly to the name of each clone. DNA from clones 11 and 14 were sent for sequencing.

IV) Discussion

The work described was realized in 4 months. At the present moment, as it is imposed by the internship report deadline, half of the project purposes have been achieved. The cloning of the *piph33* gene into the pTriex1.1 vector has been performed. Sequencing of the *piph33* gene as well as the expression of the *Lin*PIPh33-His protein in the BL21 cells has been realized too. Cloning of the *piph33* gene into the pF4X1 – 4sat – *mcherry* vector was initiated as well.

PCR amplifications successfully generated a main product with the expected *piph33* gene size. Ligation of this product with the pTriex1.1 plasmid and transformation of XL1 Blue competent cells gave rise to 8 positive colonies carrying the correct size plasmid with the correct size insert. Sequencing of the insert in plasmid DNA from two clones (clones 11 and 14) revealed a 100% identity with the nucleotide sequence of the *piph33* gene for clone 11 and just only one substitution for clone 14. The substitution is probably the result of a replication error made by the T7 RNA polymerase. However, the two amino acid sequences were also identical to the *LinPIPh33* protein (Figure 16). Western blot analysis of the His-tagged proteins expressed by the two plasmids revealed a band slightly above 30 kDa, close to the expected size for the *LinPIPh33*-His – tagged protein. The lower size band probably corresponds to a proteolytic product of the *LinPIPh33*-His protein. The bacteria could have engaged a proteolytic process to inactivate the protein. Previous work realized in the "Intracellular Parasitism" research team of the Hellenic Pasteur Institute on the *piph22* gene coding for an another atypical dual-specificity phosphatase showed similar results when expressed in *E. coli*. The cloning of the *piph33* gene with the pF4X1 – 4sat – *mcherry* did not proceed as smoothly and it was not finished before the deadline of this report. Main problems were related to the low transformation efficiency of the strain TOP10F of *E. coli* competent cells used. After several unsuccessful transformation trials of these TOP10F cells with ligation reactions performed with *piph33* gene and pF4X1 – 4sat – *mcherry* plasmid, a new batch of competent cells was prepared. This time we used another strain of *E. coli* XL1 Blue cells from another laboratory of the institute. Transformations with just the vector were realized with the former TOP10F competent cells and with the new XL1 Blue competent cells and that is how we discovered the low transformation efficiency of our competent cells. I also faced problems with the purification of the pF4X1 – 4sat – *mcherry* plasmid. The purified plasmid with the NucleoSpin® Gel and the PCR Clean-up kit (Macherey-Nagel, Düren, Germany) suffered degradation as detected by a smear when it was analysed by agarose gel electrophoresis. We then used the Costar® Spin-X® centrifuge tube filters kit (Sigma – Aldrich Corporation, Saint-Louis, USA) for purification of the digested plasmid. Interestingly the pTriex1.1 plasmid did not seem to be degraded. Ligation of the BglII – *piph33* – BglII product with the pF4X1 – 4sat – *mcherry* showed low efficiency when a small difference in the number of colonies

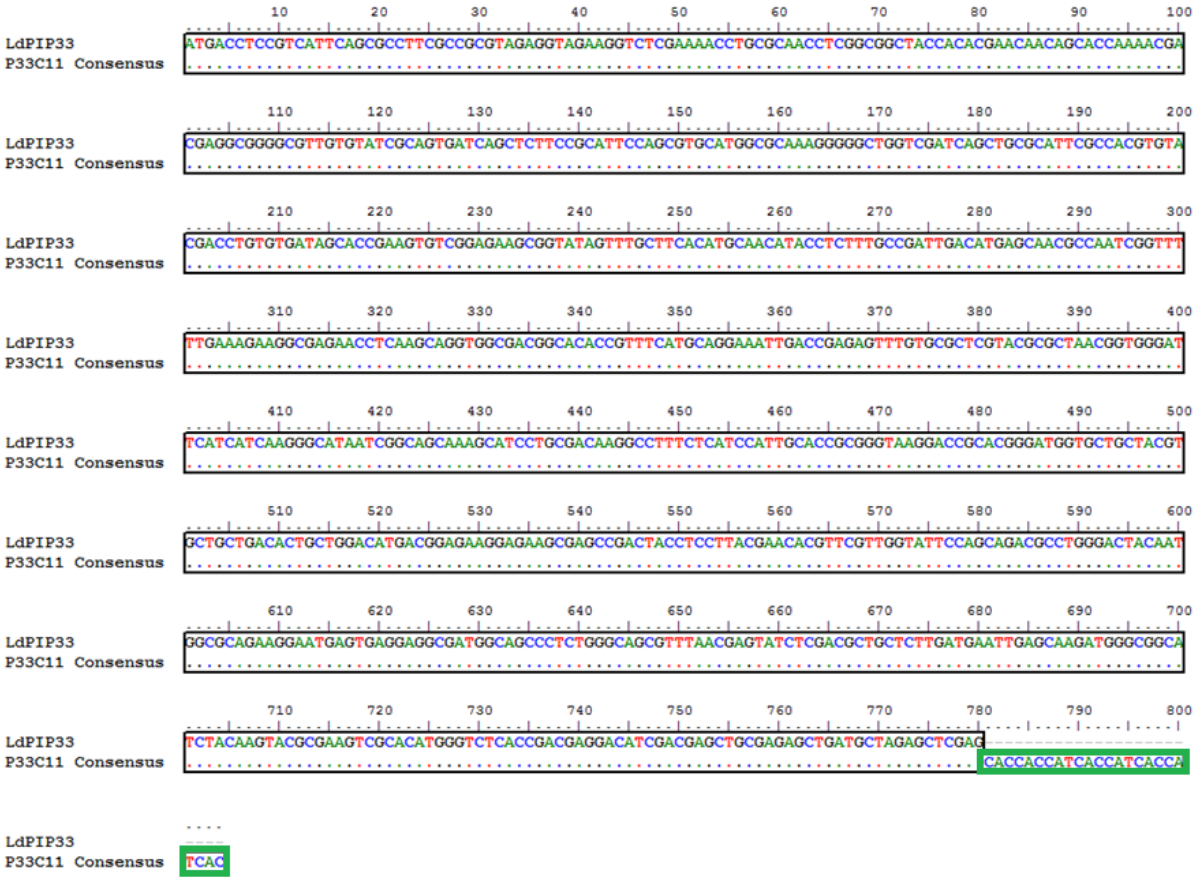


Figure 13 – **Nucleotide sequence of the *Ld*PIPh33 protein coding sequence of clone 11.** The sequence is identical to the LinJ.33.2990 gene of *Leishmania infatum*. The sequence corresponding to the histidine tag is present at the 3' end (marked by a green box).

obtained with the ligation products as compared to the control in transformations with the new batch of XL1 Blue competent cells. Therefore we tried to change the temperature and the duration of the ligation reaction. The efficiency seemed to increase with a rise of the temperature from 16°C to 25°C (RT) and a decrease of the reaction duration from 20 hours to 4 hours. This enabled us to obtain a large number of positive colonies when we used linearized and religated pF4X1 – 4sat – *mcherry* plasmid.

Cloning Remarks and Perspectives

To summarize, with regards to the pTriex1.1 – *piph33* plasmid, more experiments have to be performed to optimize the conditions of induction of the PIPh33-His protein expression in the BL21 cells. With regards to the cloning of the *piph33* gene into the pF4X1 – 4sat – *mcherry* plasmid, the problems encountered have been solved. The purified insert is available, plasmid purification has been realized efficiently, the ligation protocol has been improved and a new batch of competent cells is now available.

By the end of my training in this laboratory, until the 31th of July, I should be able to improve the expression conditions the *Lin*PIPh33-His protein using the pTriex1.1 – *piph33* plasmid, and complete the cloning of the *piph33* gene into the pF4X1 – 4sat – *mcherry* plasmid. These two plasmids will be further used in the laboratory for the characterization of the *Lin*PIPh33 protein by isolating it as His-tagged proteins from bacteria and mammalian cells and by generating transgenic *Leishmania* parasites overexpressing the *Lin*PIPh33 phosphatase.

Vector-borne diseases cause the death of one million people each year. These vector-borne diseases are also called “Neglected Tropical Diseases” (NTD). The fact is these NTDs are more and more responsible for autochthonous cases or even outbreaks in non-tropical countries. Many examples can illustrate this fact in the recent years. From 2007 to 2012, a large number of dengue and chikungunya cases were observed in France, Italy, Croatia and especially Portugal, on the island of Madeira, where more than 2.000 persons suffered from dengue fever [20]. Some years before, in 2002, a west nile virus outbreak occurred in the U.S with 4.099 confirmed cases [19]. For leishmaniasis, such events haven’t happened. However, considering the vector spreading around in Europe and the known presence of *Leishmania infantum*, we can foresee an increase of the CL prevalence in this area. Moreover, the huge flow of travelers coming back from endemic areas could permit the introduction of *Leishmania donovani* and lead to anthroponotic transmissions in new areas. Moreover, co-infection with other infectious diseases increases the risk of the illness spreading. So these NTDs, leishmaniasis included, won’t stay tropical diseases and shouldn’t be neglected as well, especially after being aware of its public health incidence in the populations of the tropical areas. Currently, many efforts are realized to find new cures, new detection methods and vaccines for leishmaniasis.

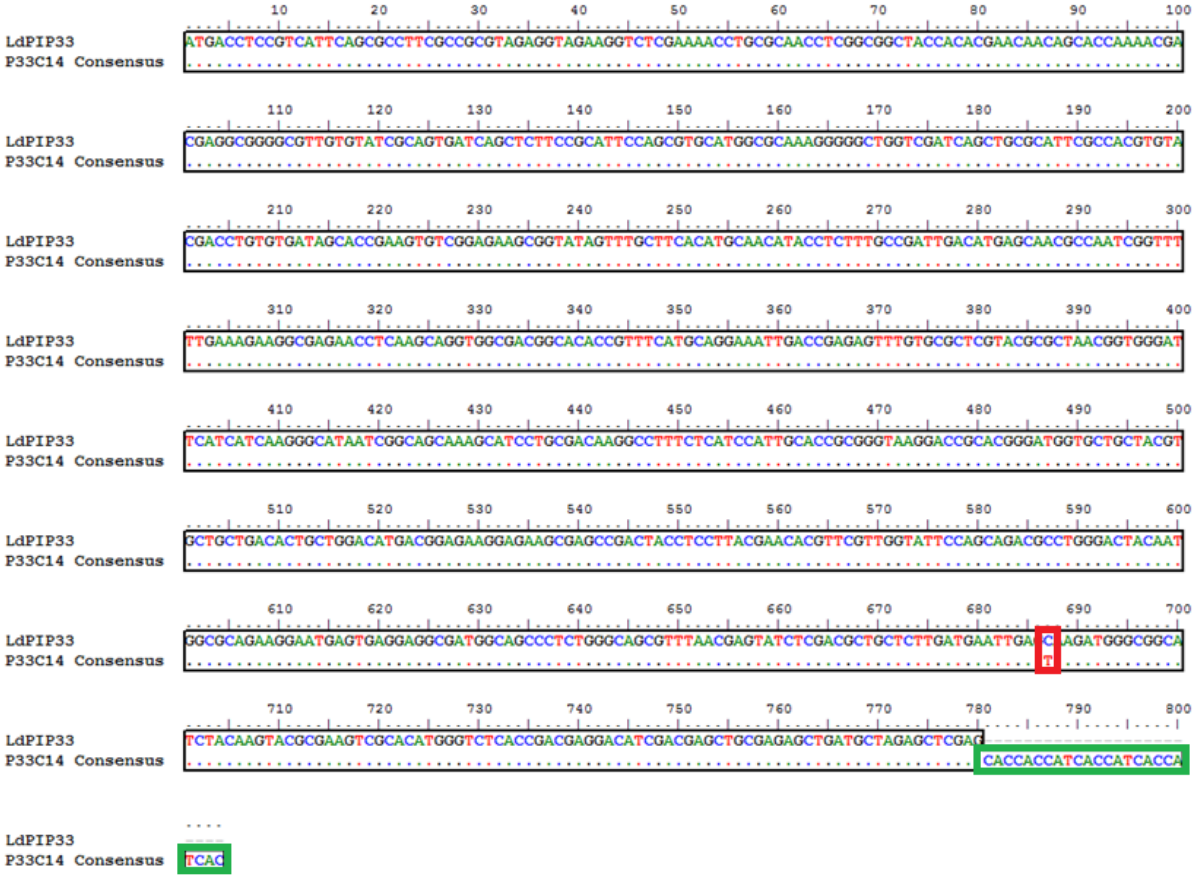


Figure 13 - **Nucleotides' sequence of the *LinPIPh33* coding sequence of clone 14.** The coding sequence has a nucleotide substitution in position 687 of a C (marked by a red box) by a T in regard to the LinJ.33.2990 gene of *Leishmania infatum*. The sequence corresponding to the histidine tag is present at the 3' end as well (marked by a green box).

However, a very little is known about the virulence factors and the weaknesses of the parasite as well as its behavior inside both the mammalian host and the insect vector. That's why it is now urgent to find efficient diagnostic techniques, efficient cures and vaccines. In order to realize all of that, it is really important to study newly discovered putative virulence factors like the aDSPs of the *Leishmania donovani* complex parasites, just as it has been realized for a few years now in the "Intracellular Parasitism" research group of the Hellenic Pasteur Institute in Athens, in the Institut Pasteur International Network and in many other research laboratories worldwide.

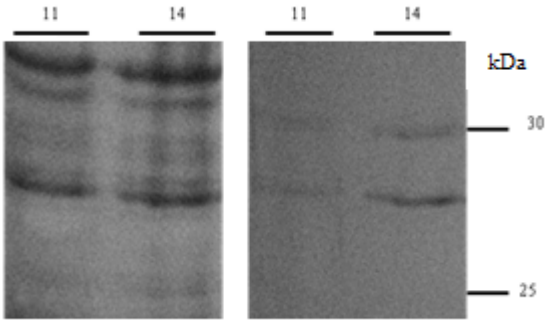


Figure 15 –**Detection of the *LinPIPh33* protein expression in BL21 clones 11 and 14 by Western Blot**. (Left) Ponceau-S stained membrane that reveals all the proteins. (Right) Western Blot that reveals the His tagged proteins. BL21 bacteria cultures from clones 11 and 14 were incubated 2hrs at 37°C in the presence of 0,5 mM IPTG ()**.** One fourth of the cells from 1ml bacteria culture centrifuged and resuspended in 1X SDS-PAGE sample buffer were loaded on each lane of a 12% w/v SDS-PAGE and the proteins were separated for 45 min at 200V. Subsequently, the proteins were transferred onto a nitrocellulose membrane for 45 minutes at 300mA. The *LinPIPh33*-His protein was detected with a primary mouse anti-histidine anti-boby and a secondary anti-mouse anti-body conjugated to Horse Radish Peroxidase (Methods section II-8).

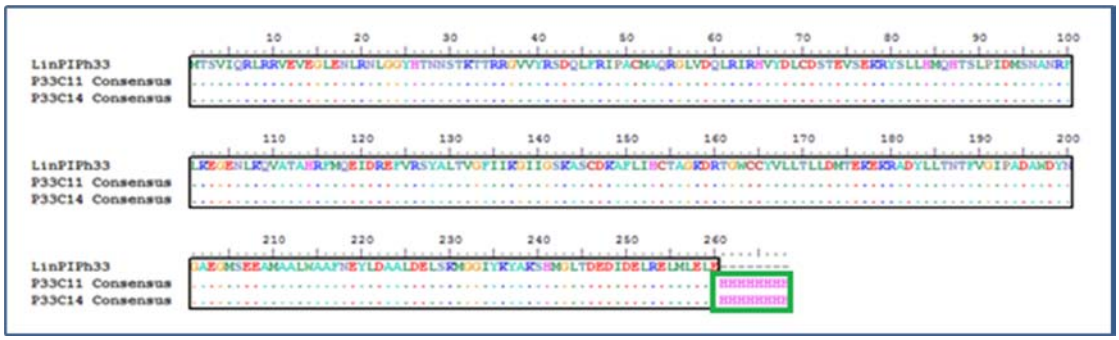
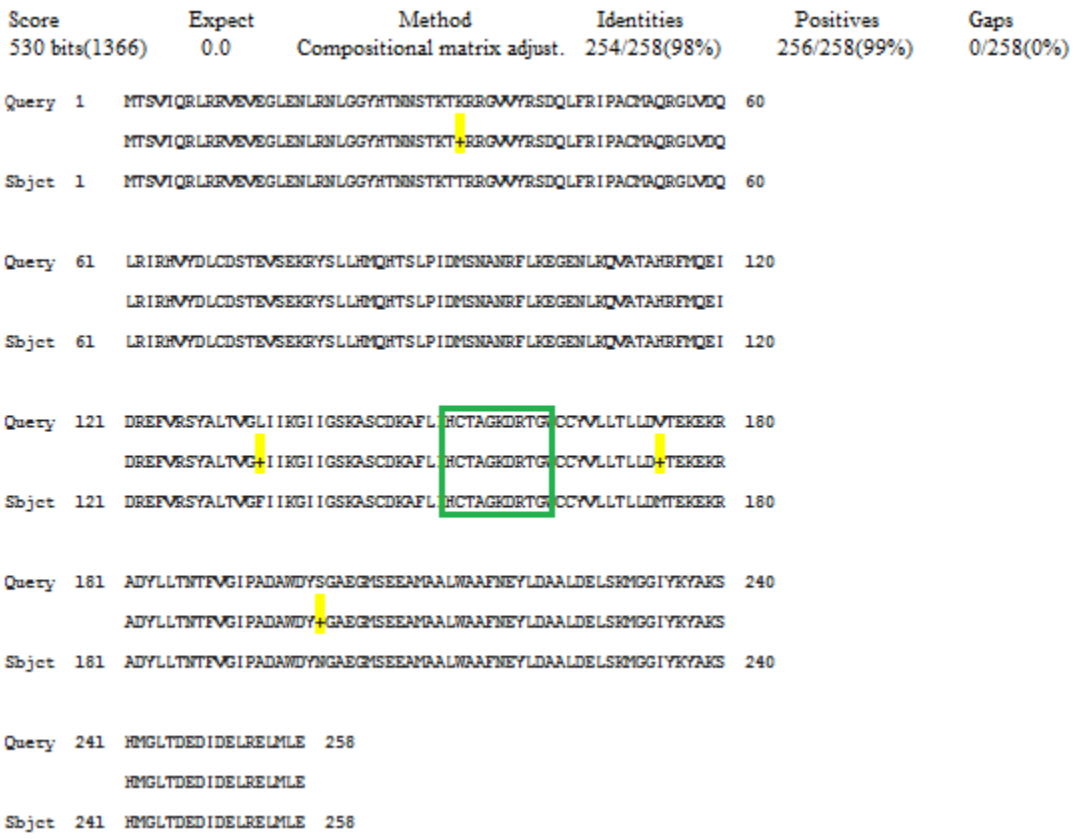


Figure 16 – **Amino acids' sequences of the *Ld*PIhP33 protein of clone 11 and clone14.** For both clones, the amino acids' sequences are 100% identical to the protein coded by the LinJ.33.2990 gene of *Leishmania infantum* with the histidine tag at the 3' ends (marked by a green box).



Annex 1 – **Protein BLAST analysis between the LDBPK_332990 gene product and its homologue in the LinJ.33.2990 in the *Leishmania infantum strain* (GenBank access number: CAM71428.1).** The protein BLAST analysis revealed only 4 amino acids' differences. None of these amino acids' substitutions are located in the P-Loop motif, the characteristic sequence of tyrosine phosphatase (marked by a green box).

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Summary

Human leishmaniasis are diseases transmitted by the bite of sandflies infected by parasites belonging to the *Leishmania* genus. Many *Leishmania* species are responsible for illnesses ranging from self-healing cutaneous lesions to visceral complication which can lead to death. More than 350 millions peoples worldwide are exposed to these pathogens. Because of the lack of cheap, non-toxic cure, easily usable diagnostic techniques and vaccine, research ais now performed on newly discovered potential virulence factors. The aim of this work was to start the characterization of one of them, the LDBPK_332990 gene product, a putative phosphoinositide/tyrosine phosphatase. For this, we cloned the *L. infantum* homologue gene into an expression vector for expression of a His – tagged – *Lin*PIP33 protein in bacteria and mammalian cells. The study of this protein is interesting in regards to its homologies with other know virulence factors of human bacteria pathogens.

Résumé

La leishmaniose humaine est une infection due à des parasites du genre *Leishmania* provoquant de simples lésions cutanées mais pouvant entamer le pronostique vitale du patient en absence de traitement. Plus de 350 millions de personnes sont exposés à ces pathogènes à travers le monde. Devant le manque de tests diagnostiques simples, de traitements non-toxiques et de vaccin, les recherches se tournent actuellement sur de nouveaux facteurs de virulence potentiels. Le but de ce travail était de commencer la caractérisation de l'un d'eux, la protéine codée par le gène LDBPK_332990, une putative phopshoinositide/tyrosine phosphatase. Nous avons cloné le gène homologue de *L. infantum* au sein d'un plasmide permettant l'expression de la protéine *Lin*PIPh33 possédant une étiquette poly-histidine dans des bactéries and des cellules de mammifères. Cette protéine est intéressante étant donnée ses homologies avec d'autres facteurs de virulence important retrouvés chez d'autres pathogènes.