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Expression for the phosphate translocator from *Toxoplasma gondii* in *Escherichia coli* and purification of the recombinant protein

Maja Jevtic

BIO- 3950, Master's thesis in Molecular environmental biology
May, 2017.

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Cover photo by

Maja Jevtic, Sample preparation for expression in different BL21 RIL clones

Table of Contents

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Abbreviations

3-PGA	3-phosphoglycerate
ATP	Adenosine Triphosphate
ACP	Acyl carrier protein
Kbp	Kilo base pairs
cDNA	Complementary deoxyribonucleic acid
dH_2O	Distilled water
DMAPP	Dimethylallyl diphosphate
DOXP	1-deoxy-D-xylulose 5- phosphate
EtOH	Ethanol
FAS II	Fatty acid synthesis Type 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Glc6P	Glucose-6-phosphate
GPI	Glucose-6-phosphate isomerase
GPT	Glucose-6-phosphate transporter
HRP	Horseradish peroxidase
IPP	Isopentenyl diphosphate
MeOH	Methanol
MVA	Mevalonate
NADPH	Nicotinamide adenine dinucleotide phosphate
PEP	Phosphoenolpyruvate
Pi	Inorganic phosphate
PPT	Phosphoenolpyruvate- phosphate transporter
pPT	Plastidic phosphate transporter
rpm	Revolutions per minute
TgAPT	*Toxoplasma gondii* apicoplast phosphate transporter
TPT	Triose phosphate transporter
tRNA	Transfer ribonucleic acid
XPT	Xylulose-5-phosphate transporter
Xyl5P	Xylulose-5-phosphate

Abstract

Plastids are plant specific cell organelles, which perform many specialized and essential functions. Integration of plastids into cellular metabolism requires an extensive exchange of metabolites between plastids and the surrounding cytosol which is catalyzed by more than 150 different transporters. One of the best-characterized families is the plastid phosphate translocator family (pPT). Proteins similar to plant pPTs (APTs) where recently identified in *Apicomplexa*, e.g. in *Plasmodium falciparum* and *Toxoplasma gondii*. Parasitic diseases caused by these species are one of the most serious medical and economic issues worldwide. The APTs show similar activities and substrate specificities as their plant counterparts. Most importantly, the disruption of the APT gene in *T. gondii* leads to immediate death of the parasite which clearly shows the importance of this transporter for growth of the parasites. Therefore, there is an ongoing project to elucidate the structure of the APT from *T. gondii* at atomic resolution and to analyze the transport mechanism at a molecular level by x-ray crystallography. To achieve that goal, the APT protein has to be expressed in a heterologous system. In this study the apicoplast phosphate transporter protein was produced by heterologous expression in *Escherichia coli*. The suitable plasmid (pET21b) was selected, covering the TgAPT gene, introduced into BL21 RIL strain of *E. coli* and the recombinant machinery was induced with IPTG. The APT protein was engineered with a Twin-Strep®-affinity tag attached to its N-terminus and was purified by affinity chromatography on Strep-Tactin® resins. The successful expression of the APT in *E. coli* offers the possibility to produce and purify the APT protein in larger quantities, and, second, to measure its transport activity directly in the bacterial cells.

Key words: *Toxoplasma gondii, E. coli* expression system, apicoplast, phosphate transporter, Twin-strep-tag

1. Introduction

1.1 The parasite *Toxoplasma gondii* causes a disease called Toxoplasmosis

Toxoplasma gondii is a member of a phylum called *Apicomplexa*. It is an obligate intercellular parasitic protozoan and the causative agent of a disease called Toxoplasmosis. Around 30-50% of the world population is infected by this parasite (Pappas 2009). It can affect any warm-blooded animal and humans. It is rarely fatal for humans, but in individuals who have a weak immune system it can be fatal and lead to death (Flegr *et al.*, 2014). Infection is often asymptomatic but immunocompetent individuals may have symptoms like fever, muscle pains, sore throat, headache and lymphadenopathy. Severe disease symptoms are usually observed only in congenitally infected children and immunosuppressed patients (Dubey 1996). Prenatally acquired *T. gondii* often infects the brain and retina causing mental retardation, while the ocular disease is the most common injury with mild symptoms consisting of slightly diminished vision. Immunosuppressed patients may have a central nervous system disease like encephalitis which is an acute infection and inflammation of the brain itself. (Tenter *et al.*, 2000).

Toxoplasma gondii causes diseases also in animals and is the major cause of abortion in sheep and goats. Cats, dogs and other pets can die of pneumonia, hepatitis and encephalitis if they were exposed to Toxoplasmosis. Humans and animals are intermediate hosts, while members of the family *Felidae* (mainly domestic cats) are the only definitive hosts, where *T. gondii* reproduces sexually (Tenter *et al.*, 2000).

The life cycle of *T. gondii* consists of three stages: sporozoites (in sporulated oocysts which are shed in feces of cats), tachyzoites (which are a rapidly multiplying form found in all tissues) and bradyzoites which are the cyst resting form (Sonar *et al.*, 2010). Infection is contracted when cats ingest either oocysts or meat containing live organisms shown in Figure 1. Bradyzoites are released in the stomach tissues containing cysts. They penetrate the epithelial cells of the small intestine and initiate the formation of asexual generations before the sexual cycle begins (Dubey 1996). The parasites then enter the intestinal epithelium and can be spread to many host tissues. Parasites infecting the intestinal epithelium produce oocysts which are shed in the feces in a non-sporulated stage. Sporulation occurs outside the body and oocyst become infectious 1-5 days after excretion. They are resistant and can survive in soil for several months (Schlüter *et al.*, 2014). Secondly, other bradyzoites in the epithelium begin to multiply as tachyzoites. Tachyzoites can enter almost any type of host cell and multiply until the host

cells are filled with parasites and finally die. Then the released tachyzoites enter a new host cell and multiply there (Dolores *et al.*, 2015).

There are three main modes of *T. gondii* transmission: congenitally, by eating raw or undercooked infected meat of sheep, goat, pigs and rabbits, and via fecal matter by ingesting food and water contaminated with oocysts from infected cat feces (Hill and Dubey 2002). The infection can also occur by organ or bone marrow transplantation (Tenter *et al.*, 2000), playing in sandboxes and school play- ground contaminated with oocysts from infected cat feces, contact with contaminated soil and gardening without gloves, contact with contaminated water, insufficient washing of contaminated vegetables and fruits (Schlüter *et al.*, 2014). Tachyzoites play the major role in the vertical transmission of *T. gondii* and they have been found in the milk of the goat. It is also assumed that the infection can occur by consumption of unpasteurized goat milk (Tenter *et al*., 2000).

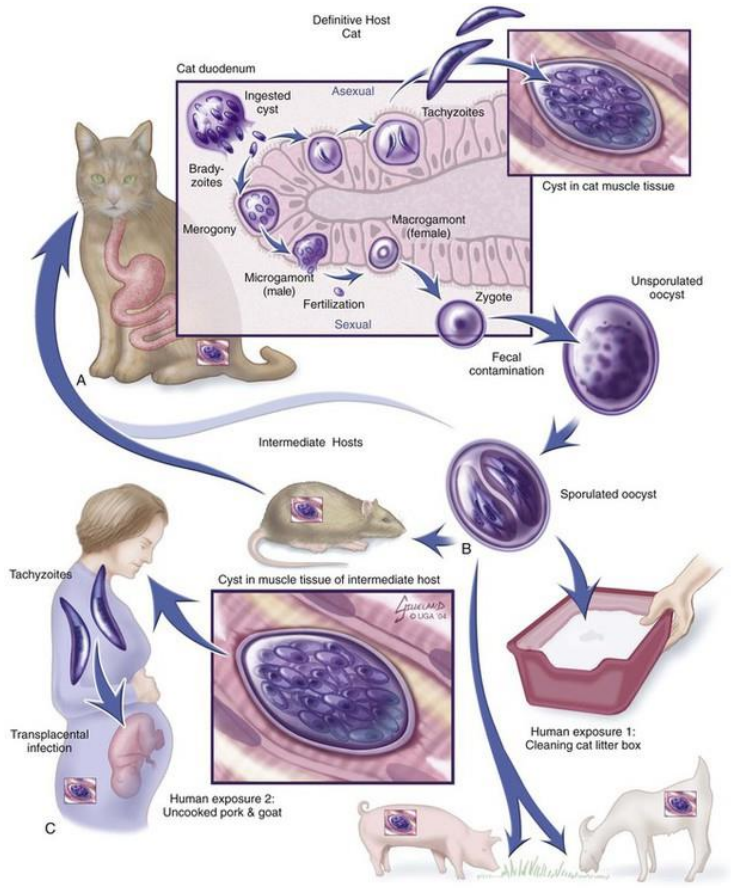

Figure 1. Life cycle of Toxoplasma gondii
A) enteroepithelial replication occurs in the cat intestine after ingestion of oocytes from fecal contamination or bradyzoites within tissue cysts. Ingested bradyzoites can also undergo merogony to form micro- and macrogamonts. An unsporulated oocyst is eventually formed after union of gamonts. **B)** the oocyst is excreted unsporulated in the feces and is noninfectious. It sporulates in the environment and becomes infectious. Then it can be ingested by a variety of intermediate hosts. **C)** muscle and tissue encystment occur in the intermediate host. In females, infected for the first time during pregnancy, congenital infection of the fetus occurs. Figure is taken from Dubey *et al*., 2009.

1.2 The phylum Apicomplexa

The phylum Apicomplexa is a large and diverse monophyletic group that comprises more than 5000 parasitic species (Figure 2) (Wiser 2011). Some of this species infect humans and several others are considered important pathogens for warm-blooded animals. Apicomplexan genera, which infect humans, are *Plasmodium* species which are the causative agent for malaria, *Cryptosporidium spp.* (cryptosporidiosis) and *Toxoplasma gondii* (toxoplasmosis) which cause benign diseases if the host has an intact immune system but can cause serious disease in immunocompromised hosts. Most important apicomplexan genera in veterinary medicine and agriculture are parasites like *Babesia spp.* and *Theileria spp*. in cattle and *Eimeria spp.* (coccidiosis) in poultry. (Beck *et al.,* 2009; Wiser 2011; Hikosaka *et al.,* 2013).

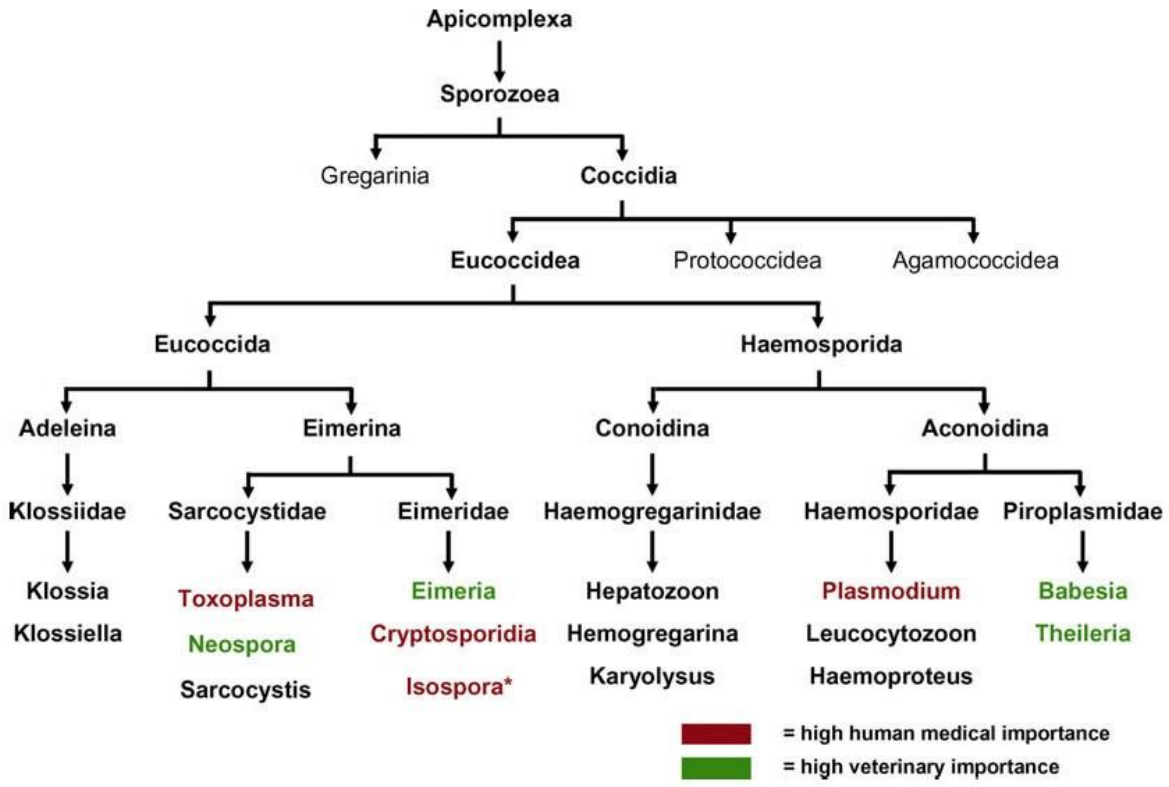

Figure 2. Phylogenetic tree of Apicomplexa. Figure is taken from Beck *et al.*, 2009.

All these parasites are obligate intracellular parasites. They live inside of host cells, which can be an animal and human cells. During their invasion, the parasites form a membrane which forms vacuole, called parasitophorous vacuole membrane in which parasites live (Lingelbach *et al.,* 1998). The cells of Apicomplexa possess the typical eukaryotic organelles like the nucleus, the Golgi apparatus, the endoplasmic reticulum, and two endosymbiotic organelles-mitochondria and plastids. The parasites also possess unique organelles like the apical complex (conoid) located at the apical end of the parasite, where the invasion occurs, and rhoptries and micronemes that play a key role in invasion (Mehlhorn 2016). The main organelles are shown in Figure 3.

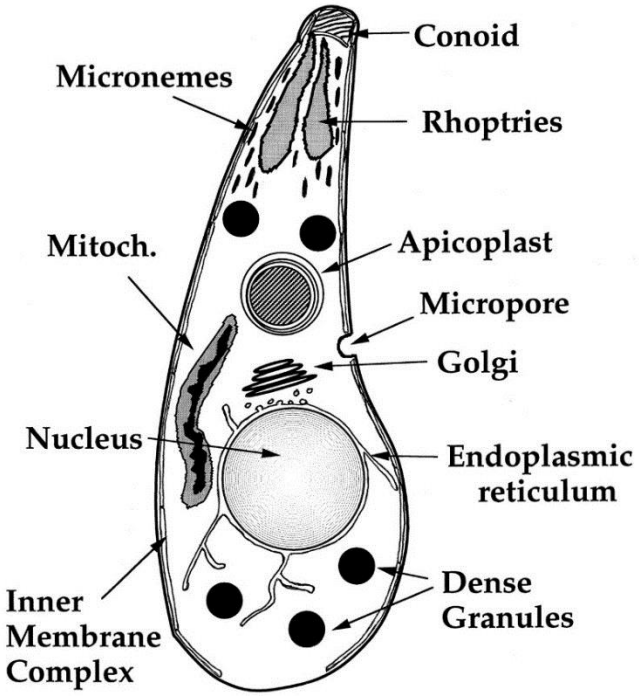

Figure 3. Main organelles in Toxoplasma gondii. Figure is taken from Black and Boothroyd 2000.

Phylogenetic analysis indicates that members of the genus *Colpodella* formed a sister group with the apicomplexan (Kuvardina 2002). Colpodellids are predatory flagellates and with the process called myzocytosis, they are feeding on unicellular algae. Myzocytosis is a feeding method, which involves attachment of the predator to the prey and sucking out the cellular content (cytoplasm) of the prey via specialized structures similar to those that apicomplexan parasites use to attach to host cells (Wiser 2011).

1.3 The apicoplast derived from a red algae chloroplast

The apicoplast is a plastid-like organelle found in parasites of the phylum Apicomplexa. It is considered to be a vestigial plastid because it is not green and it does not perform photosynthesis. The name **APICOPLAST** is derived from **APICO**mplexa + **PLAST**id (Kohler *et al.*, 1997). The apicoplast originated from a secondary endosymbiosis, i.e. a process where a eukaryotic cell engulfed another eukaryotic cell bearing plastids that had been obtained by primary endosymbiosis. In the primary endosymbiotic event, an early eukaryotic cell took up a cyanobacterium that was eventually transformed into a plastid. These primary plastids, such as those found in red and green algae, glaucophytes and plants are characterized by two surrounding envelope membranes (McFadden and Roos 1999). After primary endosymbiosis, plastids are thought to have been transferred by secondary endosymbiosis laterally into several eukaryotic lineages that normally lacked plastids (Keeling 2010). In the case of Apicomplexa, the endosymbiont was a red alga (Janouskovec *et al*., 2010).

The indication of secondary endosymbiosis is the existence of three or four membranes surrounding the plastids (Gould *et al*. 2008). For example, it has been shown that the apicoplast in *T. gondii* has four envelope membranes (Figure 4) (Kohler *et al.*, 1997; McFadden *et al.*, 2016). The four envelope membranes have different origins. First, the outer membrane of the apicoplast is homologous to the host endomembrane system. The second membrane originates from the plasma membrane of the red alga. The two inner membranes correspond to the envelope membranes of the primary plastid (Fast *et al*., 2001).

The apicoplast is considered to be essential for the survival of the parasite. Beside its basic metabolic processes such as DNA replication, transcription and translation (Brooks *et al*., 2011; Dahl and Rosenthal 2008), they also have enzymes involved in anabolic pathways like the synthesis of fatty acids, isoprenoids and haem (Ralph *et al*., 2004). These pathways are fundamentally different from the equivalent eukaryotic pathways of the animal or human hosts and that is why apicoplasts are interesting to study as potential drug targets (McFadden *et al.*, 1996; Brooks *et al*., 2011).

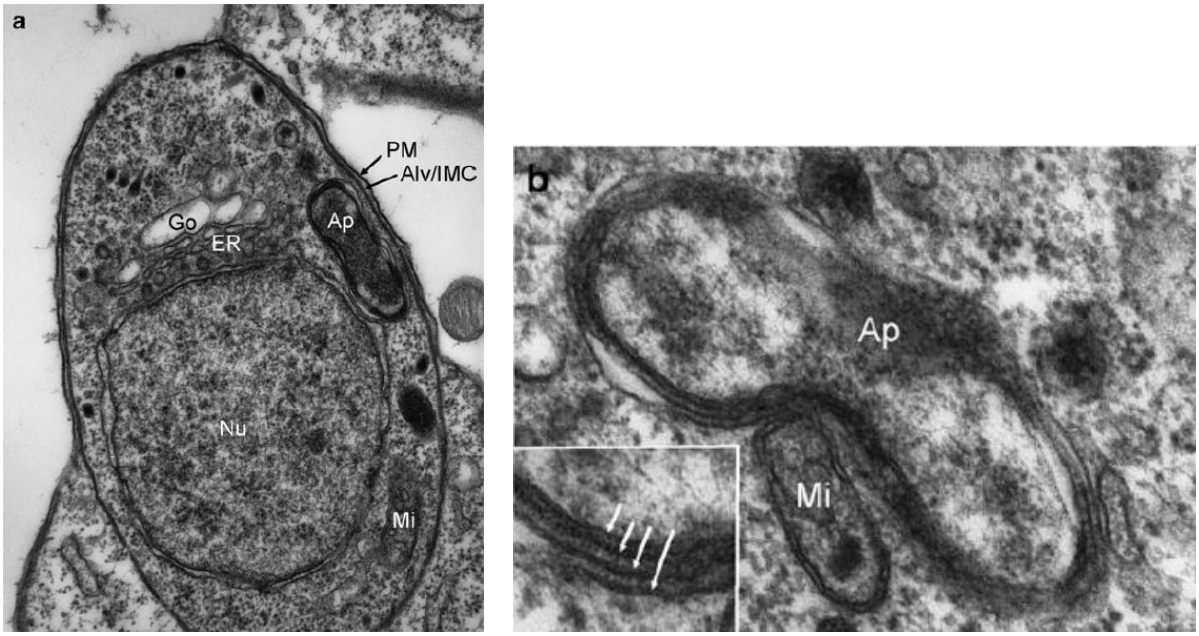

Figure 4. Toxoplasma gondii
a) is showing transmission electron micrograph of T. gondii with nucleus (Nu), endoplasmic reticulum (ER), Golgi apparatus (Go), apicoplast (Ap), mitochondrion (Mi), plasma membrane (PM) and alveoli (Alv). **b)** is showing apicoplast (Ap) and mitochondrion (Mi) in a close association and apicoplast surrounded with four membranes. Figure is taken from McFadden 2010.

An important aspect of the metabolism of intracellular pathogens is the acquisition of lipids and fatty acids. Fatty acids are playing an important role in the post-translational modification of numerous proteins and they are main building blocks of membranes and are main storage compounds (Waller and McFadden 2005; Ramakrishnan *et al*., 2012). In nature, there are two types of **fatty acid biosynthesis**, Type I found in animals and fungi, and Type II found in bacteria, plants and parasites (White *et al.*, 2005). In eukaryotes, fatty acid synthesis occurs in two subcellular compartments, namely in the cytoplasm and in mitochondria. In plants, there is another pathway in plastids. Animals and fungi use FAS type I (FASI) found in the cytosol. Bacteria uses FAS type II (FASII) complex where each enzymatic domain is a discrete polypeptide. In plants and algae, fatty acid synthesis takes place in plastids and utilizes the FASII enzyme complex. Apicoplasts, like other plastids, possess the FASII pathway (Waller *et al*., 1998; Ramakrishnan *et al*., 2012). FAS I and FASII pathways differ in structure, kinetic and susceptibility to several inhibitors. Properties that makes them different also makes them attractive targets for the development of parasite-specific drugs. It has been proven that the apicoplast FASII pathway is essential for the parasite viability (Mazumdar *et al*., 2006).

Apicoplasts have a pathway to synthesize isopentenyl diphosphate (Jomaa *et al.*, 1999) that is essential for the growth and survival of *T. gondii* (Nair *et al.*, 2011). Isoprenoids are lipid compounds with many important functions, and all of them depend on the precursors like isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These two precursors are used to synthesize a wide variety of lipids (Lim and McFadden 2010; Nair *et al.*, 2011). **Isoprenoid synthesis** in apicomplexan parasites differs from the one which occurs in animals and fungi, meaning that the common precursor IPP can be produced by two different biosynthetic routes, either via the mevalonate pathway (MVA), or the 1-deoxy-D-xylulose 5-phosphate pathway (DOXP). The DOPX pathway is used by most eubacteria, a green alga (*Scenedesmus obliquus*), liverwort and plants but is absent in mammals. Plants and some algae possess both pathways, with MVA localized in the cytosol and DOXP in plastids (Disch *et al*., 1998; Waller and McFadden 2005).

Heme metabolism is critical for parasite survival (van Dooren *et al.*, 2012). Heme is required for mitochondrial respiration and the initial step of this pathway starts in mitochondria but some subsequent reactions of the pathway are taking place in the apicoplast and it terminates in the mitochondrion. Heme is a prosthetic group of hemoglobin, myoglobin, and the cryptochromes, essential for most life on Earth. It functions in numerous cellular redox reactions like antioxidant defenses and at several stages of the electron transport chain in prokaryotes and eukaryotes and as sensor and transport molecule for oxygen (van Dooren *et al.*, 2012). The heme synthesis is described in Figure 5. The loss of photosynthesis in Apicomplexa meant that tetrapyrroles were no longer required in the plastid. The terminal steps of heme biosynthesis then shifted back to the cytosol and mitochondrion, reflecting the pathway that exists in *P. falciparum* and *T. gondiii* (van Dooren *et al.*, 2012). In animals and in fungi, heme is an end-product of the tetrapyrrole biosynthesis pathway. In plants, tetrapyrrole biosynthesis pathway produces both heme and chlorophyll (Ralph *et al.*, 2004).

All these anabolic pathways are driven by carbon sources, which are imported from the parasite's cytosolic glycolytic pathway, using transporters located in the inner membrane of the apicoplast (McFadden 2014). Non-photosynthetic plastids import fuel using specific metabolite transporters on the inner envelope of the plastid known as plastidic phosphate translocators (pPTs).

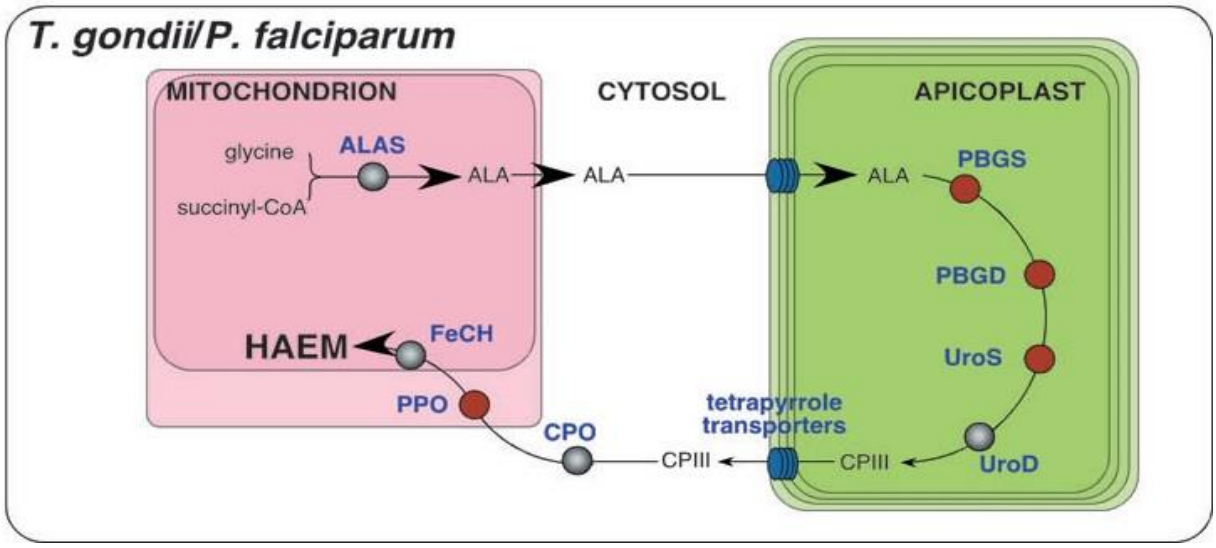

Figure 5. Heme biosynthesis in Toxoplasma gondii and Plasmodium falciparum
Heme biosynthesis begins with the aminolevulinic acid synthesis (ALAS)- catalyzed formation of aminolevulic acid (ALA) in the mitochondrion of *T. gondii* and *P. falciparum*. ALA is then transported from the mitochondrion into the stroma of the apicoplast. In apicoplast, four reactions of heme synthesis take place, leading to formation of coproporphyrinogen III. Coproporphyrinogen III is exported from the apicoplast into the cytosol where the CPO enzyme oxidizes coproporphyrinogen III forming protoporphyrinogen IX. Figure is taken from van Dooren *et al*., 2012.

1.4 The *Toxoplasma gondii* apicoplast phosphate transporter

In higher plants, carbon skeletons from photosynthesis are exported in the form of triose phosphates by the triose phosphate/ phosphate transporters (TPT) (Flügge 2003). The TPT is one of the members of a large family of plastid phosphate transporters (pPT). The other subfamilies catalyze the import of metabolites into plastids like phosphoenolpyruvate (PEP) (by the PEP transporter PPT), glucose 6-phosphate (by the GPTs) and xylulose-5-phosphate (by the XPT) (Fischer and Weber 2002). *T. gondii* possesses only one plastid phosphate transporter, suggesting that this protein is transporting more than one substrate. It is localized in the inner envelope membrane of the apicoplast and its functions as antiporter, where a sugar phosphate is translocated in exchange for inorganic phosphate (Fleige *et al.*, 2007).

Because the apicoplast is a non-photosynthetic organelle the lack carbon fixation, and ATP and NADPH production has to be compensated by the import of compounds from the cytoplasm into the apicoplast to drive this anabolism (Fischer and Weber 2002). Apicoplasts also lack hexose- or pentose-processing pathways. Therefore, apicoplasts import C3 compounds like triose phosphates and PEP from the cytosol to fuel its anabolic pathways (Ralph *et al.*, 2004).

The *Toxoplasma gondii* apicoplast phosphate transporter (TgAPT) is able to transport triose phosphates, PEP, and 3-PGA but it does not possess Glc6P transporter activity (Brooks *et al.* 2010). It is required for the activity of the FASII pathway, providing a direct link between cytoplasmic and apicoplast metabolism (Figure 6). A second pathway which depends on this transporter is the DOXP pathway (Brooks *et al.*, 2011). In addition, TgAPT is linking cytosolic and plastid isoforms of phosphoglycerate kinase, GAP-DH and triose phosphate isomerase. GAP-DH and PDH are the only enzymes identified in apicoplasts which produce redox equivalents in form of NADH or NADPH. Phosphoglycerate kinase and pyruvate kinases are the only enzymes which are producing ATP (Brooks *et al.*, 2011). The import of DHAP from the cytosol via TgAPT would then be followed by its conversion into glyceraldehyde-3-phosphate (GA3P) and via two steps into 3-phosphoglycerate (3-PGA), releasing NADPH and ATP. The 3-phosphoglycerate produced would be exported to the cytosol, via TgAPT connecting this loop with the glycolysis (Fleige *et al.*, 2007).

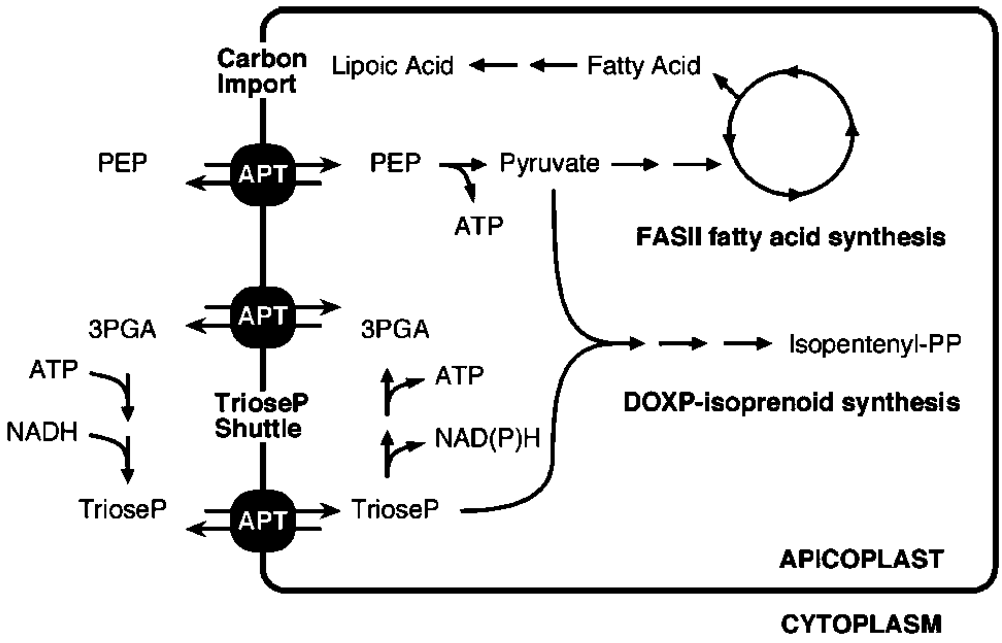

Figure 6. TgAPT links apicoplast metabolism to the parasite cytoplasm
Schematic representation of main anabolic pathways in apicoplast. Note that TgAPT serves as a hub to supply carbon (through PEP and triose phosphate) as well as energy and reduction power through the triose shuttle. TgAPT is a phosphate antiporter and Pi is not shown for simplicity. This figure is taken from Brooks *et al*., 2011.

Disruption of the TgAPT protein leads to rapid death of the parasite which clearly shows the importance of this transporter for its growth. The TgAPT delivers the carbon skeletons for fatty acid biosynthesis and the isoprenoid pathway, meaning it is considered to be the main source of carbon for these essential apicoplast anabolic pathways. In addition, it has an essential role in delivering redox equivalents and ATP to apicoplasts, suggesting that targeting this transporter could be a valuable strategy to identify antiparasitic compounds (Brooks *et al.,* 2011).

1.5 *Escherichia coli* as expression system

The expression of the APT protein from *T. gondii* was already established in yeast (Brooks *et al.,* 2011) and with our collaborators in Grenoble. However, because protein production in yeast is time-consuming and expensive, this project is focused on the production of the APT protein in a bacterial system, namely the model organism *Escherichia coli*. *E. coli* is a gram-negative, facultatively anaerobic bacterium. Gram-negative bacteria contain a distinct surface composed of inner and outer phospholipid membranes and a rigid peptidoglycan cell wall between these two layers. This structure allows them to adjust and adapt themselves to different temperatures, stress and environments (Ron 2013).

The optimal growth temperature of *E. coli* cells occurs around 37°C. They reproduce via an asexual process called binary fission in which each cell increases in size and divides into two cells (Kenweth 2002). The cells will increase in number, under optimum conditions, until they reduce the nutrients in the medium. The cells will then undergo a physiological change which is allowing them to survive in the absence of growth. This state is called stationary phase where the number of cells increases barely slow. The cell density is highest in the stationary phase where most *E. coli* strains appear to contain a high amount of plasmids. Therefore the *E. coli* cells at the stationary phase are mostly used for plasmid production. The stationary phase is then diluted with fresh medium and then the bacteria undergo another physiological change and start to divide at an exponential rate known as the logarithmic phase (log phase). This cell growth can be measured with a spectrometer by following the increase in absorption of a culture at 600 nm and by checking the optical density (OD) at different time periods. A culture with an absorbance of 1.0 OD contains approx. $2x10^8$ cells /ml (Kenweth 2002).

The heterologous expression of proteins is a commonly used technique to produce proteins for molecular biological examinations. There are different expression systems now available like yeast, insect cells, mammalian cells and amoeba (*Dictyostelium discoideum*), but

the most widely used systems are still bacteria like *E. coli*. A large number of cell lines and expression vectors are available and because the transfection and growth of this bacteria are simple, fast and cheap and it is the most commonly used system for protein production in the laboratory and industry (Doyle 2008). However, there is no universal expression system for heterologous proteins. All expression systems have some advantages as well as some disadvantages (Rai 2001). The use of expression systems such as insect or mammalian cells has their own drawbacks. In the insect system baculovirus is used to transfect the cell line and working with viruses is not as easy as with bacteria. Mammalian systems are time-consuming, expensive and difficult to perform at the large scale (Terpe 2006). Large scale production of engineered proteins is crucial for structural and functional analysis and therapeutic and *in vivo* studies. For most recombinant protein expression systems the main goal is to achieve fast, inexpensive expression of proteins which are easy to purify and could be produced in large quantities (Gräslund 2008). To increase the production of the proteins the first step is to optimize the expression conditions to avoid aggregation, degradation and improper folding. Protein expression is highly dependent on the choice of vector, cell strain and conditions like temperature and medium.

Several factors may affect protein expression. For instance, selection of a proper cell strain is an essential factor that needs to be considered during bacterial transformation and expression. There are a great variety of cell strains that can be used in order to express proteins in *E. coli*. In this study seven different strains were screened to accomplish the best expression.

1.6 Objectives of this study

The main goal of this master thesis is the expression of the TgAPT in *E. coli* for two reasons. First, the establishment of an expression and purification protocol for the APT protein paves the way towards the molecular analysis of the protein which might finally lead to the design of new drugs against Toxoplasma and Plasmodium. Second, *E. coli* cells expressing a functional APT protein could be directly used for transport experiments with different substrates, thereby characterizing the transport activities of the APT.

This program involves the following steps:

1. An *E. coli* expression vector containing the APT gene will be introduced into different *E. coli* strains. The vector with the APT gene was synthesized by our collaborator in France.
2. The different transformed *E. coli* strains will be screened for the best expression. For that, a large number of transformed cells will be analyzed for APT expression, which includes the following steps:
   a. Growth of transformed clones in liquid media
   b. Protein isolation from the cells
   c. Detection of the APT protein by a specific antibody against an N-terminal Strep-tag
3. Clones with the highest expression level will be grown at larger scale.
   A purification protocol using the Strep-tag affinity chromatography will be established.

2. Materials and Methods

2.1 Materials

Table 1. Materials used in project

Solution	Amount	Chemicals	Solution	Add	Note
Luria-Bertani liquid medium	**1 l**	Tryptone		10 g	
		NaCl		10 g	
		Yeast extract		5 g	
		dH_2O		Add to 1 l	
Solid medium for *E. coli*	**200 ml**	Peptone		2 g	
		Yeast extract		1 g	
		NaCl		2 g	
		Agar powder		2 g	
		dH_2O		Add to 200 ml	
Resuspension buffer for cell lysis	**2.5 ml**	NaPi 50 mM pH 7.8	0.5 M	2.3 ml	
		NaCl 300 mM	5 M		
		10 mM $MgCl_2$	1 M	125 µl	
		Glycerol 5%	100 %	150 µl	
		PMSF 1 mM	1000 mM	25 µl	
		DNase		2.5 µl	
Buffer used for protein purification (B1)	**15 ml**	NaPi 50 mM pH 7.8	0.5 M		
		NaCl 300 mM	5 M		
Buffer used for protein purification-washing (B2)	**10 ml**	NaPi 50 mM pH 7.8	0.5 M	9.75 ml	
		NaCl 300 mM	5 M		
		DDM 0.5 %	20 %	250 µl	
Buffer used for protein purification-elution (B3)	**2.5 ml**	NaPi 50 mM pH 8	0.5 M	1.93 ml	
		NaCl 300 mM	5 M		
		DDM 0.5 %	20 %	65 µl	
		DTB 5 mM	25 mM	500 µl	
DDM	**50 ml**	DDM	20%	10 mg	
		dH_2O		Add to 50 ml	
DTB	**50 ml**	DTB	25 mM	268 mg	
		dH_2O		Add to 50 ml	
RESOLVING GEL	**25 ml**	12% acrylamide	30% acrylamide	10 ml	SDS-PAGE
		dH_2O		5.4 ml	
		1 M TRIS pH 8.8		9.3 ml	
		10% SDS		250 µl	
		10% APS		125 µl	
		TEMED		12 µl	

STACKING GEL	**10 ml**	30% acrylamide dH_2O 1 M TRIS pH 6.8 10% SDS 10% APS TEMED		1.5 ml 6 ml 2.5 ml 100 µl 100 µl 20 µl	SDS-PAGE
5x protein loading dye	**4 ml**	1 M Tris pH 6.8	250 mM	800 µl	
		Glycerol	50 %	2 ml	
		20 % SDS solution	5 %	1 ml	
		1% Bromophenol blue in 1 M Tris pH 6.8	0.05 %	200 µl	
10x Running buffer (Leammli buffer)	**1 l**	Tris base	250 mM	30.3 g	
		Glycine	2 M	150 g	
		SDS	1%	10 g	
		dH_20		Add to 1000 ml	
1x Transfer Buffer	**1 l**	Tris base	25 mM	3.03 g	
		Glycine	192 mM	14.4 g	
		MeOH	20%	200 ml	
		dH_20		Add to 1000 ml	
10x TBS-T	**1 l**	Tris base	100 mM	12.11 g	
		NaCl	1.5 M	87.66 g	
		Tween-20	0.5%	5 ml	
		Triton-x100	2%	20 ml	
		dH_20		Add to 1000 ml	
Coomassie Blue Staining Solution	**250 ml**	Coomassie Brilliant Blue R-250		0.5 g	Dissolve coomassie completely, and then add dH_2O
		MeOH		100 ml	
		dH_20		100 ml	
		Acetic acid		20 ml	
Destaining Solution		Acetic acid	7.5 %		
		MeOH	10 %		

Table 2. Commercial kits used in project

Q5® High-Fidelity 2X Master Mix	PCR	New England BioLabs®
NucleoSpin® Gel and PCR Clean-up	Cleaning up the PCR	MACHEREY-NAGEL
NucleoSpin® Plasmid DNA purification	Purification of plasmid	MACHEREY-NAGEL
Rapid DNA Ligation Kit	DNA ligation with sticky ends	Roche
Mini-PROTEAN® TGX™ Precast gel	Precast gels	BIO-RAD
SIGMAFAST 3,3'-Diaminobenzidine tablets	Localization of peroxidase activity	Sigma-aldrich
SuperSignal® West Pico Chemiluminescent Substrate	Detection of protein after plotting	Thermo Fisher Scientific

2.2 Cloning procedures

Cloning of the TgAPT into the expression vector was done by collaborating colleagues from IBS in Grenoble. The vector used for the expression of targeted protein was pET21b+ (Appendix I) and construct of the affinity-tagged protein was designed by the GeneScript.

PCR with plasmid DNA was performed in small 200 µl tubes and with Q5® High-fidelity 2x Master Mix kit (New England BioLabs®) and primers were already pre-ordered. Master-mix was already prepared inside kit containing Mg^{2+}, deoxynucleotides and DNA polymerase. Primer concentration was 18.1 nM. For the control, a sample without the DNA was used. The PCR reaction was done according to Table 3 and everything was added into PCR tubes and program was setup like in Table 4.

Table 3. The PCR reaction

For 25 µl reaction:	
Nuclease- Free water	10 µl
Q5 High-Fidelity 2x Master Mix	1.25 µl
Forward primers	1.25 µl
Reverse primers	1.25 µl
DNA Template	1 µl

Table 4. The PCR setup

PCR setup:		
98˚C	30 sec	
98˚C	7 sec	30 cycles
66˚C	20 sec	
72˚C	42 sec	
72˚C	2 min	

The PCR reaction was analyzed by agarose gel electrophoresis. The DNA after PCR was purified with spin columns with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). Purification was done according to the manufactural manual. Restriction digestion of the PCR fragments was done with the two restriction enzymes Ndel and HindIII in Cut Smart™ buffer (New England BioLabs®) for two hours at 37˚C. After incubation, the digested fragments were

purified with NucleoSpin® Plasmid Quick Pure kit (Macherey-Nigel) and DNA fragments were separated with the agarose gel electroporation.

Plasmid purification was done according to the manufactural manual. Gel slices with good quality band were cut under the UV light and placed into new tubes and solubilized with NucleoSpin® Gel and PCR cleanup kit (Macherey-Nagel). Solubilization was done according to the manufactural manual. For the ligation of vector and insert, the DNA concentration was measured using spectrophometer (Nano Drop) because the vector and insert should be in equimolar concentration. Ligation was done with Rapid DNA ligation kit provided by Roche and according to the manufactural manual and described in Table 5.

Table 5. Ligation reaction

1. Tube- APT PCR	2. Tube- Vector
47 µl dH_2O	58 µl dH_2O
8 µl Cut Smart™ buffer	8 µl buffer
20 µl DNA	9 µl DNA
2.5 µl enzyme (Ndel)	2.5 µl enzyme (Ndel)
2.5 µl enzyme (HindIII)	2.5 µl enzyme (HindIII)

The first tube contained 40 mg/ µl and second had 10 mg/ µl of sample. To each sample 10 µl of ligation buffer was added and 2.5 µl of vector and 6.5 µl of APT insert. At the end 1 µl of T4 ligation enzyme was added, mixed well and stored on -20˚C.

2.3 Testing different strains for protein expression

To get the highest expression of TgAPT protein, different *E. coli* strains were tested. The transformation was done with the heat-shock protocol (see below) and TgAPT vector was inserted into six different *E. coli* strains (BL21 DE3, BL21 RIL, C41, C43, Rosetta, Rosetta PLys and PET21b, which contained an empty vector and was used as negative control). Cells were grown on solid agar plates with Luria-Bertani (LB) broth medium and Ampicillin (AMP; (100 µg/ml)) and Chloramphenicol (CHL; 25 µg/ml) antibiotics. Isolated colonies were transformed into small liquid cultures with 6 ml LB medium and AMP (100 µg/ml) and CHL (25 µg/ml) antibiotics. Samples were incubated at 37°C overnight in a shaking incubator. The following day, cells were transferred into a bigger culture with 250 ml LB medium and

antibiotics, and incubated on a shaking incubator at 37°C for approx. 4 hours (until an OD_{600} of 0.6-0.8 is reached which represents the middle of their log growth phase). At that time point, Isopropyl-beta-D-thiogalactoside (IPTG, 1 mM) was added to each flask and the cells were incubated at 20°C overnight in a shaking incubator. Cells were centrifuged (20 min/ 4000 rpm/ 4°C) and the supernatant was decanted. Pellets were transferred into new 50 ml falcon tubes and stored at -20°C overnight.

The pellets were resuspended in the 600 µl resuspension buffer (50 mM NaPi pH 8, 200 mM NaCl, 1 M $MgCl_2$, 100x CLAPA and 5% glycerol). Cells were lysed with a sonicator (Ultrasonicator UP50H (cycle: 0.5; amplitude: 50%)). Each sample was exposed for a total of two minutes with intervals of 10 sec sonication and 10 sec on ice. Samples were centrifuged again (20 min/ 15000 rpm/ 4˚C) to remove the insoluble cell content. The supernatant was transferred into new Eppendorf tubes and both pellets and supernatant were prepared for western blot analysis.

For the gel electrophoresis, Mini-PROTEAN® TGX stain-free™ precast gel (BIO-RAD) was used and into each well 15 µl of sample/ loading dye was added (24 µl supernatant+ 6 µl loading dye; 15 µl pellet which was resuspended in 500 µl resuspension buffer+ 15 µl loading dye). Electrophoresis was running for 50 min at 50 mA. Western blot was done with Trans-blot® Turbo™ system (BIO-RAD) and run it for 7 min with 30 ml Trans-blot® Turbo™ 5x transfer buffer (BIO-RAD) + 10 ml dH_2O + 10 ml EtOH. Nitrocellulose membranes were blocked with blocking buffer (2.5 g milk powder+ 50 ml 1x PBS and 0.03% Tween 20) and incubated with the Strep-MAB-classic-HRP antibody (IBA). Detection was done with SigmaFast™ 3.3'- Diaminobenzidine (Sigma-Aldrich).

2.4 Preparation of competent cells

Bacterial culture were chilled on ice for 10 min and the flask was swirled periodically. After centrifugation (10 min/ 3200 rpm/4°C), the cells were harvested. The supernatant containing medium was decanted and the pellets which contained the cells were gently resuspended in 1:10 volume of ice-cold 0.1 M $CaCl_2$. $CaCl_2$ is used to transform bacterial cells with the disruption of the phospholipids in the cell membrane allowing plasmid to enter the cell through the disrupted cell membrane. Cells were incubated at 4°C for 20 min. After short incubation, cells were recovered by centrifugation (10 min/ 32000 rpm/ 4°C). The supernatant was decanted and pellets were gently resuspended in 1:50 volume of ice-cold 0.1 M $CaCl_2$. Aliquot of 200 µl was removed into cold Eppendorf tubes and stored on ice.

2.5 Transformation of *E. coli* cells

Competent *E. coli* cells were thawed on ice. The ligation mixture of the APT plasmid DNA (10 ng) was added to the 200 µl BL21 RIL competent cells and incubated for 30 min on ice, allowing the plasmid DNA to bind to the cellular membrane. The cells were then subjected to heat shock at 42˚C for 90 seconds in a heat block and placed back on the ice for 2 min before adding 1 ml of LB medium. The cells were then incubated at 37˚C for 1 hour in a heating block, and gently inverted every 15 min. This is allowing cells to recover from the heat shock and for the transformed cells to express antibiotic resistance genes.

Transformed cells were plated into two different volumes (100 µl and 400 µl) on agar plates with Carbenicillin (CARB (20 µg/ ml)) and Chloramphenicol (CHL (100 µg/ ml)) antibiotics. Inoculation was done overnight at 37˚C.

2.6 Growth of bacterial cells and induction of protein expression

Bacteria with inserted plasmid was introduced into liquid media with appropriate antibiotics and everything was done in sterile conditions. Into small glass tubes, 5 ml of LB, 10 µl of CHL (100 µg/ ml) and 10 µl of CARB (20 µg/ ml) was added. Using a sterile toothpick, a single colony from the plate was picked and mixed together with medium and antibiotics. Bacterial culture was incubated overnight at 37˚C with constant shaking (200 rpm).

Next day the samples had to be diluted and re-grown in bigger culture. Into new 50 ml glass flasks 10 ml of medium was added together with 500 µl of small culture and 4 µl of CHL and 4 µl of CARB antibiotics. Incubation was done for 2 hours at 37˚C in a shaking incubator. Again, everything was done under sterile conditions.

After approx. two hours, when the OD_{600} (SmartSpec. 300 – BIO-RAD) was between 1 and 1.6, the cells were induced with IPTG (1 mM). Only the sample which was used as a negative control was not induced. All samples were incubated overnight at 20˚C with constant shaking (200 rpm).

2.7 Cell lysis and membrane preparation

After overnight induction, the samples with were centrifuged (10 min/ max. speed/ 4˚C) and the supernatant was decanted and pellets where stored at -20°C. After one hour, pellets were resuspended in buffer (described in Materials) and transferred into new Eppendorf tubes and kept on the ice through the whole procedure.

Cells were lysed with a sonicator **(**10 seconds on, 1 minute break (Branson Sonifier 250; cycle 50%; output control 5)). After the cells were disrupted, samples were centrifuged (10 min/

13500 rpm/ 4°C) to remove cell fragments. Pellets were resuspended in 300 µl buffer (50 mM NaPi pH 7.8+ 300 mM NaCl) and booth, pellets and supernatant were taken for further solubilization and purification.

2.8 Solubilization

Membrane proteins were solubilized by adding 1% w/v DDM detergent and 500 µl Strep-Tactin® Sepharose® (IBA) resin was added. The resin was previously washed three times with B1 buffer and centrifuge (1 min/ 5 000 rpm/ 4˚C), each time removing the supernatant was. Samples were then incubated for 45 min on a rotating wheel at 4°C.

2.9 Affinity chromatography purification on Strep-Tactin® resin

First, the gravity column was washed one time with 500 µl of B1. Next, the samples were loaded into column and flow trough was collected into new Eppendorf tubes. Second, the column was washed 10x with 500 µl of B2 buffer. Last, the columns were eluted with 800 µl of B3 buffer and incubated for 5 min. after incubation, the elution was collected into new Eppendorf tubes. All buffer concentrations are described in Materials.

2.10 SDS -PAGE, Coomassie staining and Western blot

Proteins were separated by **SDS-PAGE** on 12% polyacrylamide gels performed according to the Laemmli protocol from 1970. The resolving gel was prepared (described in Materials), allowing to polymerize on the bottom of the cast before pouring the stacking gel (preparation described in Materials) on top. Due to the higher acrylamide percentage in the separating gel, the proteins will be concentrated on the interface between the two gels resulting in increased protein resolution. After complete polymerization, the chamber was assembled and running/ Laemmli buffer (described in Materials) was poured into the well. Each membrane pellet was solubilized in 300 µl buffer (50 mM NaPi pH 7.8+ 300 mM NaCl). Aliquot of 60 µl of sample was placed into new Eppendorf tubes and 20 µl of loading die (2x SDS-PAGE loading buffer) was added. Samples were loaded into the gel (20 µl per lane. In this study, PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, #SM0671) were used as size markers. The electrophoresis was run for 1 hour at 15 mA and after one hour, it was increased for 5 mA (per gel) with the constant 250 V.

The protocol is based on staining and destaining method with the **Coomassie blue dye** R-250. The SDS gel was first washed with dH_2O to remove the SDS and the salts from the buffer, which interfere with a binding die to the protein and discard the rinse by decanting. The

gel was stained with Coomassie blue staining solution (described in Materials) overnight on a shaker at RT. Following day, staining solution was removed and the gel was quickly washed with water and the destaining solution (described in Materials) was added. Incubation was done for 30 min on shaker at RT. Dye that is not bound to protein diffuses out of the gel during destaining steps (Biji *et al.*, 2012). Second washing and destaining step was repeated and incubation was done under the same conditions only for 1 hour. All liquid was removed and the gel was washed with water and equilibrated in the water so it could return to its original dimensions. The proteins were detected as blue bands on a clear background

In this study, the protein samples were separated by SDS-PAGE before they were transferred to a Polyvinylidene Fluoride (PVDF) membrane (#162-0177, BIO-RAD) with the **western blotting**. The proteins were detected with SuperSignal® West Pico (#34077, Thermo Fisher Scientific) and Strep-Tactin HRP enzyme conjugates. The blot was built from the anode side starting with three sheets of filter paper soaked in transfer buffer (described in materials). On the top of that filter paper the membrane, which was incubated in MeOH was placed. The SDS-PAGE gel was put on top of the membrane and covered with three more filter papers, also soaked in the transfer buffer. The system was closed and current was applied for 24 min and 1 mA per 1 cm^2 membrane. After blotting, the membrane was transferred into blocking solution (50 ml of 1xTBS-T + 2 % milk powder) and incubated overnight at 4˚C on a shaker. Following, the membrane was washed twice for 10 min in 1xTBS-T buffer. The buffer was decanted and antibody solution was added (1% milk powder + 50 ml of 1x TBS-T + 10 µl Strep-Tactin HRP conjugated) and the membrane was incubated for 2 hours on a shaker at RT. The membrane was washed twice with 1xTBS-T buffer for 10 minutes. Detection was done with Super Signal® kit (Thermo Fisher Scientific) and equal volumes of stable peroxide and enhancer solution (400 µl) were mixed and added on the membrane. The membrane is placed then, between two transparent foils and the signal was detected with ChemiDoc™ MP imaging system (BIO-RAD).

3. Results

3.1 Overview about the study

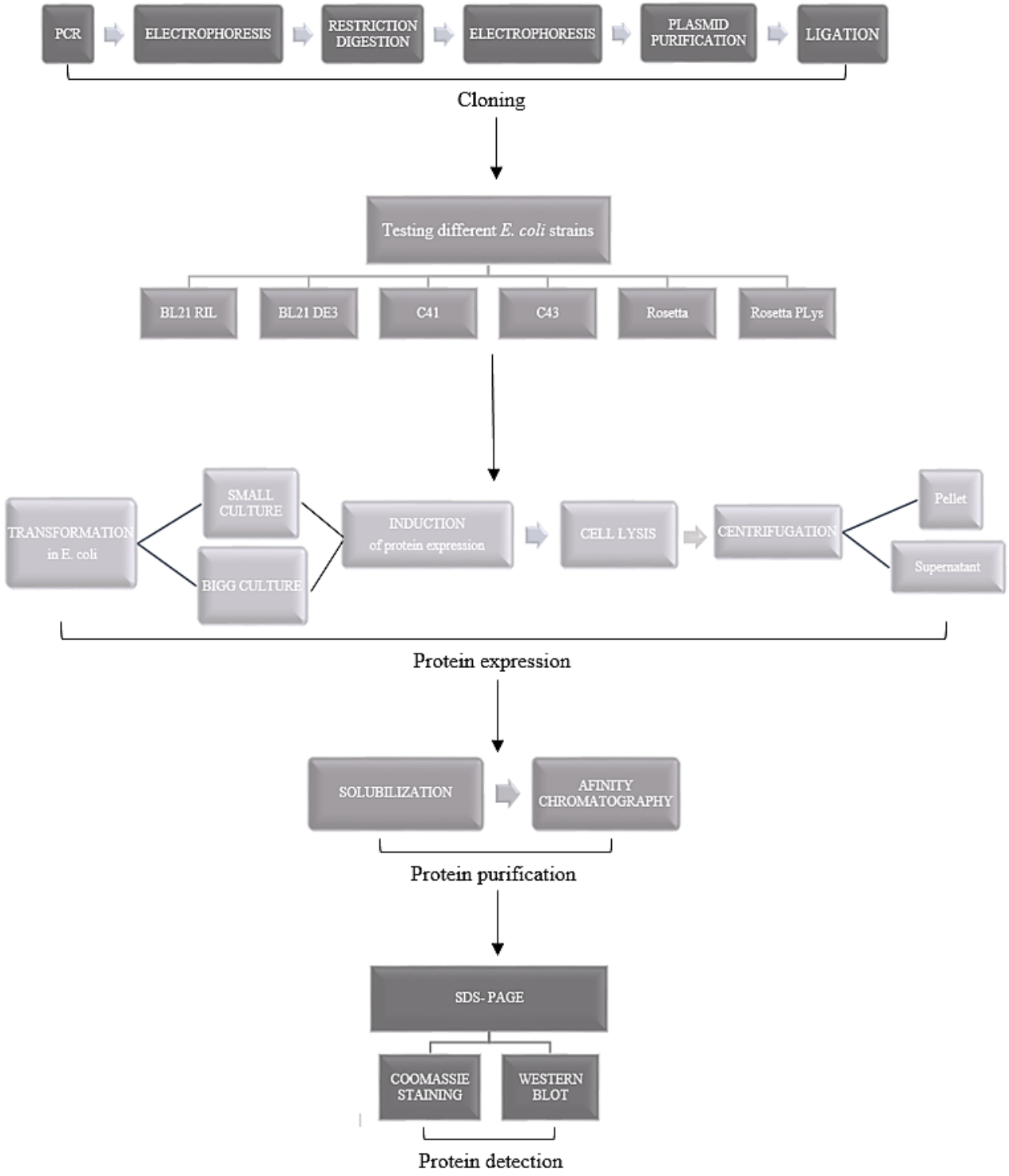

Figure 7. Flow chart displaying main steps in methodology, describing all the methods performed at various steps during this project.

Figure 7 shows an overview about the project. In order to achieve the expression of the *Toxoplasma gondii* apicoplast phosphate transporter (TgAPT), the cDNA, which encodes the transporter protein with the N-terminal Twin-Strep®-tag sequence was cloned into the *E. coli* expression vector pET21 b+ (Novagen). To achieve the best heterologous expression, the new plasmid was transferred into different E. *coli* strains. The best-expressing *E. coli* strain BL21 RIL was used for protein production. Cells were disrupted by sonication and the extract was separated into soluble and insoluble fractions by centrifugation. The proteins were solubilized with the detergent n-Dodecyl-β-D-Maltoside (DDM) and purified using affinity chromatography on a matrix carrying an engineered streptavidin (Strep-Tactin®). Detection of the APT protein was achieved by western blots with Strep-Tactin® labeled horseradish peroxidase-conjugate.

3.2 Cloning of the new TgAPT construct

The TgAPT had been expressed before in yeast with two affinity tags, a His-tag and a Strep-tag (see Figure 8; Brooks *et al.*, 2010). Because the purification by streptavidin affinity chromatography turned out to be more efficient than by NTA chromatography, a new construct for the expression in *E. coli* was designed with two Strep-tags attached to the N-terminus of the APT (Figure 8).

Figure 8. Schematic presentation of a) old and b) new TgAPT gene
His6 and Strep are the two affinity tags, TEV is a protease cutting site; Strep-tag II® is a new double tag, Ser-Ala represents a short amino acid sequence inserted as a spacer between the APT protein sequence and the tags.

A cDNA encoding the new construct, cloned into a vector, was synthesized by the GenScript company. For cloning into the *E. coli* expression vector pET21b+ (Novagen; for a map see Appendix 1), the APT DNA was amplified by PCR. A strong band of the expected length of 1kb (Figure 9) was cut out of the gel and digested with the restriction enzymes Ndel (5'-end) and HindIII (3'-end). The APT DNA was then inserted into the expression vector which had been cut with Ndel and HindIII. The resulting plasmid was named pET-APT.

In the case of the pET21b+ vector, induction of protein expression is achieved with Isopropyl-beta-D-thiogalactoside (IPTG) which is a non-metabolizable lactose derivate. Its structure mimics lactose and it is used to induce protein expression driven by a lactose inducible promoter (King *et al*., 2013). When IPTG is present, it dissociates the lac repressor and that allows the T7 RNA polymerase to bind to its binding site thereby starting transcription of the inserted gene. If the IPTG is not present, the lac repressor will remain bound to the promoter, preventing the T7 RNA polymerase from transcribing the inserted gene (Wurm *et al.*, 2016).

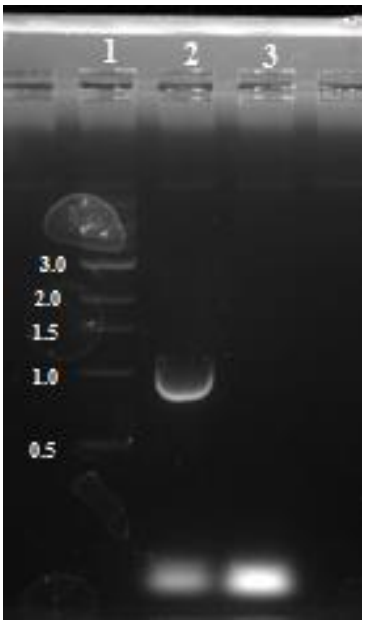

Figure 9. Analysis of PCR product by agarose gel electrophoresis
Lane **1)** marker Quick-Load® Purple 1 kb DNA Ladder (NEB); lane **2)** PCR with plasmid DNA template; lane **3)** Control sample without DNA template.

3.3 Screening of protein expression in different *E. coli* strains

Protein expression is highly dependent on the type of protein, cell strains, temperature, medium and vector of choice. Because the expression of membrane proteins in *E. coli* is difficult to achieve several different strains of *E. coli* have been tested for the expression of the APT. The BL21 DE3 strain contains a T7 RNA polymerase gene that is integrated into the genome and that is under control of the *lacUV5* promoter, which is an IPTG inducible system (Kortmann 2015). The BL21 RIL cells contain extra copies of the *argU*, *ileY*, and *leuW* tRNA genes. These genes encode tRNAs that recognize the arginine codons AGA and AGG, the isoleucine codon AUA, and the leucine codon CUA, respectively (Agilent technologies manual). They are important for difficult protein expression, especially when codon bias is a problem. Rosetta strains are derived from BL21 *lacZY* and tuner strains but they carry a pRARE derived plasmid (conferring chloramphenicol resistance) that encodes several "rare" *E. coli* tRNA genes. Some of the rare codon genes are AGG/AGA (arginine), AUA (isoleucine), and CCC (proline), (Novagen® user protocol). Therefore, these strains are designed to increase the expression of heterologous proteins whose genes encode numerous rare *E. coli* codons. The pLysS strain has a plasmid encoding a T7 lysozyme gene that allows the expression of more toxic proteins in greater amounts since that gene is a natural inhibitor of the T7 RNA polymerase and therefore, the protein expression is tightly regulated. Some membrane-bound proteins have been successfully expressed in the C41 and C43 *E. coli* strains. The strain has a mutation in the T7 RNA polymerase gene that reduces the transcription from downstream recombinant genes (Mulrooney 2000).

To achieve the best heterologous expression, the different *E. coli* strains (BL21 RIL, BL21 DE3, C41, C43, Rosetta, Rosetta PLys) were transformed with the plasmid pET-APT. Clones from each transformation were grown in liquid culture. For membrane preparation, cells were grown overnight at 37°C to an OD_{600} of 0.8–1.0. Expression was induced by adding IPTG. The cells were harvested the next day after overnight incubation at 20°C. After cell lysis, the samples were centrifuged to separate insoluble from soluble material (Roy 2015). Phenylmethylsulfonyl fluoride (PMSF) protease inhibitor was added to the buffer to avoid protein degradation.

The proteins samples, both supernatant and pellet, were analyzed by SDS-PAGE followed by a Western blot. In this study, protein samples from the SDS-PAGE gels were transferred to a Polyvinylidene Fluoride membrane (PVDF, BIO-RAD). For the detection of the tagged APT protein, Strep-Tactin® labeled with horseradish peroxidase (IBA) was used.

The horseradish peroxidase (HRP)-conjugate is used for direct detection of Strep-tagged proteins in Western blots without the need of a secondary antibody.

The highest expression level of the TgAPT protein was observed in the BL21 RIL strain (Figure 10b) while the other strains show low or no expression (Figure 10a). The empty vector pET21 was used as a negative control and showed no expression. The strongest band runs at about 35 kDa which is the expected size for the APT (see black arrow in Figure 10b). This protein band was present both in the supernatant and in the pellet. The high background is suggesting that there was a problem with the antibody.

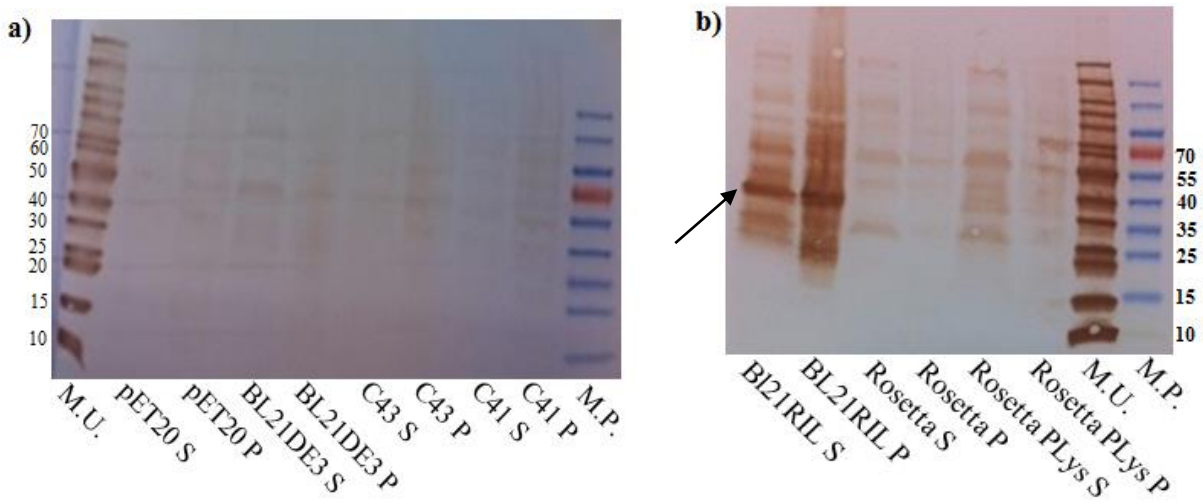

Figure 10. Western blot analysis of TgAPT expression in various E. coli strain
M.U. - unstained marker (Thermo Fischer #26614); M.P - prestained marker (Thermo Ficher #26616). The names of the different *E. coli* strains are shown below the blot. S- supernatant; P- pellet

To confirm the results of the first screening, cell pellets from the best-expressing strain (BL21 RIL) and the lowest-expressing strain (Rosetta) were analyzed in a second experiment (Figure 11). The effect of glycerol on the membrane preparation was also tested. With this analysis, it was confirmed that the best expression was achieved with the BL21 RIL strain (see black arrow on Figure 11). There was no difference between membrane preparations with glycerol or without. Again, a strong background was present, probably due to the specific antibody sample that was used.

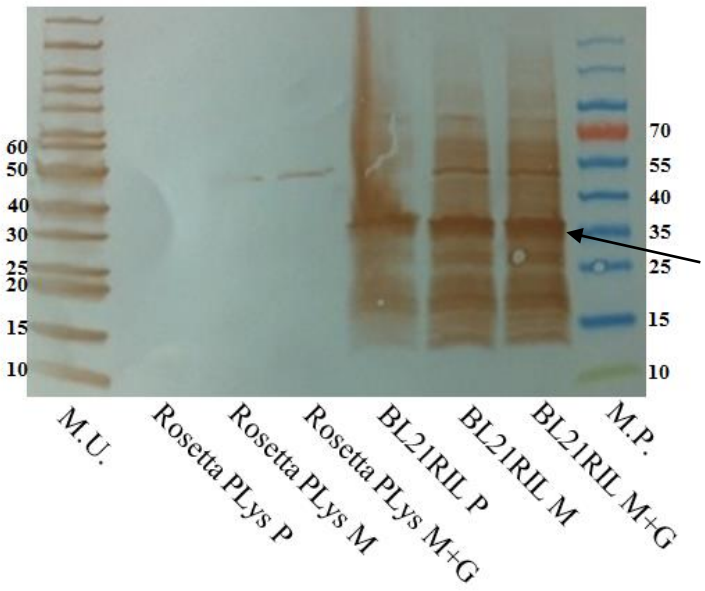

Figure 11. Western blot analysis with Strep-MAB-classic-HRP antibody
M.U. - unstained marker (Thermo Ficher #26614); M.P. - prestained marker (Thermo Ficher #26616). The names different *E. coli* strains are shown below the blot. P- pellet; M- membrane; G- with the addition of the 25% of glycerol.

3.4 Screening for expression in different BL21 RIL clones

The *E. coli* Bl21 RIL was found to be the only strain expressing the APT. Therefore competent cells of this strain were prepared for new transformations. The pET-APT vector was transformed into BRL21 RIL cells and more than 10 clones were tested for APT expression. As it is shown in Figure 12 the protein expression was achieved in clone number 23 and 25 in the pellet while there was no expression or only poor expression found in the other clones. Coomassie gels was done with the same samples and protein band cannot be observed probably because the protein amount was to low (Figure 13).

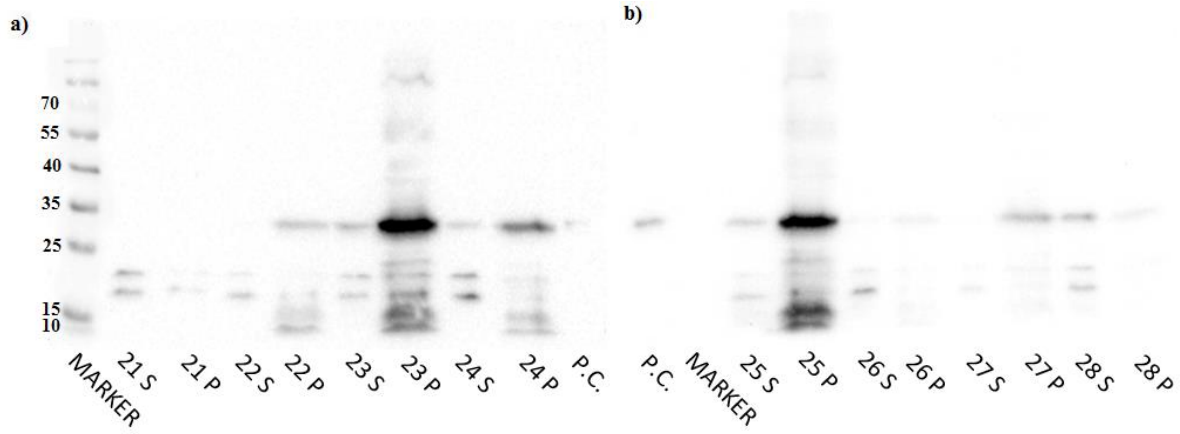

Figure 12. Western blot of supernatant and pellet of different isolated clones after cell lysis and SDS-PAGE
The marker was PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, #SM0671) and for the positive control (P.C.) the sample from the previous experiment was used. S- supernatant; P- pellet.

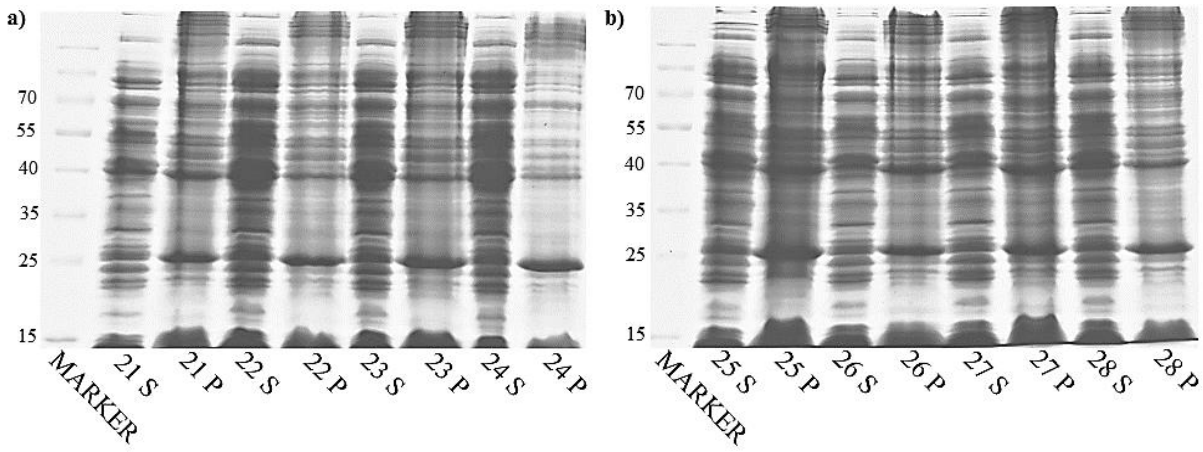

Figure 13. Coomassie blue staining with the same samples as in Figure 12
The marker was PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, #SM0671).
S - supernatant; P- pellet.

Protein separation can be used to determine the molecular weight of a protein of interest by comparing its migration in the gel with that of marker proteins of known size (Figure 14 right). For that, the migration distance of the protein marker is plotted against the logarithm of the molecular weights (MW) of the proteins as shown in Figure 14 left. This leads to a standard curve showing a linear relationship which was used to determine the MW of the APT protein (Figure 15). The mobility of the TgAPT protein was found to be 1.48 and the MW of the protein was therefore 30 kDa.

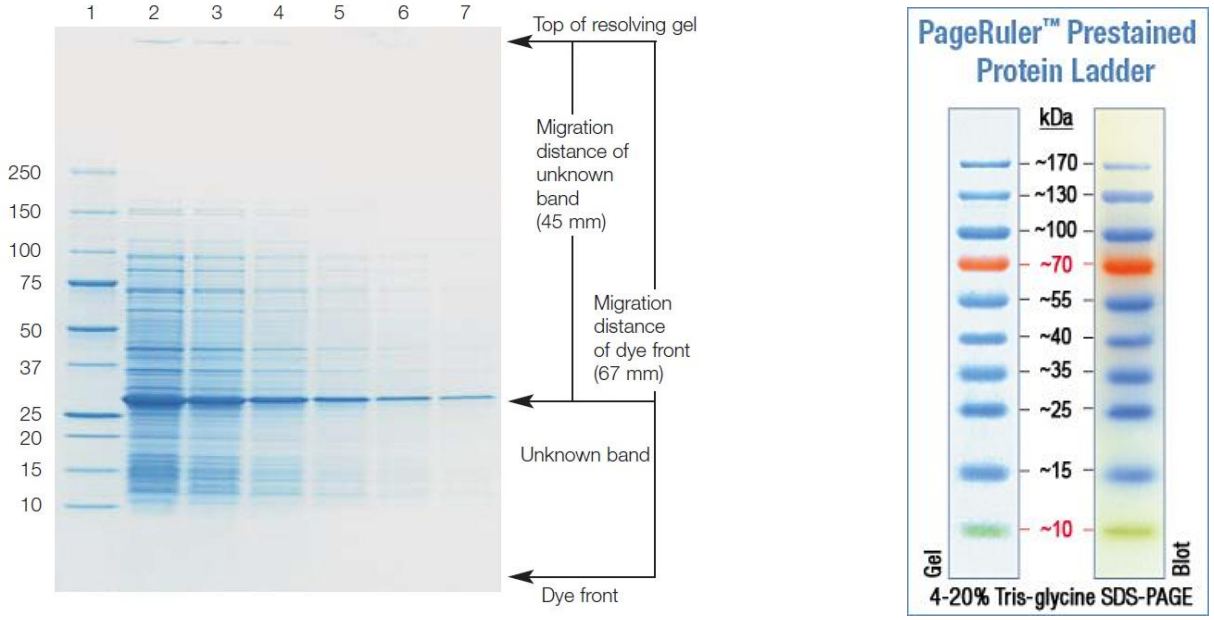

Figure 14. Calculation of MW
The left picture is showing the example how to calculate the MW of unknown proteins (BioRad); **The right** picture is showing PageRuler™ prestained protein ladder (Thermo Fisher Scientific, #SM0671)**,** used to monitor protein separation during SDS-PAGE and as a standard curve for calculating the MW.

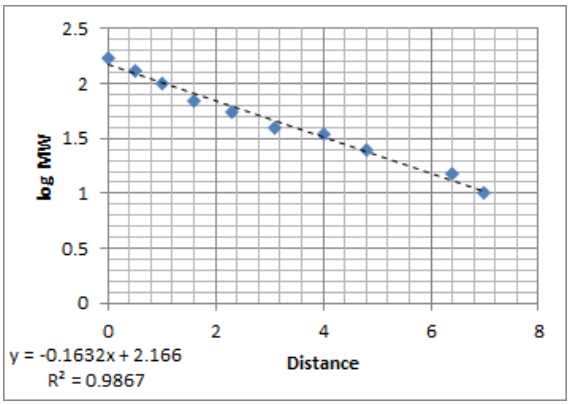

Figure 15. Determination of MW of the TgAPT protein
Using the formula in the figure, the MW of the APT protein was calculated.

3.5 Localization of the APT in pellet and supernatant

The best expression was achieved with clones 23 and 25. The next step was to repeat the expression experiments with only these two clones to confirm the previous results. In addition, the distribution of the APT protein between pellet and supernatant was analyzed in more detail. The clones were re-grown in liquid culture and, induced with IPTG. There was again an APT protein band in the extracts of clones 23 and 25 which was found almost exclusively in the pellet fraction (Figure 16) while there was no band in the negative control. The positive control of the yeast sample did not show a signal, probably because the sample was too old.

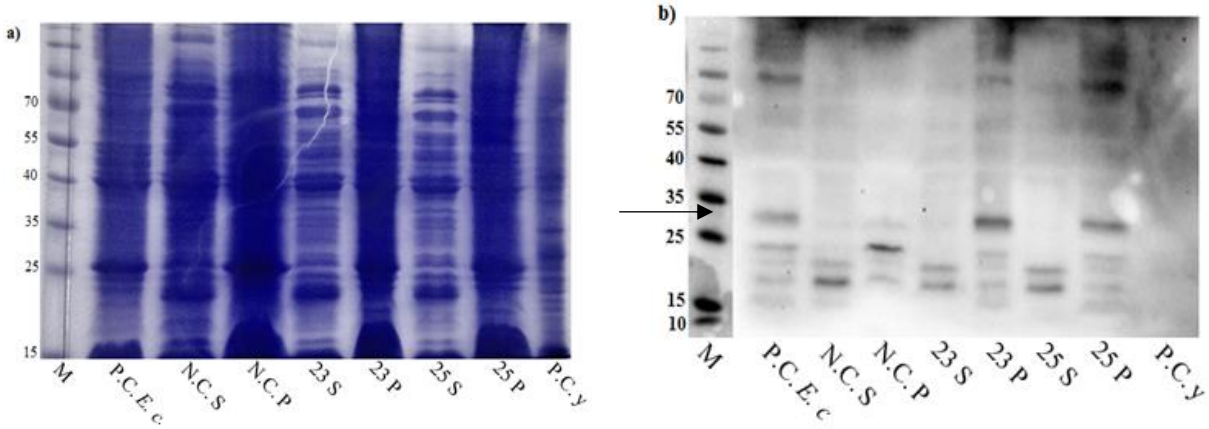

Figure16. Coomassie staining and Western blot analysis with clone 23 and 25 after lysis
Gel **a)** Coomassie stained SDS-PAGE; Gel **b)** Western blot;,**M** - marker PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, #SM0671); **P.C.E.c** - positive control *E. coli* expression; **N.C.** - negative control non-induced clone 25; **P.C.y** - positive control yeast cell expression. Numbers 23 and 25 represent the corresponding *E. coli* clones; S - supernatant; P - pellet. The arrow points to the APT band.

The distribution of the APT protein was analyzed in a second experiment. As shown in Figure 17a, some of the APT protein was found in the supernatant, while most of it is still in the pellet.

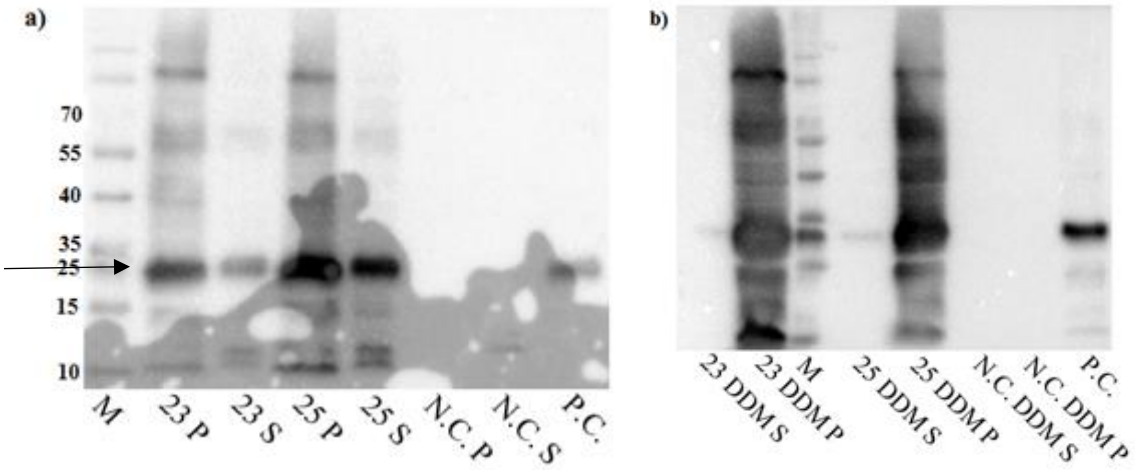

Figure 17. Western blot analysis
Blot **a)** analysis after cell lysis; M – marker, PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, #SM0671); N.C. - negative control non-induced clone 25; P.C. - positive control expression from previous experiments; numbers indicate the clones 23 and 25; S - supernatant; P - pellet;
Blot **b)** analysis after solubilization with DDM. The arrow points to the APT band.

As these results confirmed that the APT protein is present in the pellet and in the supernatant, the next step was to solubilize the proteins by using a detergent. Both the supernatants and the pellets of clones 23 and 25 were solubilized with 1% n-Dodecyl-β-D-Maltoside (DDM) and analyzed by Western blot. The signal in positive control and no signal in the negative control confirmed that the analysis was correct. A strong signal in pellets in both clones and weak signal in the supernatant what we expected according to the previous experiment (Figure 17b).

3.6 Purification of the TgAPT from cell lysate on Strep-Tactin® resin

To achieve affinity purification of APT, the protein sample of the pellet which contained more APT protein was solubilized with DDM and bound to a Strep-Tactin® matrix according to the manufacturer's instruction (IBA, Germany). The bound TgAPT was eluted in ten 0.5 ml fractions with the elution buffer containing 25 mM desthiobiotin (DTB). The elution fractions were analyzed by SDS-PAGE and Western analysis with a Strep-Tactin® HRP-conjugated antibody.

Figure 18 shows that no signal could be detected in the samples of the affinity purification, neither in the flow through nor in the eluted sample (Figure 18, lanes 25 F and 25 E), although the APT could be detected in the supernatant and the pellet after cell lysis (Figure 18, lanes 25 S and 25 P).

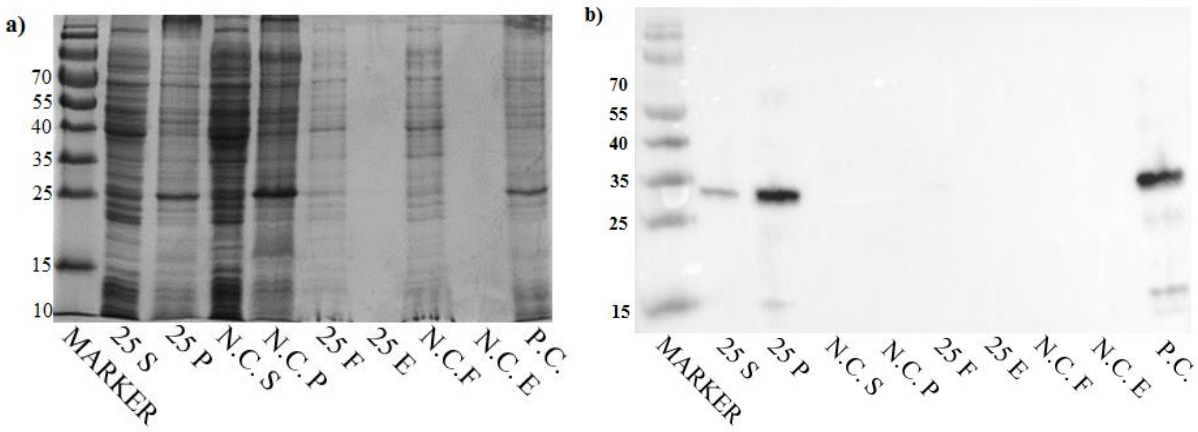

Figure 18. SDS-PAGE and Western blot analysis of a clone 25 after lysis and purification
The marker was PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, #SM0671); N.C. - negative control non-induced clone 25; P.C. - positive control is the sample from the previous experiment; number 25 indicates the clone 25; P- pellet; S - supernatant; F - flow through; E – elution.

Therefore, the affinity purification was repeated with the protein pellet of an extract from newly induced *E. coli* cells of clone 25. Figure 19 shows that part of the APT protein was detected both in the flow through and the elution of the affinity chromatography (lanes 25 F and 25 E).

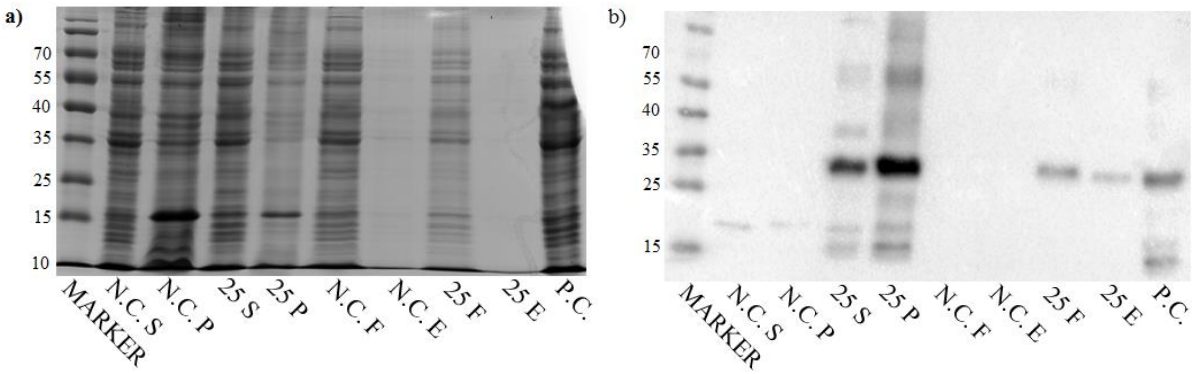

Figure 19. a) Coomassie and b) Western blot analysis of a clone 25 after lysis and purification
The marker was PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, #SM0671); N.C. - negative control non-induced clone 25; P.C.- positive control is the sample from the previous experiment; number 25 indicates the clone 25; P- pellet; S - supernatant; F - flow through; E - elution

The next purification of the APT was done with the supernatant obtained after cell lysis of the clones 23 and 25 (Figure 20). No signal was detected in the flow through of the affinity purification, but a weak signal in the eluted samples. This indicates, that the APT protein in the supernatant could be solubilized by DDM and binds effectively to the affinity column.

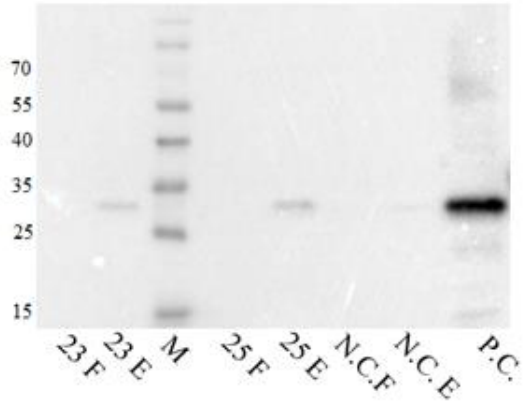

Figure 20. Western blot analysis after purification on Strep-tactin resin; *M - marker was the* PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, #SM0671); N.C. - negative control non-induced clone 25; P.C.- positive control is the sample from the previous experiment; numbers indicate the clones 23 and 25; F - flow through; E – elution

4. Discussion

Around 30-50% of the world population is infected by the parasite *Toxoplasma gondii* (Pappas 2009), which causes the disease Toxoplasmosis. It can affect any warm-blooded animal and humans. It is rarely fatal for humans, but in individuals who have a weak immune system it can be fatal and lead to death (Flegr *et al*., 2014). *T. gondii* belongs to the group Apicomplexa, which also includes *Plasmodium* species, which are the causative agent for malaria, and *Cryptosporidium spp.* Which causes cryptosporidiosis. Most important apicomplexan genera in veterinary medicine and agriculture are parasites like *Babesia spp.* and *Theileria spp*. in cattle and *Eimeria spp.* (coccidiosis) in poultry. (Beck *et al.,* 2009; Wiser 2011; Hikosaka *et al.,* 2013). *T. gondii,* as all other Apicomplexa, possesses a special plastid-like organelle called apicoplast discovered by Kohler in 1997. It is a vestigial plastid because it is not green and it does not perform photosynthesis (Kohler *et al.,* 1997).

The apicoplast is considered to be essential for the survival of the parasite. Beside its basic metabolic processes such as DNA replication, transcription and translation (Brooks *et al*., 2011; Dahl and Rosenthal 2008), they also have enzymes involved in anabolic pathways like the synthesis of fatty acids, isoprenoids and haem (Ralph *et al*., 2004). These pathways are fundamentally different from the equivalent eukaryotic pathways of the animal or human hosts and that is why apicoplasts are interesting to study as potential drug targets (McFadden *et al.,* 1996).

The malaria parasite possesses two APT transporters that are differentially localized to the inner and outer membrane of the apicoplast (Mullin et al., 2006). The *T. gondii* on the other hand has only one APT (TgAPT) (Karnataki et al., 2007; Fleige et al., 2007) that most likely localizes to the multiple membranes of the apicoplast. The TgAPT delivers carbon units, triose phosphates and phosphoenolpyruvate, for at least two different anabolic processes in the apicoplast, namely fatty acid synthesis and the DOXP pathway. It also has an essential role in indirectly supplying the apicoplasts with ATP and redox equivalents. The APT is therefore a metabolic hub that links cytosolic metabolism with essential processes in the apicoplast (Mullin et al., 2006; Brooks et al., 2010; Karnataki et al., 2007; Lim et al., 2010).

The TgAPT had already been expressed in yeast (Brooks *et al.,* 2010) and because protein production in yeast is time consuming and expensive we also wanted to produce the APT protein in a bacterial system, namely the model organism *Escherichia coli* and to establish the purification protocol for the protein. To obtain the best APT expression, the APT protein was expressed in six different *E. coli* strains (BL21 RIL, BL21 DE3, C41, C43, Rosetta PLys and

Rosetta). These cell strains have different properties, especially in the translation system. However, all chosen strains have a T7-based expression system. Here, the T7-RNA polymerase gene is under the control of the *lacUV5* promoter and is induced by the addition of IPTG. The induced T7 polymerase is then transcribing the APT gene that is regulated by a T7 promoter. The results presented here clearly show that not all transformed *E. coli* strains are capable of expressing the protein (Figure 10). Only the BL21 RIL strain but none of the other strains expressed the APT protein. The BLR21 RIL strain possesses additional copies of the thre tRNA genes *argU*, *ileY*, and *leuW*. Thus, a lack of sufficient amounts of these tRNAs might explain the missing APT expression in the other strains. However, a more detailed analysis of the expression of the APT in several independently transformed BLR21 RIL cells showed that different clones differed significantly in their APT expression level, with a number of clones showing no expression at all while two (clones 23 and 25) showed a high expression level (Figure 12). These data reveal that the expression of membrane proteins in *E. coli* is difficult to achieve and that it is necessary to screen several strains and large number of independent clones for expression.

For protein isolation, the *E. coli* cells were disrupted by sonication and the extract was centrifuged at low speed. The pellet represents in the insoluble material which is mainly unbroken cells and cell debris. It also contains unfolded and insoluble proteins which are concentrated in large "inclusion bodies". Heterologously expressed proteins are often found in these inclusion bodies. In contrast, the supernatant contains the soluble proteins and the membranes released from the cell. A total of 10 protein extraction experiments were performed (not all data shown). In each experiment the APT protein was present both in the pellets and the supernatants after cell lysis. One of the reasons for the occurrence of the APT protein in the pellet could be that the cells were not disrupted completely. The second reason is that part of the APT did not correctly fold into its native conformation and therefore is found in inclusion bodies. For example, during sonication much heat is produced causing protein denaturation. Therefore, the conditions during the process of cell lysis should be optimized or different techniques to break the cells like a French press or a beat beater should be used.

One of the many properties that proteins have is the ability to bind to specific ligands. This property enables to separate specific proteins of interest from others in a mixture. Affinity chromatography is a type of liquid chromatography which is based on the highly specific interaction of a protein (or part of it) with another molecule that is attached to a matrix. The Strep-tag® system uses the Strep-tag® II tag which is a synthetic peptide consisting of eight amino acids (**WSHPQFEK**). This peptide sequence exhibits native affinity towards Strep-

Tactin®, a specifically engineered streptavidin. It binds to the biotin binding pocket, enabling mild competitive elution with biotin or biotin derivates like desthiobiotin (Schmidt 2013; see Figure 21).

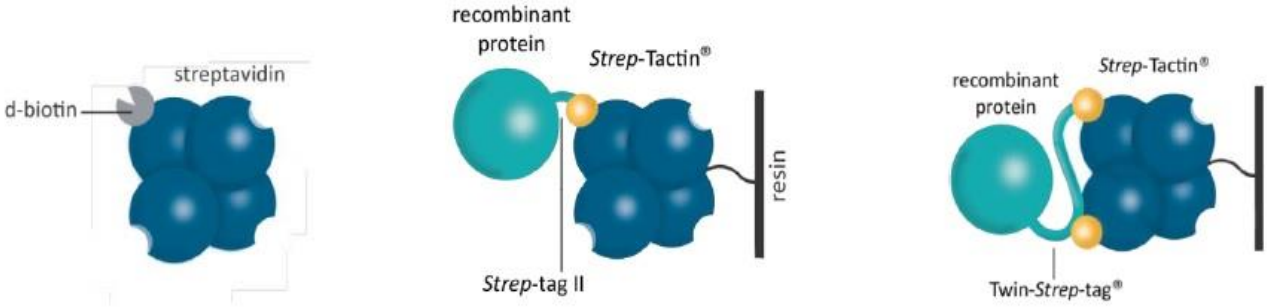

Figure 21. Schematic view of:
1. biotin and streptavidin, **2.** a protein with Strep-Tag II and Strep-Tactin® , **3**. a protein with a Twin-Strep-tag and Strep-Tactin® (IBA 2012).

To achieve an affinity based purification, a new construct of the APT protein was used. Purification by streptavidin affinity chromatography turned out to be more efficient than NTA chromatography (unpublished data by Karsten Fischer). Therefore, the his6-tag and Strep-tag® II of the APT that had been expressed in yeast were replaced with the new Twin-Strep-tag®. This Twin-Strep-tag® (WSHPQFEK-GGGSGGGSGG-SAWSHPQFEK) is a short synthetic peptide which consists of two Strep-tag II moieties connected by a short linker and two amino acid spacer between the protein and the tag (see Introduction). The chromatography is then performed in three steps: sample loading, washing, and elution of the protein by a high concentration of a free ligand like biotin (Hage et al., 2013).

In a first experiment, no APT signals were detected in a western blot analysis of the affinity chromatography, neither in the flow through nor in the eluted samples, although the APT protein was expressed in the *E. coli* cells (Figure 18). This suggests that there were problems with the solubilization of the membrane proteins and/or with the affinity purification.

The affinity purification was repeated with the newly induced *E. coli* clone 25 and part of the APT protein was detected in the flow through and elution (Figure 19), indicating that part of the total APT protein content in the pellet has been successfully solubilized by the detergent DDM. However, only less than 50% of the solubilized protein was bound to the affinity column.

The reason could be that in part of the protein the affinity tag is not accessible because it is covered by the detergent.

The APT protein from the supernatants of the clones 23 and 25 was also purified by affinity chromatography. Here, no signal was detected in the flow through and a week signal in the elution samples (Figure 20), indicating that APT protein in the supernatant can be solubilized by DDM and that it binds effectively to the affinity column.

5. Conclusion and future work

Determination of the structures of membrane proteins is a challenging task that is essential to understand biological function at the molecular level. In order to provide insights into its biochemical properties, we tried to overexpress and purify the APT protein of *Toxoplasma gondii*. At present, the protein could not be completely purified. Therefore, several problem have to be solved in the future. These are the optimization of cell lysis, solubilization of the protein and the affinity purification.

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Appendix

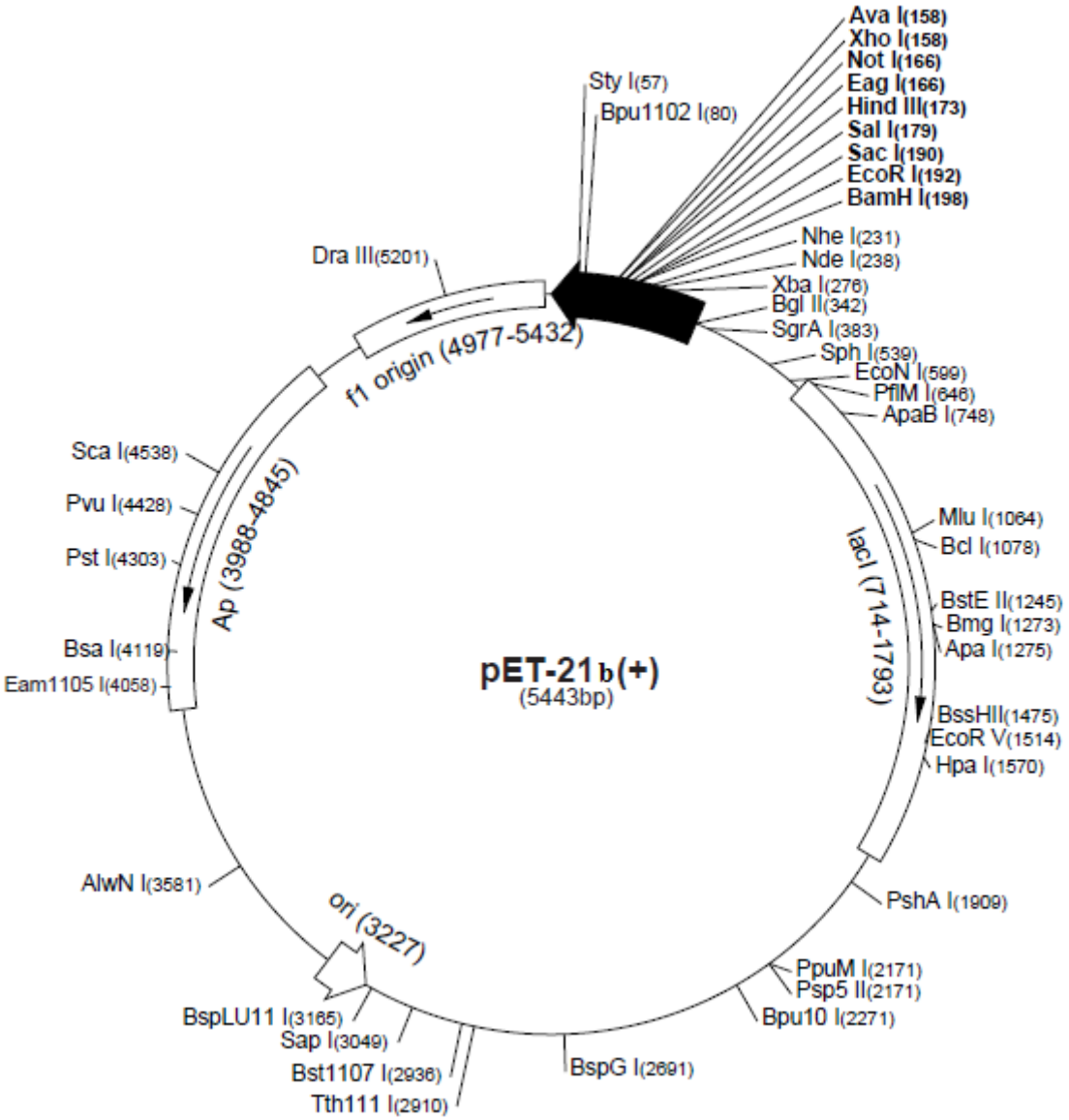

Figure 22. The systemic diagram of the pET-21 b (+) vector

The pET-21 b (+) vectors carry an N-terminal T7-Tag® sequence plus an optional C-terminal His-Tag® sequence. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer. Black arrow indicates the multiple cloning site; Ap- ampicillin resistance gene; ori- bacterial origin of replication; *lacI*- lactose operon repressor. Figure taken from Novagen pET system manual.