CLONING, EXPRESSION, AND CHARACTERIZATION OF A POLYPRENYL

DIPHOSPHATE SYNTHASE FROM *TOXOPLASMA GONDII*

by

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(Under the Direction of Silvia N. J. Moreno)

ABSTRACT

Toxoplasma gondii is an opportunistic protozoan parasite of fetuses from infected mothers and of immuno-compromised patients. Limitations of current treatments and unavailability of a suitable vaccine necessitate discovery of novel targets and development of new drugs. The isoprenoid pathway of *T. gondii,* being unique and essential, could be exploited for therapeutic intervention. Recently, we have characterized a polyprenyl diphosphate synthase from *T. gondii* (TgPPS)*,* as a target of bisphosphonates*.* We cloned the *TgPPS* gene homologous to polyprenyl synthases from other organisms, expressed it in *Escherichia coli*, and characterized the recombinant-TgPPS. Our results show that TgPPS is a soluble protein and localizes to the cytoplasm of the parasite. We propose that TgPPS is a heptaprenyl diphosphate synthase, and may synthesize the isoprenoid side chain of ubiquinone. Our *in vitro* enzyme inhibition study demonstrates that TgPPS is inhibited by many bisphosphonates, which justifies the potential of *TgPPS* as a putative drug target.

INDEX WORDS: *Toxoplasma gondii (T. gondii)*, Toxoplasmosis, Isoprenoid Pathway, Polyprenyl diphosphate synthase (TgPPS), Cloning, Characterization, Bisphosphonates (BPs)

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B.V.Sc. & A.H., Orissa University of Agriculture and Technology, India, 2004

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment

of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2009

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December 2009

DEDICATION

I dedicate the thesis to my beloved parents.

ACKNOWLEDGEMENTS

First of all, I would like to thank my advisor, Dr. Silvia Moreno, and Dr. Roberto Docampo for giving me an opportunity to do research in their lab, although I was a novice to the world of experimental molecular biology. I thank my committee members; Dr. Boris Striepen, Dr. Kojo Mensa-Wilmot, and Dr. Walter Schmidt, for their valuable suggestions for my research. I sincerely thank Dr. Zhu-hong Li, who has been extremely helpful in my experiments. I also thank Dr. Marcus Fechheimer and Dr. Mark Farmer for their kind support and advice during my study. I thank all the faculties and staffs of the department for their help on different occasions. I thank my wife Rebati for her unconditional love and support all the time. I also thank my best friend Animesh for being very helpful to me whenever needed. Last but not least, I thank all my lab mates for helping me in many different ways.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

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1. *Toxoplasma gondii*

1.1 Overview

Toxoplasma gondii, an intracellular pathogenic apicomplexan parasite, is the causative agent of toxoplasmosis. Among three distinct genotypes (I, II and III) of *T. gondii* described so far, type I strain has been found to be most virulent[1]. The target host range among vertebrate animals is very broad and includes humans as intermediate hosts[2]. Felines (*e.g.* domestic cats) are the only known definitive hosts and harbor the sexual stages of the parasite.

Prevalence rate could be as high as 70 % in some places, which clearly denotes *T. gondii* as a flourishing parasite worldwide[3]. Transmission occurs mostly through ingestion of contaminated food and/or water. As a zoonotic infection, there are considerable variations in the seroprevalence of *T. gondii* among human population from different geographic areas[4].

Toxoplasmosis, in case of immuno-competent individuals, is limited by the host's immune system and therefore, the disease remains asymptomatic. However, the manifestation could be very severe in developing fetuses from recently infected pregnant women, immune deficient individuals like AIDS patients, and organ transplant recipients.

The currently available treatments have many limitations like drug intolerance and toxicity in patients. An effective vaccine is still not available to prevent this disease.

Therefore, efforts are being made to understand the biological features of *T. gondii*, which will facilitate the discovery of druggable targets and development of novel drugs.

1.2 Life Cycle and Disease Transmission

T. gondii progresses through a complicated life cycle involving both definitive (primary) and intermediate (secondary) hosts. As far as the hosts are concerned, the definitive host range is very narrow and confined to felines (*e.g.* domestic cats) only. In contrast, the intermediate host range is very broad and includes humans, rodents, birds, dogs and many more. Tachyzoites (the fast replicating form), bradyzoites (the slow replicating form in tissue cysts) and sporozoites (in oocysts) are the three developmental stages of the *T. gondii* life cycle. The sporulated oocyst form of the parasite is highly infectious and a very small dose is sufficient to establish an infection[5]. Cats are capable of releasing millions of noninfectious unsporulated oocysts in their feces for a few weeks after acquiring the infection. The oocysts become infective after sporulation within 1-21 days depending on the environmental conditions[6].

Humans acquire infection through ingestion of undercooked infected meat (containing tissue cysts), drinking of contaminated water, contact with feces from infected cats, and rarely through organ transplantation and blood transfusion from infected donors[7,8,9].Nearly 38% of the meats available in the market in the UK are infected as reported in one study[10]. *T. gondii* has been listed as category B priority pathogen by the US National Institute of Health (NIH) because of its moderately easy transmission through contaminated food and (or) water. Also, *T. gondii* causes moderate morbidity and low mortality. Upon ingestion, sporozoites are released from the oocysts and transform into

tachyzoites in the intestinal epithelium of the host[85]. Tachyzoites undergo rapid division inside cells causing massive tissue destruction and further spread of infection.

The developing fetuses are at risk of infection with tachyzoites through vertical transmission from infected pregnant mothers[7]. Congenital toxoplasmosis has drawn significant attention in the past[11]. Contact with infected cat's faces or consumption of improperly cooked meat have been noted as the risk factors for those mothers who gave birth to infected children in one study[12]. Eventually, tachyzoites transform into bradyzoites and localize to tissue cysts in muscles and the central nervous system (CNS). Usually, after two - three weeks of infection, the division of the parasite is slowed down by the immune system and a protective membrane is formed around the cell containing the parasite (bradyzoites) which is called the tissue cyst. Formation of cyst is supposed to be a protective host immune response against the parasite for controlling the spread infection[6]. Tissue cysts may persist for the entire life of the hosts and can spread the infection if ingested without proper processing.

1.3 Pathogenesis and Outcomes of *T. gondii* Infection in Human

Pathogenesis of *Toxoplasma* infection is due to the parasite's ability to enter nearly any type of nucleated cell and produce infection. The disease, in case of healthy individuals may be mild and often remains undetected. However, symptoms like fever and swelling of lymph nodes can be observed at times. Incidence of ocular toxoplasmosis characterized by single or multiple necrotic retinitis, resulting in partial or complete loss of vision is rare[13,14].

The outcome of *T. gondii* infection can be quite severe in immunodeficient patients (e.g. patients with AIDS), congenitally infected newborns, and organ transplant recipients.

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In AIDS patients severe toxoplasmic encephalitis may occur, which still remains a cause for high morbidity and mortality[2,12,15]. Also, the incidence of myocaridits and pneumonitis has been reported in AIDS patients with toxoplasmosis[16].

Congenital toxoplasmosis is characterized by hydrocephalus, retinochoroiditis, and diffuse intracerebral calcification in neonates from infected mothers. Also, involvement of the central nervous system and other organs like heart, liver and lungs is very frequent[15,17]. Organ transplant recipients acquire infection from exogenous source has been a serious cause for concern[18]. The organ transplant recipients are administered with immunosuppressive drugs to accommodate the foreign organ. Therefore, *T. gondii* is capable of establishing a productive infection in organ transplant recipients similar to immuno-compromised individuals. The effects of cardiac toxoplasmosis depend on multiple factors, such as degree of myocarditis, level of muscle fiber necrosis, and presence of tachyzoites in the cardiac myocytes. Cardiac arrest resulting in death of the individual is the ultimate outcome[19].

1.4 Current Chemotherapy and Future Perspective

Treatment and prevention of *T. gondii* infection has been very challenging due to the following two reasons. First, it's very hard to find safe drug targets because the parasite shares many common metabolic pathways with the mammalian hosts. Second, severe infection occurs mostly in immunocompromised patients, which makes the development of an effective vaccine very difficult. The treatments currently available are unsatisfactory and have many pitfalls. Also the bradyzoite stages of the parasite are resistant to several drugs.

Drugs, which are currently in use for the treatment of toxoplasmosis, primarily target the metabolic pathways of *T. gondii*. Sulfonamide and pyrimethamine, which target the folic acid metabolism of the parasites, are in clinical use[20]. Atovaquone has been shown to disrupt the mitochondrial electron transport of *T. gondii*[21,22]. Other drugs against *T. gondii* include azithromycin, roxithromycin, clarithromycin, dapsone and trimetrexate[23]. The standard therapy, pyrimethamine with sulfadiazine, has been reported to be toxic with continued use[24]. Other reasons include drug intolerance by the patients, adverse side effects of the drugs, and drugs inhibiting multiple targets[20].

Severity of toxoplasmosis warrants further exploration of the parasite's biology in search of novel therapeutic targets and potential drugs. In this scenario, biosynthetic pathways, which are essential for the parasite but different from the hosts, have been the focus of drug discovery related studies. Isoprenoid biosynthesis pathway in apicomplexan is one such pathway which could be exploited for therapeutic interventions with potential drugs.

2. Isoprenoid Biosynthesis Pathway

2.1 Isoprenoids

Isoprenoids, also known as terpenes, constitute a family of organic compounds. They are essential components of all organisms and derived from the five carbon isoprene (2-methyl-1, 3-butadiene) unit. Functional groups like carbonyl, hydroxyl etc. are attached to the carbon backbone, which imparts diversity to isoprenoids with respect to structure and function. Some isoprenoids like sterols are cyclic where as others (squalane, phytol etc) are acyclic and have linear structures. Isoprenoids are named according to the numbers

of isoprene units present. Monoterpene has two isoprenes, sesquiterpene has three, and diterpene has four and so on.

Steroid hormones, vitamins (A, E and K), cholesterol, dolichols, pigments like retinoid and carotenoid, ubiquinone and prenylated proteins are few examples of isoprenoids having different biological roles in eukaryotes, bacteria and archea. Sterols are the components of the cellular membrane structures and dolichols are necessary for glycoprotein biosynthesis[25]. Pigments like carotenoids are essential for photosynthesis and retinoids are important for vision. Ubiquinones (coenzyme Q) are involved in the oxidative phosphorylation and energy generation in the mitochondria. Prenylated proteins like Ras, G-proteins etc. have roles in signal transduction pathways[26,27]. Evidence shows the presence of dolichols[28] ubiquinones[21], and prenylated proteins[29] in *T. gondii.* This suggests that the isoprenoid pathway of *T. gondii* might be important from biological point of view.

2.2 Isoprenoid Biosynthesis

Isopentenyl diphosphate (IPP) is the physiological isoprene unit in all living organism and central compound of the isoprenoid biosynthesis pathway. IPP along with its isomer, DMAPP (dimethylallyl diphosphate) gives rise to all isoprenoids through a series of consecutive condensation reactions catalyzed by different prenyl diphosphate synthase enzymes. There are two pathways of IPP biosynthesis described so far, namely, the classical mevalonate (MVA) pathway and the non-mevalonate [1-Deoxy D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (DOXP/MEP) pathway][30,31]. The pathways have been named according to their key intermediates, mevalonate and DOXP/MEP (Figure-1.1[35]).

The MVA pathway has been well established in mammals, bacteria, higher plants, trypanosomatids and yeast[32]. The MVA pathway begins with three molecules of acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) which is reduced to mevalonate (MVA) by HMG-CoA reductase. Mevalonate is subjected to double phosphorylation to produce mevalonate diphosphate (MDP) in two reactions catalyzed by mevalonate kinase and phosphomevalonate kinase respectively. Subsequently, mevalonate diphosphate is decarboxylated by phosphomevalonate decarboxylase (MDD) in an ATP-dependent reaction to form isopentenyl pyrophosphate (IPP), which is the precursor of all isoprenoids (Figure-1.2[79]).

The non-mevalonate pathway was discovered in bacteria and plant species during isotope labeling studies[30]. The nonmevalonate pathway predominates in higher plants, several algae, bacteria, and notably, in apicomplexans[33,34]. 1-Deoxy D-xylulose 5-phosphate (DOXP) synthase catalyzes the first step of the nonmevalonate pathway to make DOXP from pyruvate and glyceraldehyde-3-phosphate. DOXP is converted to 2-C-methyl-D-erythritol 4-phosphate (MEP) in the second step by DOXP reductoisomerase. MEP is converted to 4-diphosphocytidyl-2C-methyl-D-erythritol-phosphate (CDP-MEP) by MEP cytidyltransferase. 4-Diphosphocytidyl-2C-methyl-D-erythritol (CDP-ME) kinase catalyzes the synthesis of 4-Diphosphocytidyl-2C-methyl-D-erythritol diphosphate (CDP-ME2P) from CDP-MEP. 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate (MECDP) synthase use CDP-ME2P to produce 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate, which is catalyzed by 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBDP) synthase to make 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate. HMBDP, the immediate precursor of IPP, is catalyzed by HMBDP reductase to make IPP (Figure-1.3[78]).

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Following synthesis of IPP by either pathway, IPP is converted into its isomer DMAPP. As mentioned earlier isoprenoid synthesis follows a condensation reaction of IPP and DMAPP which generates geranyl diphosphate (GPP), a ten carbon isoprenoid chain. GPP gives rise to farnesyl diphosphate (FPP), a 15 carbon compound. FPP is the key intermediate which gives rise to other isoprenoids. The enzyme farnesyl diphosphate synthase (FPPS) catalyzes the synthesis of both GPP and FPP. Chain elongation of isoprenoids is carried out by the addition of IPP molecules to the allylic substrates by a family of enzymes known as prenyl diphosphate synthases or prenyl chain elongating enzymes. Isoprenoids are synthesized via the non-mevalonate pathway in *T. gondii* and *Plasmodium falciparum*[33,34].

2.3 Prenyltransferases: Enzymes of the Isoprenoid Pathway

2.3.1. Nomenclature and Classification

Broadly the enzymes of the isoprenoid pathway are called prenyl transferases. However these enzymes can be further classified in many different ways.

Class-I: <u>**Prenyl diphosphate synthases**</u> are prenyltransferases which determine the linear carbon chain lengths of the prenyl diphosphate products. According to the stereochemistry of the products they may be *cis-* or *trans-* prenyl diphosphate synthases[36]. *Cis*-prenyl synthases have been found in bacteria[37,38], plants[39] and yeasts[40]. *Trans*-prenyl diphosphate synthases are widely found in higher eukaryotes. In general, the stereoisomerism is not considered for naming these enzymes[41].

Another way of classifying prenyl diphosphate synthases is based on the quaternary structure and linear chain length of the products as short, medium or long chain prenyl diphosphate synthases[42]. Short chain prenyl diphosphate synthases produce an isoprenoid

chain of 15-25 carbons and include farnesyl diphosphate synthase (FPPS) and geranylgeranyl diphosphate synthase (GGPPS). The chain length of the products of medium chain prenyl diphosphate synthases ranges from 30-50 carbons. This includes hexaprenyl diphosphate synthase (C_{30}), heptaprenyl diphosphate synthase (C_{35}), octaprenyl diphosphate synthase (C_{40}), solanesyl diphosphate synthase (C_{45}), and decaprenyl diphosphate synthase (C_{50}). Long chain prenyl diphosphate synthases product has more than 50 carbons like undecaprenyl diphosphate synthase (C_{55}), and others[42].

Class II: <u>Protein Prenyltransferases</u> are a group of prenyltransferases that facilitate the transfer of prenyl group into a cysteine residue of a peptide or protein[43]. Protein farnesyl transferase (PFT) and protein geranylgeranyl transferases I (PGGT I) are examples of this class of enzyme and transfer farnesyl and geranylgeranyl group to CAAX motif of the protein substrate[44,45]. However, another member of this class of enzymes, Rab geranylgeranyl transferase or geranylgeranyl transferase II transfers two geranylgeranyl groups to the cysteine residues at the C-terminus of Rab proteins[86]. The Rab proteins lack the CaaX motifs. So, the geranylgeranyl group transfer is facilitated by Rab escort protein (REP) with the characteristic XXCC, XCXC, or CCXXX motifs[80].

Class III: <u>Terpenoid/ Isoprenoid Cylcases</u> are a class of prenyltransferases that catalyzes the cyclization of isoprenyl diphosphate products[46]. Squalene synthase, a terpenoid cyclase, synthesizes squalene from cyclization of two molecules of FPP[47].

Prenyltransferases are essential enzymes of the isoprenoid pathway. Some of them have been asserted as compelling drug targets in apicomplexan parasites to discover lead inhibitors, and for designing new drugs[54,57,81]. Therefore, a thorough understanding of

different enzymes of this pathway in *T. gondii* will be significantly helpful for drug discovery.

2.3.2 Characterization of Prenyl diphosphate synthases

All prenyl diphosphate synthases (short, medium and long chain) are regarded as polyprenyl diphosphate synthases. More commonly, the term polyprenyl diphosphate synthase is used to describe the medium and long chain prenyl diphosphate synthases. Many prenyl diphosphate synthases have already been characterized in different organisms. Farnesyl diphosphate synthase (FPPS) catalyzes the first committed step in the pathway and produce FPP. Geranylgeranyl diphosphate synthase (GGPPS) makes geranylgeranyl diphosphate (GGPP) by adding one molecule of IPP with FPP.

Genes encoding FPPS and GGPPS have been characterized from rat[48], human[49], yeast[50], and plants[51,52]. FPPS have been cloned and characterized from different parasitic kinetoplastids[53,54,55] as well as from apicomplexan parasites[56]. *T. gondii* FPPS has been described as the first bifunctional FPPS/GGPPS in eukaryotes[56].

Some polyprenyl diphosphate synthases characterized so far include solanesyl-diphosphate synthase from *Trypanosoma cruzi*[57], octaprenyl pyrophosphate synthase from *Plasmodium falciparum*[58]*,* solanesyl and decaprenyl diphosphate synthases from mice and human[59], solanesyl diphosphate synthase from *Arabidopsis thaliana*[60], heptaprenyl diphosphate synthase from *Bacillus subtilis*[61].

It has been proposed that polyprenyl diphosphate synthases essentially define the length of the side chain of ubiquinone[62] and therefore, the type of ubiquinone in an organism. Ubiquinones are essential components of the mitochondrial electron transport

chains. Owing to the significant roles played by the isoprenoid products in the biology of an organism, many of the enzymes, which make them, are indispensable.

2.3.3 Structural Features and Activity of Prenyl diphosphate synthases

As mentioned earlier, different prenyl diphosphate synthases have been cloned and characterize from different organisms. Alignment of the deduced amino acid sequences from different organisms revealed a strong similarity[63]. Also there are seven conserved domains (I-VII) and two aspartate rich (DDXXD) motifs, namely First aspartate rich motif (FARM) and second aspartate rich motifs (SARM)[63]. This suggests that the prenyl diphosphate synthases might have a common origin. The FARM and SARM were identified from three dimensional structural studies[64] and further supported by site directed mutagenesis studies[65,66]. It was found that these aspartate rich motifs are responsible for binding of substrates and cofactors like Mg^{+2} and catalysis[41,67].

The structures of several prenyl diphosphate synthases have been successfully resolved from different species using X-ray crystallography such as FPPS from *T. brucei*[41], *T. cruzi*[68], human[69], GGPPS from *S. cerevisiae*[70], *Plasmodium vivax* (Unpublished), human[71], undecaprenyl diphosphate synthase from *Mycobacterium tuberculosis*[72] etc. The avian FPPS was the first in this regard[64].

Analysis of the FARM and SARM demonstrated that all the aspartate residues are equally important except the last aspartate residue in the SARM[73]. Also it was discovered that the FARM is the binding site for allylic substrates like GPP, FPP and GGPP where as the SARM provides the site for interaction and binding of IPP[67,74].

Regarding the chain length determination of the product, the amino acids at position 4th an 5th of the N-terminus to the first aspartate rich motif were found to be

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important. Along with this the two amino acids in the FARM are also important[74,75]. The amino acid sequence spanning the FARM and up to 5 amino acids to the N-terminus of the FARM is considered as the chain length determination domain (CLD). It has been reported that substitution of amino acids with bulky side chains at position fourth and fifth can prevent elongation of the isoprenoid product[74].

2.3.4 Isoprenoid Pathway of *Toxoplasma gondii* as a Potential Target

Unavailability of ideal chemotherapy for treatment of toxoplasmosis in immuno-compromised patients warrants discovery of novel drug target and development of effective drugs. Therefore, the metabolic pathways, which are essential for the parasite and different from the hosts, have been the focus of drug discovery related studies. Isoprenoid biosynthesis pathway in *T. gondii* is one such pathway, which has been proposed as a druggable target[35]. Isoprenoid derivatives like dolichols, ubiquinone and prenylated protein are present in the parasite. This suggests that the parasite isoprenoid pathway might be essential from the biological point of view. Different groups have reported inhibition of the parasitic isoprenoid pathway with bisphosphonates (BPs) in parasites such as *Plasmodium*[76], *Trypanosome*[54,57], *Leishmania*[77] and *Toxoplasma*[56].

3. Bisphosphonates

Considering the huge costs and extended time from the point of drug discovery to the approval for clinical use, efforts are being made to search for suitable candidates from the repertoire of existing drugs and to test against promising targets in parasites. Bisphosphonates represent such a class of drugs, which can be employed to control parasitic infections in humans and animals.

Bisphosphonates are stable pyrophosphate derivatives in which a carbon atom replaces the central oxygen atom between the two phosphorous atoms of the pyrophosphate. The central carbon atom imparts diversity to bisphosphonates because of its ability to have variable side chains. Many of them are potent inhibitors of bone resorption and are the treatment of choice for various diseases[76]. Broadly, bisphosphonates are classified into simpler non-nitrogen containing bisphosphonates (clodronate, etidronate etc) and the nitrogen containing bisphosphonates (pamidronate, alendronate, risedronate etc). The mechanisms of actions are also different for different BPs. Some of them are converted into stable (resistant to enzymatic hydrolysis) ATP analog by mammalian cells and produce a cytotoxic effect[82]. Certain nitrogen containing BPs have been shown to target and inhibit the mevalonate pathway[83,84].

The activities of bisphosphonates have been tested against the replication of *T. gondii in vitro* and *in vivo*. Two n-alkyl bisphosphonates containing long hydrocarbon chains were most active compared to the nitrogen containing BPs[81]. These results suggest that alkyl-bisphosphonates, targeting isoprenoid pathway, are promising compounds for further studies against *T. gondii*.

4. Summary

T. gondii is an opportunistic human pathogen and infects a wide range of vertebrate hosts. The disease, although mild in immune individuals, can be life threatening in immune compromised patients and infected newborns. Currently available treatments are unsatisfactory for many reasons. Also, no vaccine is available for prevention of the disease. Therefore discovery of new drug targets and development of effective drugs are of paramount importance. The isoprenoid pathway of *toxoplasma gondii* represents a

promising target. A thorough understanding of the different steps of the pathway might lead to discovery of novel therapeutic approach. Bisphosphonates are currently in clinical use for treatment of a number of diseases. It has been reported that bisphosphonates target the isoprenoid pathway in different parasites. Investigation and synthesis of more potent bisphosphonate will lead to discovery of better drugs against parasites like *T. gondii*.

Figure 1.1: Schematic Overview of the Isoprenoid Pathway (Adopted[35]). IPP is synthesized via the Mevalonate pathway (bacteria and most eukaryotes) and the DOXP/MEP pathway (bacteria, algae, higher plants, and apicomplexan parasites). FPP is the key intermediate which gives rise to ubiquinones, heme a, sterols, dolichols and geranylgeranyl diphosphate (GGPP). FPP synthase (FPPS) catalyzes the synthesis of FPP. Protein farnesyl transferase (PFT) and protein geranylgeranyl transferase (PGGT) are responsible for protein prenylation. Statins and fosfidomycin inhibit the Mevalonate Pathway and DOXP/MEP pathways respectively. Bisphosphonates inhibiting FPPS has also been shown.

Figure 1.1:
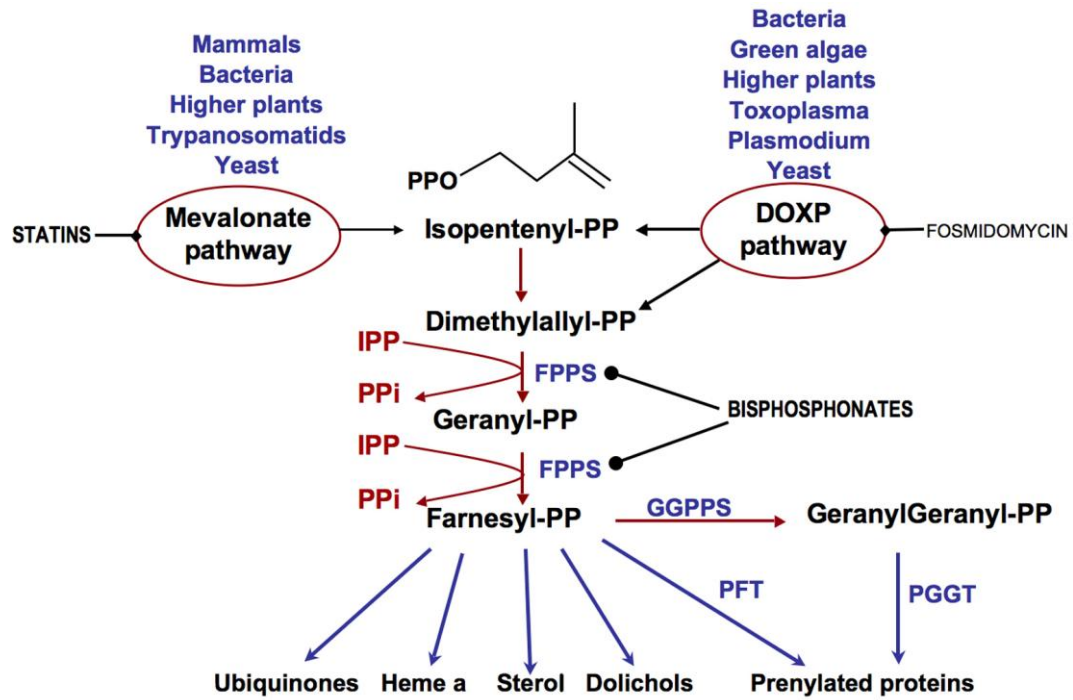
Figure 1.2: The Mevalonate Pathway (Adopted and modified[79]): IPP is synthesized from Acetyl-coA in the Mevalonate pathway. Structures of different intermediates have been shown. The enzymes which catalyze the different steps of the pathway have been mentioned as AAS - acetoacetyl-CoA synthase; HMGS-HMG-CoA synthase; HMGR-HMG-CoA reductase; MK - mevalonate kinase; PMK - phosphomevalonate kinase; PMD - mevalonate diphosphate decarboxylase.
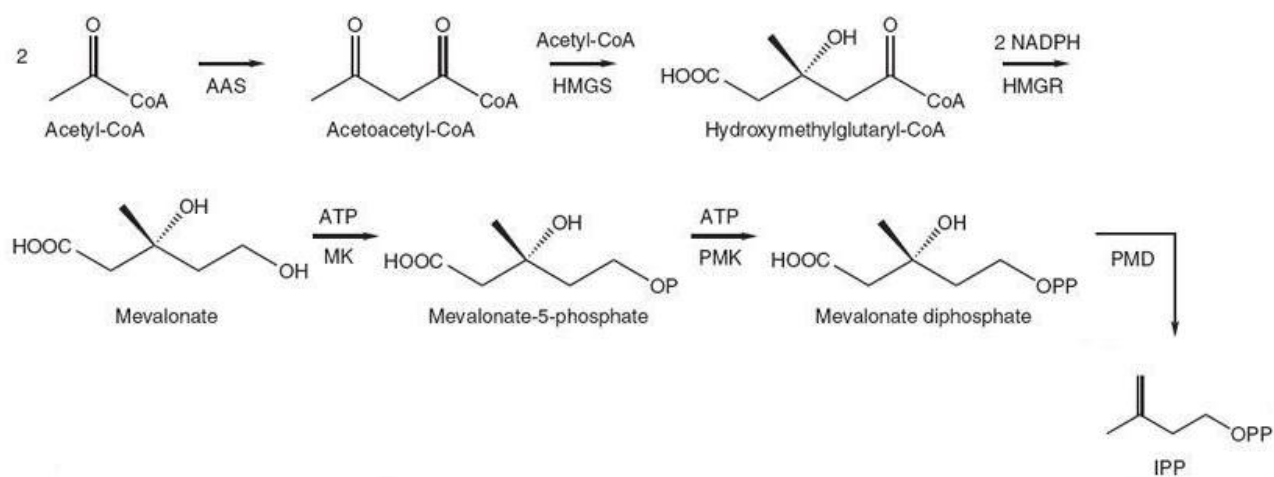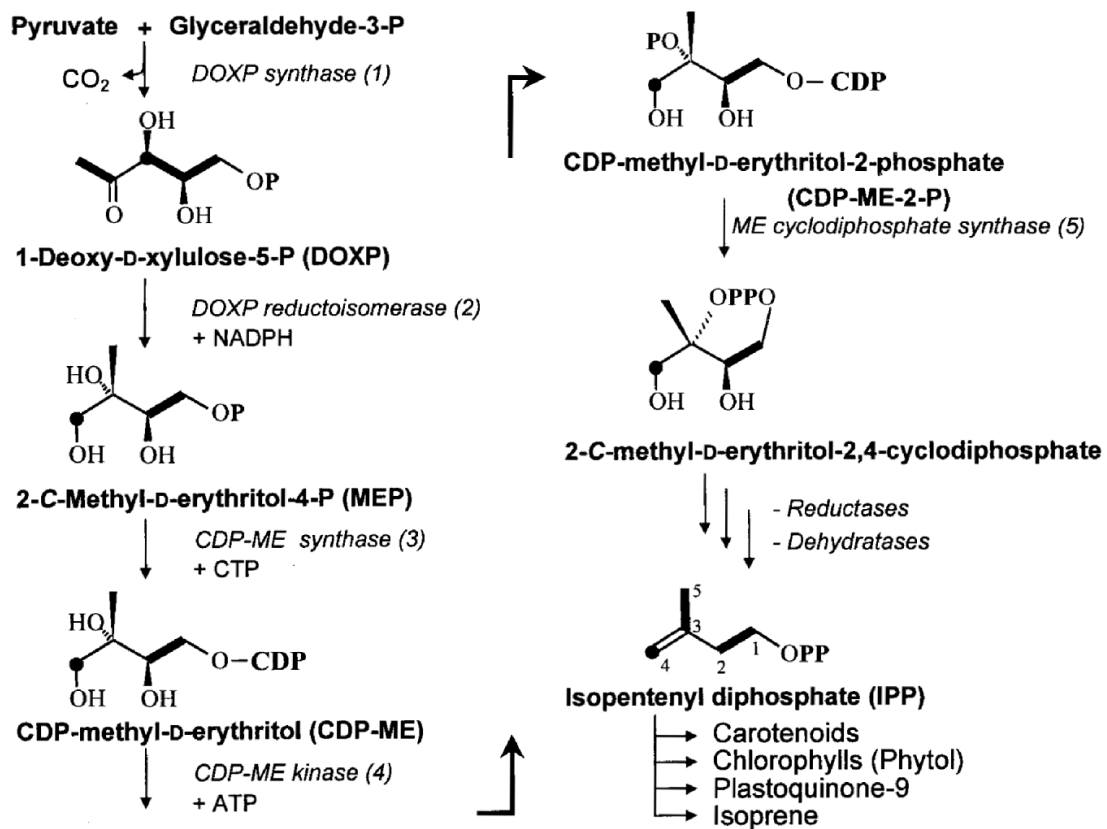
Figure 1.2:

Figure 1.3: The Non-mevalonate Pathway (Adopted and modified[78]). The five important enzymes of the nonmevalonate pathway have been shown (1-5) along with the structure of the intermediates. Subsequent to the synthesis of 2-C-methyl-D-erythritol-2,4-cyclodiphosphate, there are two enzymes, namely 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBDP) synthase and HMBDP reductase which catalyze the synthesis of IPP. Structure of HMBDP is not shown.

Figure 1.3:

Pyruvate + Glyceraldehyde-3-P

CO_2 — DOXP synthase (1)

1-Deoxy-D-xylulose-5-P (DOXP)

DOXP reductoisomerase (2)
+ NADPH

2-C-Methyl-D-erythritol-4-P (MEP)

CDP-ME synthase (3)
+ CTP

CDP-methyl-D-erythritol (CDP-ME)

CDP-ME kinase (4)
+ ATP

CDP-methyl-D-erythritol-2-phosphate (CDP-ME-2-P)

ME cyclodiphosphate synthase (5)

2-C-methyl-D-erythritol-2,4-cyclodiphosphate

- Reductases
- Dehydratases

Isopentenyl diphosphate (IPP)

→ Carotenoids
→ Chlorophylls (Phytol)
→ Plastoquinone-9
→ Isoprene

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CHAPTER II

CLONING, EXPRESSION, AND CHARACTERIZATION OF A POLYPRENYL DIPHOSPHATE SYNTHASE FROM *TOXOPLASMA GONDII*

1. Abstract

Polyprenyl diphosphate synthases catalyze the synthesis of varied chain length isoprenoids through a series of condensation reactions involving isopentenyl diphosphate and an allylic diphosphate substrate. Depending upon the carbon chain length of the products they may be short (C_{15}-C_{25}), medium (C_{30}-C_{50}) or long chain prenyl diphosphate synthase ($>C_{50}$).

We report the identification, cloning and functional characterization of a medium chain prenyl diphosphate synthase (TgPPS) from *Toxoplasma gondii*. The *TgPPS* gene encodes for a 526 amino acid protein (molecular mass ~56.76 kD) homologous to polyprenyl diphosphate synthases from other organisms. All seven conserved motifs characteristic of the polyprenyl diphosphate synthase family are present in TgPPS. Recombinant TgPPS purified from *Escherichia coli* was enzymatically active and was used for biochemical characterization. Radiolabeling and thin layer chromatography (TLC) show that the final product of TgPPS activity is made up of seven isoprene units. Hence, we propose that TgPPS is a heptaprenyl diphosphate synthase. Ubiquinone analysis from *T. gondii* by HPTLC revealed the presence of ubiquinone-7 in the parasite. Immuno-fluorescence microscopy with *T. gondii* cells expressing TgPPS-3myc shows a cytoplasmic distribution of TgPPS both in the intracellular and in the extracellular parasites. TgPPS was detected in the soluble fractions by western blot and subcellular fractionation analysis.

Our *in vitro* drug inhibition studies demonstrate that TgPPS is inhibited by a number of bisphosphonates. This evidence preliminarily suggests that TgPPS might have potential as a putative drug target.

2. Introduction

Current treatment for toxoplasmosis faces many challenges like drug resistance in the parasites (bradyzoites) and drug intolerance in the immuno-compromised patients. Many drugs have toxic effects and can't be employed for prolonged use[7,8]. Development of an effective vaccine for prophylactic measures has been more troublesome as severe infection occurs mostly in immuno-deficient patients. Therefore, discovery of new targets and development of effective drugs are of paramount importance to fight against toxoplasmosis. In this regard, the isoprenoid pathway, being essential to the parasite and different from the hosts, represents a novel target for chemotherapy.

Isoprenoids are biologically important macromolecules derived from repeating five carbon isoprene units. Isopentenyl diphosphate (IPP), the physiological isoprene unit, is synthesized via the 1-Deoxy D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (DOXP/MEP) pathway in *T. gondii*[9,10]. Isoprenoid derivatives like dolichol[11], ubiquinone[12], and prenylated proteins[13] are found in this parasite and have important biological roles. Biosynthesis of these products is catalyzed by prenyl diphosphate synthases which constitute a family of homologous enzymes and can be classified into short chain (C_{15}-C_{25}), medium chain (C_{30}-C_{50}) or long chain prenyl diphosphate synthases ($>C_{50}$) depending on final chain length of their products[14]. Enzymes of the *T. gondii* isoprenoid pathway studied so far includes farnesyl diphosphate synthase (TgFPPS)[15] and

protein farnesyl transferase[13]. TgFPPS has been described as the first bifunctional FPPS/ geranylgeranyl diphosphate synthase (GGPPS) in eukaryotes[15].

The information available regarding the polyprenyl synthases in apicomplexan parasites is very limited. The Octaprenyl pyrophosphate synthase from *Plasmodium falciparum* is the only such enzyme from apicomplexan characterized so far[16]. Some other homologous polyprenyl diphosphate synthases already characterized include solanesyl-diphosphate synthase from *Trypanosoma cruzi*[17,] solanesyl and decaprenyl diphosphate synthases from mice and human[18], solanesyl diphosphate synthase from *Arabidopsis thaliana*[19], heptaprenyl diphosphate synthase from *Bacillus subtilis*[20], etc.

It has been proposed that 'Polyprenyl diphosphate synthases essentially define the length of the side chain of ubiquinone[21] and therefore, the type of ubiquinone in an organism. Ubiquinones are essential components of the mitochondrial electron transport chains. Polyprenyl diphosphate synthases are indispensable for their role in the synthesis of the ubiquinone and can be exploited as potential drug targets in pathogenic parasites.

Bisphosphonates (BPs) are stable pyrophosphate derivatives in which the central oxygen atom between the two phosphorous atoms of the pyrophosphate is replaced by a carbon atom. The central carbon atom imparts structural and functional diversity to bisphosphonates. Many of them are in current use for treatment of bone related disorders and other diseases[22]. Two broad classes of bisphosphonates are simpler non-nitrogen containing BPs (clodronate, etidronate, etc) and the nitrogen containing BPs (pamidronate, alendronate, risedronate, etc).

Independent research studies have reported inhibition of the parasitic isoprenoid pathway with bisphosphonates in *Plasmodium*[22], *Trypanosomes*[23,17], *Leishmania*[24], and

33

Toxoplasma[15]. The TgFPPS has been described as a target of BPs supported by enzyme inhibition study as well as parasite growth inhibition study *in vitro* and *in vivo*[25]. Two n-alkyl BPs containing long hydrocarbon chains were more active when compared to the nitrogen containing BPs[25]. These results suggest that alkyl-bisphosphonates are promising compounds for further studies against *T. gondii*.

In the present study, we report the identification, cloning, and characterization of a polyprenyl diphosphate synthase homolog from *Toxoplasma gondii* (TgPPS). We demonstrated that TgPPS is a soluble, medium chain heptaprenyl diphosphate synthase and localizes to the cytoplasm of the parasite. TgPPS was heterologously expressed in *Escherichia coli*. We purified and used the recombinant TgPPS for biochemical characterization and determination of kinetic parameters. Ubiquinone analysis from *T. gondii* revealed that the parasite makes CoQ-7. Our *in vitro* drug inhibition studies suggest that TgPPS could be a novel target of bisphosphonates and other drugs.

3. Materials and Methods

3.1 Materials

Oligonucleotide primers were obtained from Sigma, Integrated Biotechnology Laboratories, and Integrated DNA Technologies. *Taq* DNA polymerases were from Denville Scientific Inc. and Invitrogen. TRIzol reagent, SuperScript ™ III Reverse Transcriptase, TOPO TA cloning kit, and GeneRacer™ Advanced RACE Kit were from Invitrogen and dNTP were from New England BioLab Inc. Restriction enzymes were from New England BioLab Inc. and Promega. Plasmid miniprep kit, Gel extraction kit and DNA purification kit were from Qiagen Inc. Pierce ECL Western Blotting Substrate and BCA protein assay kits were from Thermo Fisher Scientific. IPP, DMAPP, GPP, FPP, GGPP

were from Sigma. [4-^{14}C] Isopentenyl diphosphate triammonimum salt (55.0 mCi/mml) was from PerkinElmer Life Sciences. Silica gels HPTLC were from Analtech and Nitrocellulose membranes from Bio-Rad. All other reagents were analytical grade or better.

3.2 Parasite Cultures

T. gondii RH strain tachyzoites were cultured using human HFB and purified as described earlier[26]. Host cells were grown in Dulbecco's modified minimal essential medium supplemented with 10 % fetal bovine serum. Cell cultures were maintained at 37 ^{0}C with 5 % CO_2.

3.3 RNA isolation, RT-PCR, and 5'RACE

Total RNA from *T. gondii* tachyzoites was extracted with TRIzol reagent as per the manufacturer's instructions. First strand DNA/ cDNA was synthesized using the total RNA by reverse transcription using oligo-dT primers and SuperScript III reverse transcriptase. Three sets of degenerate oligonucleotide primers were designed based on the coding sequence information available for the putative TgPPS gene in the in *ToxoDB*. Standard PCR was carried out using the cDNA as template and the gene specific primers. The GeneRacer™ kit from Invitrogen was used to perform 5'RACE. Total RNA from the parasite was converted to RACE-ready cDNA according to the manufacturer's protocol. 5'RACE PCR was performed using GeneRacer™ 5'primer and reverse gene specific primer(5'ATCACCTCTGTCGAGCCGCAC3').The 5'RACE PCR product was subjected to amplification using GeneRacer™ 5′ Nested primer and Nested gene specific primer(5'TCGTTTGGCTCTTTGCGCTGAAC3').The full length cDNA obtained was designated as TgPPS.

3.4 Cloning of Full-length Coding Sequence of *TgPPS* Gene

The entire coding sequence of the *TgPPS* gene was PCR- amplified using the gene specific primers; Forward: 5'ACTG<u>CCATGG</u>GAATGTCCATCGCCTCGTCCCCTG3' and Reverse: 5'GGC<u>GCGGCCGC</u>CCCAGACCGCCGCTGGGAGAG3'.The underlined regions are the restriction sites for *NcoI* and *NotI* respectively. The PCR mixture included 200 ng of cDNA template, 1 µM of both Forward and Reverse primers, 10 X PCR buffer, 2 mM $MgCl_2$, 200 µM dNTP and 0.02 unit/µL platinum *Taq* DNA polymerase High Fidelity (Invitrogen).The standard thermocycling conditions were: Initial denaturation at 94 ^{0}C/2 min, 30 cycles of 94 ^{0}C/30 Sec, 56 ^{0}C/30 sec, 72 ^{0}C/1 min, and final incubation at 72 ^{0}C/10 min. The resulting band of PCR product (1578 bp) was gel purified and cloned into pCR-2.1-TOPO vector as per the prescribed protocol. The positive clones with the insert were screened by direct colony PCR and restriction enzyme digestion.

3.5 Sequencing and Sequence Analysis

Four of the positive clones were sent for sequencing at DNA Analysis Facility at Yale University. Sequencing reactions were set up with ~500 ng of purified plasmid and 3-5 p mols of primers (T7 promoter primer and M13 reverse primer). Sequencing was performed in both directions using Big Dye® Terminator v3.1 Cycle Sequencing Kits at 1/8 concentration on a 96-capillary 3730 *xl* DNA Analyzer (Applied Biosystems). The sequencing results obtained were analyzed using Vector NTI Advance ^{TM} 10 software from Invitrogen. By using Clustal W program multiple sequence alignment was executed with polyprenyl diphosphate synthase homologs from different organisms for comparison. The sequences were retrieved from orthoMCL (version 1.0) and National Center for Biotechnology Information (NCBI) databases using default BLAST parameters. GeneDoc

(version 2.6.002) Multiple Sequence Alignment Editor and Shading Utility programs were used to optimize the alignment.

3.6 Construction of TgPPS Expression Cassette

In order to carry out heterologous expression of the TgPPS protein in *E.coli* expression system, we engineered an expression cassette with *pET-28a (+)* vector. The TgPPS coding region was released from the pCR-2.1-TOPO vector by restriction enzyme digestion with NcoI and NotI and ligated into the *NcoI* and *NotI* sites of the *pET-28a (+)* vector. The resultant *pET-28a (+)-TgPPS* expression construct has the TgPPS coding sequence aligned and configured with a C-terminal 6x His-tag in frame without a stop codon to produce a His-tagged fusion protein . The construct was again sequenced for confirmation.

3.7 Expression and Purification of Recombinant TgPPS in *E.coli*

The expression cassette was transformed into *E.coli* BL2-Codon (+) cells. The transformed *E.coli* positive clones were selected with Kanamycin and were grown in LB medium. Expression of the recombinant protein was optimally induced by adding 0.4 mM isopropyl β-thiogalactopyranoside (IPTG) to the culture at an OD_{600} of 0.8-0.9. The bacterial culture was allowed to grow overnight at 18 ^{0}C. A HisBind 900 cartridge from Novagen was used to purify the recombinant protein as per the manufacture's instruction. The purified protein was desalted using a His Trap Desalting columns from GE Healthcare according to the prescribed protocol and was stored at -80 ^{0}C with 40 % glycerol.

3.8 TgPPS Antibodies Production

http://bioweb.pasteur.fr/seqanal/interfaces/antigenic.html was used for prediction of antigenic sites. Primers were designed (*TgPPS* _ Ab F: 5'ACTG<u>CCATGG</u>AAATGC

GTCTCCCCAAGTAC3' and *TgPPS* _ Ab R: 5'CCG<u>CTCGAG</u>TGCCGTCTTGTTGC

AGAAG3') to amplify 600 bases of *TgPPS* gene encoding 200 amino acids encompassing

multiple antigenic sites. *TgPPS* _ Ab F and *TgPPS* _ Ab R have restriction sites

(underlined) for *NcoI* and *XhoI* respectively which are essential for direct insertion of the

PCR products into *pET-28a (+)* expression vector. The 200 amino acids antigenic peptide

was expressed in the *E.coli* expression system with a C terminal 6X His-tag. Protein

expression was induced with 0.5mM IPTG at 37 ^{0}C for 6 hrs. The predicted recombinant

protein, *TgPPS* _ Antigen, of ~ 21.15 kD remained in the insoluble fraction. According to

the manufacturer's instructions, the peptide antigen was purified from the inclusion body

under denaturing conditions using a His Bind Quick 900 Cartridge (Novagen), after

solubilizing with 6 M Urea. Nearly 650 µgm of the purified TgPPS _ Antigen was used to

inoculate two rats for antibody generation at Cocalico Biologicals Inc.

3.9 SDS-Gel Electrophoresis and Western Blot Analysis

Protein samples were prepared using 20 µg proteins with equal volume of 1X

Laemmli loading buffer and boiled for 5 min. The samples were loaded into a 10 % SDS-

polyacrylamide gel. The protein bands were separated electrophoretically and stained with

coomassie blue for visualization. For Western blot analysis protein bands were transferred

to a nitrocellulose membrane and blocked overnight at 4 ^{0}C with 5 % nonfat milk in PBS-

T (0.1 % Tween 20 in PBS). Incubation with primary and secondary antibodies was for 1hr

each, with three washes of 15 min with PBS-T in between. After final incubation and

wash, the protein bands were exposed by autoradiography on an X-ray film using an ECL

detection kit.

3.10 Measurement of Enzymatic Activity

To measure the enzymatic activity, a standard protocol for medium/long chain prenyl diphosphate synthase was followed as described previously[17]. The enzyme activity was measured by determination of the amount of [4-^{14}C] IPP incorporated into butanol-extractable polyprenyl diphosphates. The standard assay mixture contained, in a final volume of 100 µL,100 mM Tris-HCl; pH- 7.4, 100 µM of any of the allylic substrates FPP/GGPP/GPP, 1 mM MgCl$_2$,1 mM DTT, 1 % v/v Triton X-100, 100 µM [4-^{14}C]-IPP (1 µCi/µMol) and 500 ng of purified protein. Reactions were incubated at 37 ^{0}C for 30 min unless otherwise indicated. The radioactive prenyl products were extracted with 1-butanol, washed with NaCl-saturated water and activities were calculated from the DPM values using a liquid scintillation counter.

3.11 Determination of Kinetic Parameters

Kinetic parameters for the recombinant TgPPS were calculated from the activity obtained by varying the concentration of the allylic substrates (GPP, FPP, GGPP and IPP) and keeping concentration of counter substrates at a saturating level. The nonlinear regression analysis in Sigma plot 10.0 was used to calculate the Kinetic parameters.

3.12 Product Analysis by Reverse Phase TLC

The radioactive prenyl diphosphate products were extracted from 500 µL reaction and hydrolyzed to their corresponding alcohols (polyprenols) by using potato acid phosphatase. The enzymatic hydrolysis reaction was set up at 37 ^{0}C for more than 12 hours as described before [27]. The resultant products were extracted with n-pentane and separated on a reverse phase TLC plate with acetone: water (19: 1 v/v) as the solvent system. The positions of the polyprenol standards were visualized by iodine vapors and marked with

pencils. The radioactive polyprenol products were exposed and visualized by autoradiography.

3.13 Inhibition of TgPPS Activities with Bisphosphonates

To test the effect of bisphosphonates on TgPPS activity, enzymatic reactions were set up, as described in this section, in the presence of different bisphosphonate compounds. Preliminary screening for potential inhibitors was carried out at a final concentration of 1 µM and 10 µM respectively and IC-50s were calculated for the promising ones as described earlier for *in vitro* drug testing[25].

3.14 Generation of TgPPS-3myc Overexpressing Parasites

TgPPS coding sequence was amplified using sequence specific primers, with restriction sites *Bgl II* and *Avr I,* for the forward and reverse primers respectively, cloned into pCR-2.1-TOPO and sequenced. The coding region was finally cloned into *Avr II and Bgl II* of *pDTM* vector, which has a pyrimethamine resistance cassette for selection of stable transfected parasites, a tubulin promoter to drive the expression of the cloned gene, and a triple Myc tag. The *pDTM-TgPPS* construct was transfected into *T. gondii* RH strains parasites using a standard electroporation protocol. The stable transfectants were selected using 1 µM pyrimethamine.

3.15 Indirect Immunofluorescence Assays (IFA)

IFA were performed with both intracellular and extracellular *T. gondii* cells. Cover slips were prepared with human fibroblasts (HFB) attached to them in 12-well plates. Transient transfected parasites were allowed to invade and grow in HFB for 24 hours and fixed with 4% formaldehyde for IFA. To carry out the IFA with the extracellular parasites, *T. gondii* tachyzoites were released by lysing the HFB and fixed in 4% formaldehyde for

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1hr at room temperature. Parasites were adhered to the poly-L-Lysine coated cover slips.

In both cases parasites were permeabilized with 0.3 % Triton X-100 for 30 min and

blocked with the blocking buffer (3 % Bovine serum albumin, 1 % fish gelatin, 50 mM

NH_4Cl, and 5 % goat serum in PBS). Incubations with primary and secondary antibodies

were for 1 hr each. Primary anti-myc antibodies were used at a dilution of 1: 500, anti-

soluble pyrophosphatase antibodies at 1: 200, anti TgPPS antibodies at 1: 200. Secondary

antibodies, in all cases, were used at a concentration of 1: 1000. Cover slips with parasites

were mounted on glass slides with Antifade-DAPI. A Delta Vision fluorescence

microscope was used to observe the cells and a Photometrics coolsnap camera was used to

capture images. Deconvolved images were obtained by using softWoRx deconvolution

software.

3.16 Cell Lysate Assay

    T. gondii cells were collected and washed with 1X Buffer-A with glucose (116 mM

NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 50 mM HEPES, and 5.5 mM glucose) by

centrifuging at 1700 RPM for 10 min. The pellet was resuspended, at a final concentration

of $5x10^8$cells/ml in a buffer containing 20 mM Hepes-Tris, pH- 7.4, 100 mM Sucrose, and

1: 500 protease inhibitor. Cells were collected by centrifuging at 1000 x g 5min and

removing all supernatant, leaving the pellet as dry as possible. Cells were broken by three

cycles of Freeze (5min, on dry ice and 100 % Ethanol bath) and thaw (1min at 37 ^{0}C) and

resuspended in 500 μl of the same buffer. Finally, pellet and supernatant were separated by

centrifuging at 100,000 x g for 1 hr at 4 ^{0}C. Supernatant was transferred into another tube

and pellet was resuspended in the same buffer. Protein concentration was measured using

BCA protein quantification kit. Both the supernatant and pellet fractions were used for enzymatic assays and western blot analysis as mentioned.

3.17 Ubiquinone Extraction and Analysis

Ubiquinone from *T. gondii* was isolated using a standard protocol as follows. Parasites were released by disrupting the host cells, filtered using 8 μM, 5 μM and 3 μM filters, and washed twice with Buffer A with glucose. Freeze and thaw method, as described, was used to break the cell pellet. One volume of water and three volume of 1-propanol were added to the pellet. The sample was vortexed for 30 sec at room temp and allowed to stand for a minute. Two volumes of n-Hexane was added, vortexed for 30 sec at room temperature, and centrifuged at 14000 x g for 5min. The upper phase containing ubiquinone was collected into a glass tube. The sample was analyzed by HPTLC to determine the type of ubiquinone present in *T. gondii*.

4. Results

4.1 Identification, Cloning and Sequence Analysis of a Gene Encoding a Putative

T. gondii **Polyprenyl diphosphate synthase (TgPPS):**

To identify the gene encoding *Toxoplasma gondii* polyprenyl diphosphate synthase (TgPPS), we performed BLAST in the *ToxoDB* Release 4.2, a genome database for the apicomplexan parasite *T. gondii*. A putative polyprenyl synthetase domain containing protein on chromosome VIII (TGME49_069430) was found for the *T. gondii* ME49 strain. As per annotation it has a genomic size of 6481 bp and coding sequence of 1611 bp. Oligonucleotide primers were designed to amplify the *T. gondii* cDNA by RT-PCR based on the *TgPPS* annotated coding sequence. However, a partial amplification of 1492 bp was obtained with a truncated 5' end. The RT-PCR product was cloned into pCR-2.1-TOPO

vector and sequenced. Two sequences were obtained with a difference of 163 bp, indicating the possibility of alternative splicing. Also a sequence of 51bp, annotated as a part of the CDS, was found missing which could be a part of the intron. To determine the 5'end with the first initiation codon, rapid amplification of 5' complementary DNA ends (5'RACE) was performed using RACE primers and gene specific primers. Sequence analysis of the RACE product indicated nine exons instead of ten (annotation data from ToxoDB). The full length TgPPS coding sequence constitutes of 1578 bp encoding a protein of 526 amino acids. The full length TgPPS was PCR-amplified, cloned into pCR-2.1-TOPO vector, and sequenced.

To confirm the alternative splicing in *TgPPS* gene, genomic DNA (gDNA) and cDNA PCR were carried out. Primers were designed flanking the anticipated splicing site (*TgPPS* –genomic Forward - 5'TCGCCGAATGAGTCGTCCGCA3' / *TgPPS* - genomic Reverse - 5' ACCTGG CTGTGTGAATGAGCTCC 3' for gDNA template and *TgPPS* - Alt. Sp Forward-5' ATTCGCGG GGGACATCGACCCGTT 3'/ *TgPPS* - Alt. Sp Reverse - 5' ATCACCTCTG TCGAGCCGCAC 3' for cDNA template. PCR amplifications revealed a single 600 bp band with gDNA template, and two bands of 534 bp and 371 bp using cDNA as template (data not shown). Together with the restriction enzyme experiments and sequencing analysis, it was concluded that the *TgPPS* gene encodes at least two transcripts, variant 1 and 2. Only one of the transcripts (variant 1) encodes the full open reading frame of *TgPPS*. Alternative splicing of 163 bp disrupts the open reading frame (ORF) for the *TgPPS* variant 2. So, investigation into the functionality of the variant 2 transcript was not studied further.

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We constructed a multiple sequence alignment (Figure-2.1), from amino acid sequences of representative polyprenyl diphosphate synthases from different organisms, using Clustal-W. The sequences were obtained from the *orthoMCL* and *NCBI* databases. We were able to identify all the seven conserved domains (I-VII), characteristic of the poly prenyl diphosphate synthases in TgPPS. The regions II and VI containing the aspartate rich motifs (DDXXD) are highly conserved. The FARM (first aspartate rich motif) and SARM (second aspartate rich motif) domains were identified. They are responsible for binding to substrates and cofactors, and for catalysis.

4.2. Purification and Biochemical Characterization of Recombinant TgPPS

We expressed the recombinant TgPPS in *E.coli*, *BL-21*codon (+) as a recombinant protein with an N-terminal poly Histidine tag using *pET-28a (+)* vector. Optimal induction was obtained with 0.4 mM IPTG at an incubation temperature of 18 ^{0}C. TgPPS was purified as a soluble protein with a molecular weight of 56.76 kD (Figure-2.2) using His-bind columns (His tag affinity chromatography).

The specific activity of the purified TgPPS was measured following an established protocol[17]. The protocol was optimized for TgPPS activity for substrate specificity, pH, Mg^{+2} concentration, and incubation period (Figure-2.3).

Four different allylic substrates, one at a time, [Dimethylallyl diphosphate (DMAPP), Geranyl diphosphate (GPP), Farnesyl diphosphate (FPP), and Geranylgeranyl diphosphate (GGPP)] were used along with 4-^{14}C [IPP] at a concentration of 100 μM, to investigate the best substrate for TgPPS. The enzyme is capable of utilizing all these allylic substrates. However, highest enzymatic activity for TgPPS was obtained with FPP as the substrate (Figure-2.3A). This suggests that TgPPS has a higher preference for FPP as the

allylic substrate when compared to other allylic substrates. Therefore, TgPPS might be responsible for further chain elongation reaction downstream of FPP.

TgPPS, like other prenyl diphosphate synthases, strongly requires divalent cation like Mg^{+2} for activity. To determine the effect of different concentration of Mg^{+2} on enzymatic activities, the final concentration of $MgCl_2$ in the reaction mixture was varied. Optimal activity was obtained in the presence of 1 mM $MgCl_2$ as shown in Figure-2.3B. In the absence of Mg^{+2}, the enzyme has almost no activity.

TgPPS activity was tested with in a pH range of 7.0 - 8.5 with Tris-HCl buffer. No significant difference in enzymatic activity was seen between pH- 7 and 8.0 (Figure-2.3C). Therefore, a pH of 7.4 was maintained in all enzymatic reactions for activity analysis.

Different incubation periods at 37 ^{0}C were tested to get the optimal TgPPS activity and found that beyond 1hr the enzyme activity is significantly reduced. By 30 minutes more than 80 % of the optimum activity was observed (Figure-2.3D). Therefore, incubation period of 30min was used for all enzymatic assays.

4.3 Kinetic Analysis of Recombinant TgPPS

For kinetic studies, the concentration of the allylic substrates like GPP, FPP, and GGPP were varied, and the counter substrate concentration was kept at a saturating level. The Kinetic parameters Km and Vmax were calculated by fitting the data to the Michaelis-Menten equation, using nonlinear regression analysis in Sigma plot 10.0. The kinetic parameters obtained have been summarized in Table-1 along with the supporting graphical data for FPP and GGPP (Figure-2.4).

The Km values for FPP and GGPP are similar indicating that TgPPS binds to both FPP and GGPP with equal affinity. There is no significant difference in the Km values for

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IPP when the allylic substrate was GPP, FPP, or GGPP. However, the Kcat/Km value is nearly three-fold higher with FPP as the substrate when compared with GPP and GGPP. The Kcat/Km values for GPP and GGPP are nearly equal. The Kcat/Km for IPP is higher when the primer substrate is FPP compared to GPP and GGPP. Also, Vmax is highest when FPP is the allylic substrate. These results indicate that TgPPS most efficiently utilize FPP than GPP and GGPP. Hence, FPP is the most preferred substrate for TgPPS.

4.4 Functional Analysis of TgPPS

The hydrolyzed radioactive poly-prenol products were prepared as described under materials and methods. The products were separated by reverse phase thin layer chromatography using an acetone: water (19: 1 v/v) system. Using authentic poly-prenol standards, it was found that TgPPS catalyzes the synthesis of a 35 carbon product (Figure-2.5).

The role of polyprenyl diphosphate synthases for ubiquinone biosynthesis has been reported previously [21]. These enzymes are responsible for determining the type of CoQ in an organism. With the anticipation that TgPPS might be responsible for the synthesis of the side chain of Ubiquinone (CoQ), ubiquinone samples from *T. gondii* were analyzed, by HPLC. We extracted the ubiquinone samples from *T. gondii* expressing TgPPS at endogenous level and at over-expression level following the standard protocol as described in the materials and methods. The samples were dried under nitrogen gas and sent to Kawamukai's Lab, Shimane University, Japan. The ubiquinone samples were analyzed by HPLC and the results as reveled from the retention time confirmed that *Toxoplasma gondii* produces CoQ-7 (data not shown).

4.5 Subcellular Localization of TgPPS

To investigate the sub-cellular localization of TgPPS, *T. gondii* cell line expressing TgPPS with a c-terminal 3x-myc tags were prepared by transfecting 3x-myc tagged TgPPS cDNA into the parasites. Indirect Immunofluorescence microscopy was performed using anti 3x-myc antibodies (antibodies specific to c-myc epitope tag), anti SP1 antibodies (antibodies against soluble pyrophosphatase 1) anti SAG1 (antibodies to the major surface antigen). TgPPS showed co-localization with anti-SP1 (Figure-2.6 C, D) a known cytosolic marker, but didn't co-localize with anti-SAG (Figure-2.6 B), a marker for plasma membrane. Also in intracellular parasites, the cytoplasmic distribution of the enzyme is clearly indicated (Figure-2.6 A).

4.6 TgPPS is a Soluble Protein in *Toxoplasma gondii*

In order to investigate the solubility of TgPPS in the parasite, we performed Western blots as well as enzymatic assays, with both soluble and pellet fractions obtained from whole cell lyses. Both RH and TgPPS over-expressing (OE) parasites were used for this study. The polyclonal antibody raised against TgPPS was used for the Western blot. The endogenous TgPPS enzyme (~56.76 kD) is found to be enriched in the supernatant (S) fraction (Figure-2.7 A) indicating that TgPPS is a soluble protein in *T. gondii*. Also this result was supported by the enzymatic activity assay as shown in Figure-2.7 B. The soluble fractions from OE parasites have higher enrichment of TgPPS compared to the wild type fractions.

4.7 Inhibition of TgPPS by Bisphosphonates

We tested the effect of a series of bisphosphonate compounds on the enzymatic activities of recombinant TgPPS. The IC-50s of different compounds have been

summarized in Table-2. Also, the *T. gondii* growth inhibition data have been shown for comparison. It was found that the alkyl side chain containing bisphosphonates were better inhibitors of TgPPS with IC-50s as low as 0.01-0.02 μM as in case of compound 19. The IC-50s for enzyme inhibitions and *T. gondii* growth inhibitions are of the same order for compounds 22A and 23A. This result strongly suggests that TgPPS might be target for these compounds in the parasite. TgFPPS has also been described as a molecular target of bisphosphonates[15]. So, we believe that different bisphosphonates could be used in combination to have a synergistic inhibitory effect on *T. gondii*.

5. Discussion

In this study we report the functional characterization of a polyprenyl diphosphate synthase from *T. gondii* (TgPPS) which belongs to the family of medium chain polyprenyl diphosphate synthases. Sequencing analysis of the cloned TgPPS exposed alternative splicing which was confirmed by genomic and cDNA-PCR. This feature is not uncommon in *T. gondii*. TgFPPS has been found to be encoded by a single copy gene producing two transcripts[15]. Some other genes showing alternative splicing in *T. gondii* are genes encoding myosin B and C[28], heat shock protein HSP 60[29], and hypoxanthine-xanthine-guanine phosphoribosyltransferase[30]. In case of TgPPS coding sequence, the shorter transcript (variant 2) differs from the full length transcript by 163 bp, which disrupts the open reading frame (ORF). At this point we have no indication for a biological role of the alternative transcript of TgPPS variant 2.

TgPPS gene encodes for a protein of 526 amino acids with an approximate molecular weight of 56.76 kDa (approximate and with 4 significant figures). No N-terminal targeting signal sequence was predicted. Multiple sequence alignment indicates

the presence of all seven conserved domains characteristic of medium/long-chain prenyl synthases in TgPPS. Also the two aspartate rich motifs (DDXXD), first (FARM) and second (SARM) are present in regions II and IV respectively which have been reported as the substrate binding sites. The FARM serves as the site of interaction and binding for FPP where as the SARM is responsible for binding with IPP. Taking into account the level of conservation, TgPPS is nearly 31 % identical with the human HsaDPS1, 46 % identical with TcrSPPS, 32 % identical with AthSPPS1, 37 % identical with DdiPPS, 36 % identical with EcoOPPS, and 35 % identical with the *Plasmodium falciparum* octaprenyl pyrophosphate synthase. TgPPS also has an extended N-terminal end similar to *Dictyostelium discoideum* along with two insertions. One insertion is between domains I and II and other between VI and VII. Contrast to TgFPPS the TgPPS insertions and N-terminal extensions are not serine-rich. Why *T. gondii* has these extra sequences is not fully understood.

Heterologous expression of recombinant TgPPS in *Escherichia coli* yielded a soluble protein, which was enzymatically active. Similar to *TcSPPS* this enzyme also requires divalent cation like Mg^{+2} for activity[17]. Comparing the specific activities, we concluded that TgPPS is a more active enzyme than TgFPPS and has a preference for FPP over GGPP and GPP. The kinetic parameters *(Vmax, Kcat and Kcat/Km)* further support that TgPPS more efficiently catalyzes FPP compared to other allylic substrates. However, the affinity for both GGPP and FPP is nearly equal as suggested by the *Km* values.

Our TLC data reveals that TgPPS catalyzes the synthesis of an isoprenoid chain containing seven isoprene units. Based on this we propose that TgPPS is a heptaprenyl

diphosphate synthase. We demonstrated that TgPPS is functional by itself and without being associated with other proteins.

Ubiquinones are important components of the mitochondrial electron transport chain and comprise of a benzoquinone ring and an isoprenoid side chain. Synthesis of the two direct precursors of ubiquinone takes place in two different pathways[34]. The ubiquinones are named according to the number of isoprene units contained in their side chains. It has been reported that the isoprenoid side chains of ubiquinones in different organisms are essentially made by polyprenyl diphosphate synthases[21]. To explore whether TgPPS has a similar role, we analyzed the ubiquinone samples from the parasite by HPLC and ubiquinone-7 was detected. In the past, a mutant *E.coli* strain *(KO229)* has been employed for complementation study of polyprenyl diphosphate synthases[35]. This study facilitates to correlate the role of a candidate polyprenyl diphosphate synthase for ubiquinone synthesis by determination of ubiquinone type following complementation. The mutant *E.coli* strain has a defective *ispB* gene which encodes for an octaprenyl diphosphate synthase under normal condition. Homologs from *S. cerevisiae, Haemophilus influenza, Synechocystis sp* and *T.cruzi* have successfully complemented the role of *isp-B*[21,36]. However, we were unsuccessful in complementing the *ispB* of mutant *E.coli* using TgPPS gene (Data not shown).

The isoprenoid pathway enzymes from different organisms are localized to different subcellular compartments such as FPPS from *T. cruzi*, *T. brucei*[6] and *Leishmania*[5] in the cytoplasm, *T. cruzi* SPPS in the glycosomes[17], mevalonate kinase in peroxisome of animals[4], *A. thaliana* SPS1 in the endoplasmic reticulum[31] and SPS2 in the plastid[31], etc. The TgFPPS has been found in the mitochondria[15]. Investigation of the

subcellular localization demonstrated that TgPPS localizes to the cytoplasm of the parasite. This is apparent from the fact that TgPPS lacks an obvious targeting signal sequence. However, the rationale behind cytoplasmic localization of TgPPS is not fully understood. TgFPPS synthesizes FPP in the mitochondria which must be transported to the cytoplasm to be utilized by TgPPS. Again, in the case where TgPPS synthesizes the side chain of ubiquinone, the final product of TgPPS needs to be transported back into the mitochondria. This is because previous observation indicates the presence of ubiquinone in the mitochondria of *T. gondii*. Therefore, we believe that there must be a very effective mechanism for transportation of isoprenoids across the mitochondrial membrane.

Bisphosphonates are pyrophosphate analogs and are currently in use for the treatment of bone resorption disorders and other diseases. It has been demonstrated that bisphosphonates target and competitively inhibit farnesyl diphosphate synthase *(FPPS)* and other prenyl transferases in apicomplexan parasites[1] and trypanosomatids[23]. N-alkyl bisphosphonates are more effective *T. gondii* growth inhibitors *in vitro* and *in vivo* over nitrogen containing bisphosphonates[25]. We showed that TgPPS is inhibited by a number of bisphosphonate compounds. In agreement with the previous finding TgPPS is more potently inhibited by alkyl side chain containing bisphosphonates. We hypothesize that TgPPS is a novel target of bisphosphonates and other drugs.

Figure 2.1: Multiple sequence alignment of polyprenyl diphosphate synthases from different organisms. The numbers along the right hand side margin indicate the amino acid numbers for respective prenyl diphosphate synthases as mentioned in the left hand side margin. TgPPS has 526 amino acids. The seven conserved motifs characteristic of long-chain prenyl synthases have been labeled as I-VII. The two aspartate rich motifs (DDXXD), first (FARM) and second (SARM) are indicated with in dotted boxes. TgPPS, *Toxoplasma gondii* polyprenyl synthetase; TcrSPPS, *Trypanosoma cruzi* solanesyl diphosphate synthase; HsaDPS1, *Homo sapiens* decaprenyl synthase-1; AthSPPS1, *Arabidopsis thaliana* solanesyl diphosphate synthase1; DdiPPS, *Dictyostelium discoideum* polyprenyl synthetase; EcoOPPS, *Escherichia coli* octaprenyl diphosphate synthase. The black shadow represents 100 % conservation of the amino acid sequence where as the dark gray and the light grey show 80 % and 60 % conservation respectively.

Figure 2.1:

Figure 2.2: Expression (Induction profile) and purification of recombinant TgPPS.

SDS-PAGE showing the TgPPS induction profile in *E.coli* BL21codon (+) culture [lane 2

represents non- induced state, lane 3 shows overnight induction with 0.4 mM IPTG at

18 ^{0}C] and the purified protein fractions-2, 3 (Lane 4 and 5) after His tag affinity

chromatography purification. TgPPS displayed the expected molecular weight of

56.76 kD. The Bio-Rad Broad Range protein standard (Lane-1) was used to determine the

protein size.

Figure 2.2:
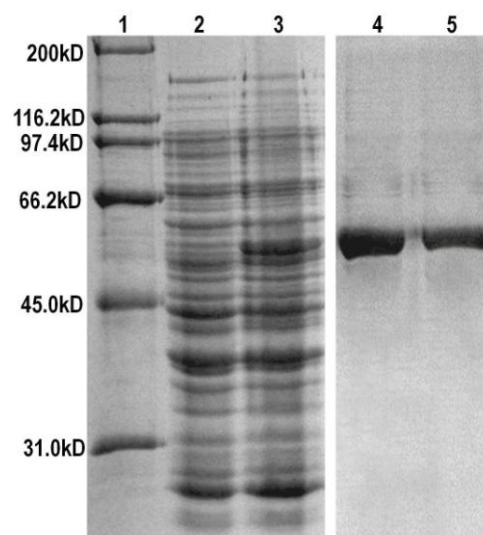
Figure 2.3: Specific activity assays of recombinant TgPPS. Results shown here are from two independent experiments performed in duplicate. The error bars indicate the standard errors (S.E) of different experiments. **(A) Specific activity with different allylic substrates.** The activity of the enzyme was measured in a 100 μL reaction volume containing any of the allylic substrates (FPP/GGPP/GPP), 100 mM Tris HCl (pH-7.4),1 mM $MgCl_2$,1mM DTT, 1 % v/v Triton X-100, 100 μM [4-^{14}C]-IPP (1 μCi/μMol) and 500 ng of purified protein. Reactions were incubated at 37 ^{0}C for 30 min and were stopped by chilling on ice. The radioactive prenyl products were extracted with 1-butanol, washed with NaCl-saturated water and activities were calculated from the DPM values. FPP is the most preferred substrate followed by GGPP. Therefore, FPP was used as the allylic substrates for all subsequent experiments. **(B) Effect of $MgCl_2$ concentration on TgPPS activity.** TgPPS activity was measured with different concentration of $MgCl_2$ in the reaction medium as described earlier. The enzyme requires $MgCl_2$ at a final concentration of 1 mM for optimum activity. **(C) Effect of pH on TgPPS activity.** Activity of the purified TgPPS was measured in reaction mixture containing 100 mM Tris-HCl, pH 7.0 - 8.5. The pH for optimum enzyme activity is 7.4 as shown. No significant difference in the enzyme activity was noticed within a pH range of 7.0 - 8.0. **(D) Time course experiments for the TgPPS activity.** The activity was followed during varying time periods (minutes) at 37 ^{0}C. Maximum activity was observed at 60 min.
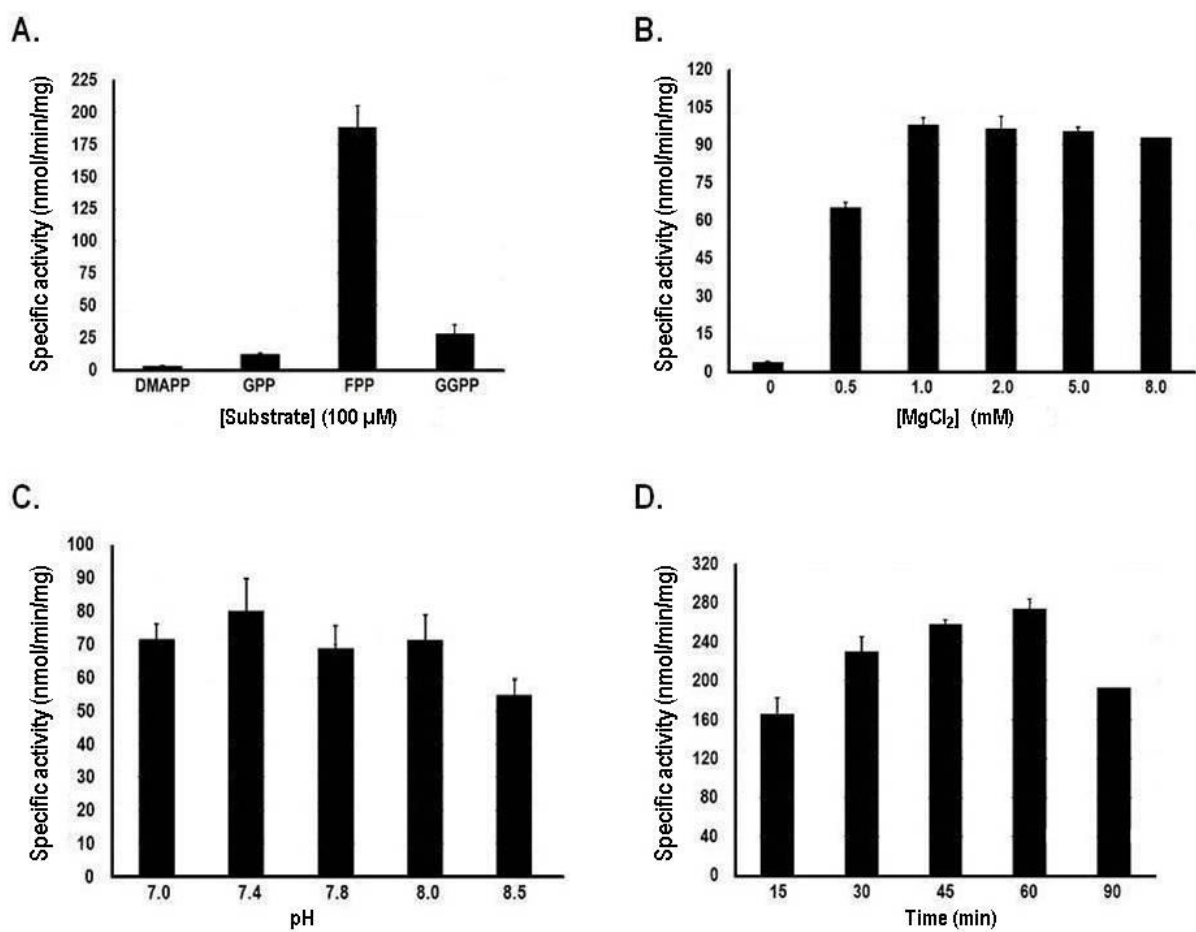
Figure 2.3:

Table-1: Kinetic parameters of recombinant TgPPS: To determine the kinetic parameters, enzyme activity was determined under optimized conditions with variable concentration of substrates (GPP, FPP, GGPP, and IPP) and saturating level of the counter substrates. Data shown are mean ± Standard Deviation of two independent experiments performed in duplicate. The nonlinear regression analysis in Sigma plot 10.0 was used to calculate the Kinetic parameters Km and Vmax. Kcat/Km indicates the catalytic efficiency of the enzyme.

Table-1:

Substrate	Counter Substrates	Km (μMolar)	Vmax (nmoles/min/mg)	Kcat (min^{-1})	Kcat/Km (min^{-1} x mM^{-1})
GPP	IPP (100 μM)	0.88 ± 0.60	6.16 ± 0.45	0.012 ± 0.0009	13.63
FPP	IPP (100 μM)	12.37 ± 1.83	226.13 ± 7.56	0.452 ± 0.015	36.54
GGPP	IPP (100 μM)	14.05 ± 1.43	82.98 ± 2.51	0.165 ± 0.005	11.74
IPP	GPP (150 μM)	145.48 ± 1.18	28.39 ± 0.16	0.057 ± 0.0003	0.39
IPP	FPP (100 μM)	139.11 ± 17.68	560.54 ± 46.09	1.12 ± 0.092	8.05
IPP	GGPP(50 μM)	141.89 ± 35.75	372.92 ± 65.53	0.745 ± 0.131	5.25

Figure 2.4: Graphical data supporting the Km and Vmax for TgPPS

(**A**) FPP was used as the allylic substrate and (**B**) GGPP was used as the allylic substrate. In both the cases, the concentration of the counter substrate was kept at 100 µM. The Lineweaver-Burk Plot from the Sigma Plot-10 was used to calculate the Km and Vmax values.

Figure 2.4:
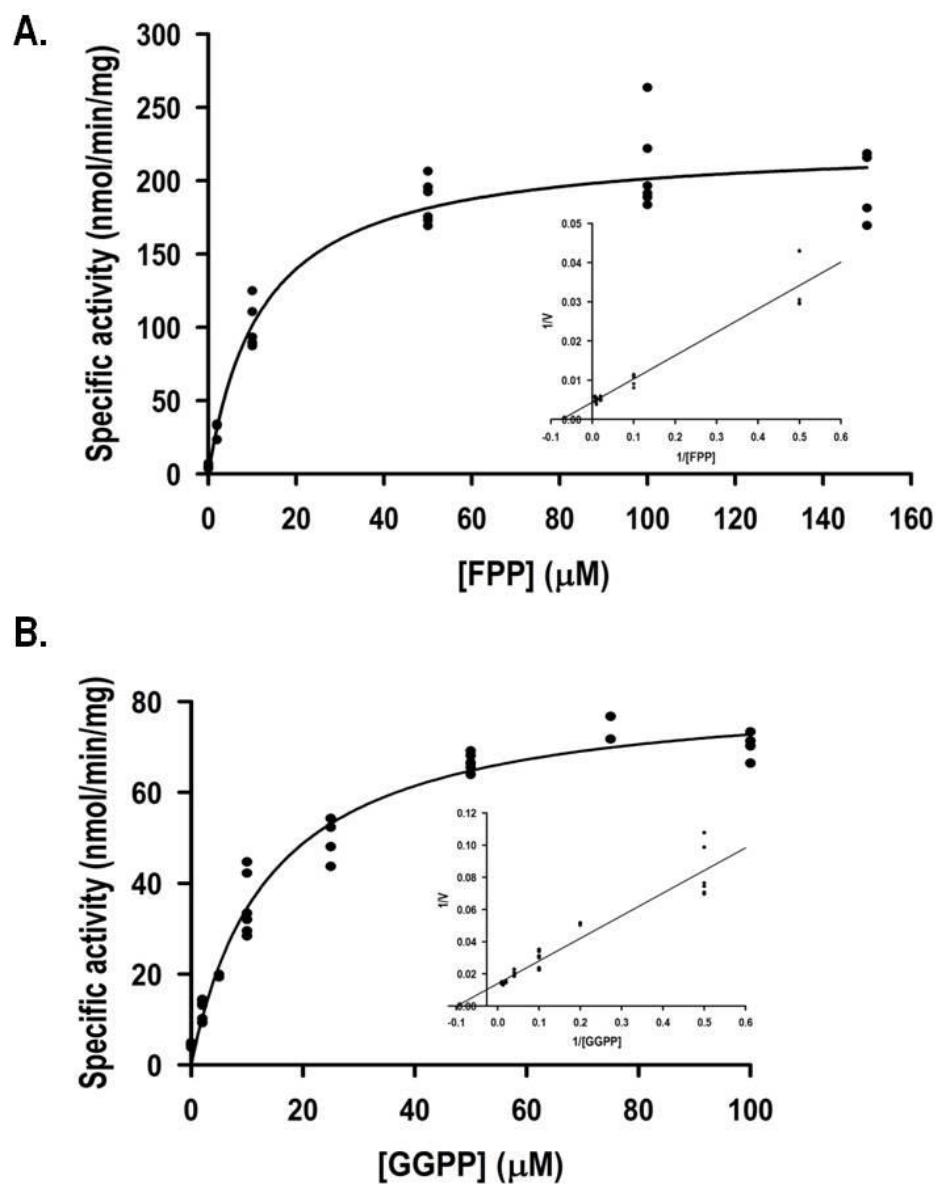
Figure 2.5: Thin-layer chromatogram of hydrolyzed products of TgPPS (lane-1) and TcrSPPS (lane-2): The enzyme reactions were carried out with [^{14}C]-IPP and FPP as substrates. The products were hydrolyzed into corresponding alcohols with potato acid phosphate and were analyzed by running on TLC plates with acetone-water solvent. The standards were exposed by iodine vapor and position of the test samples were visualized by autoradiography. The arrows in the picture represent the positions of the respective standards (lane-3). S.F is the solvent front and Origin is the loading level of the samples. The alcohol product of *T. cruzi SPPS* in lane-2 was used as a positive control.

Figure 2.5:
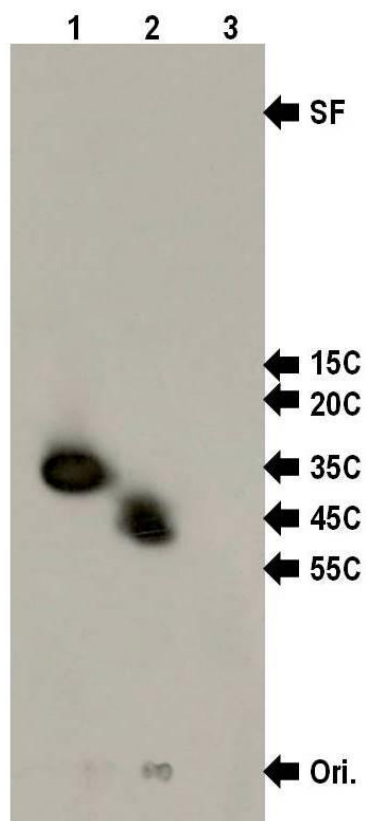
Figure 2.6: Subcellular localization of TgPPS: *T. gondii* mutant parasites overexpressing TgPPS were fixed and stained with anti-3xmyc antibodies, anti-SP1 antibodies and anti-SAG antibodies. DAPI was used to stain the nuclei. **(A) IFA with intracellular parasites. (B), (C), and (D) IFA with extracellular parasites.** Differential Interface Contrast (DIC), images with antibodies and DAPI, and overlay images are shown. Scale bars are indicated as lines in the pictures.

Figure 2.6:
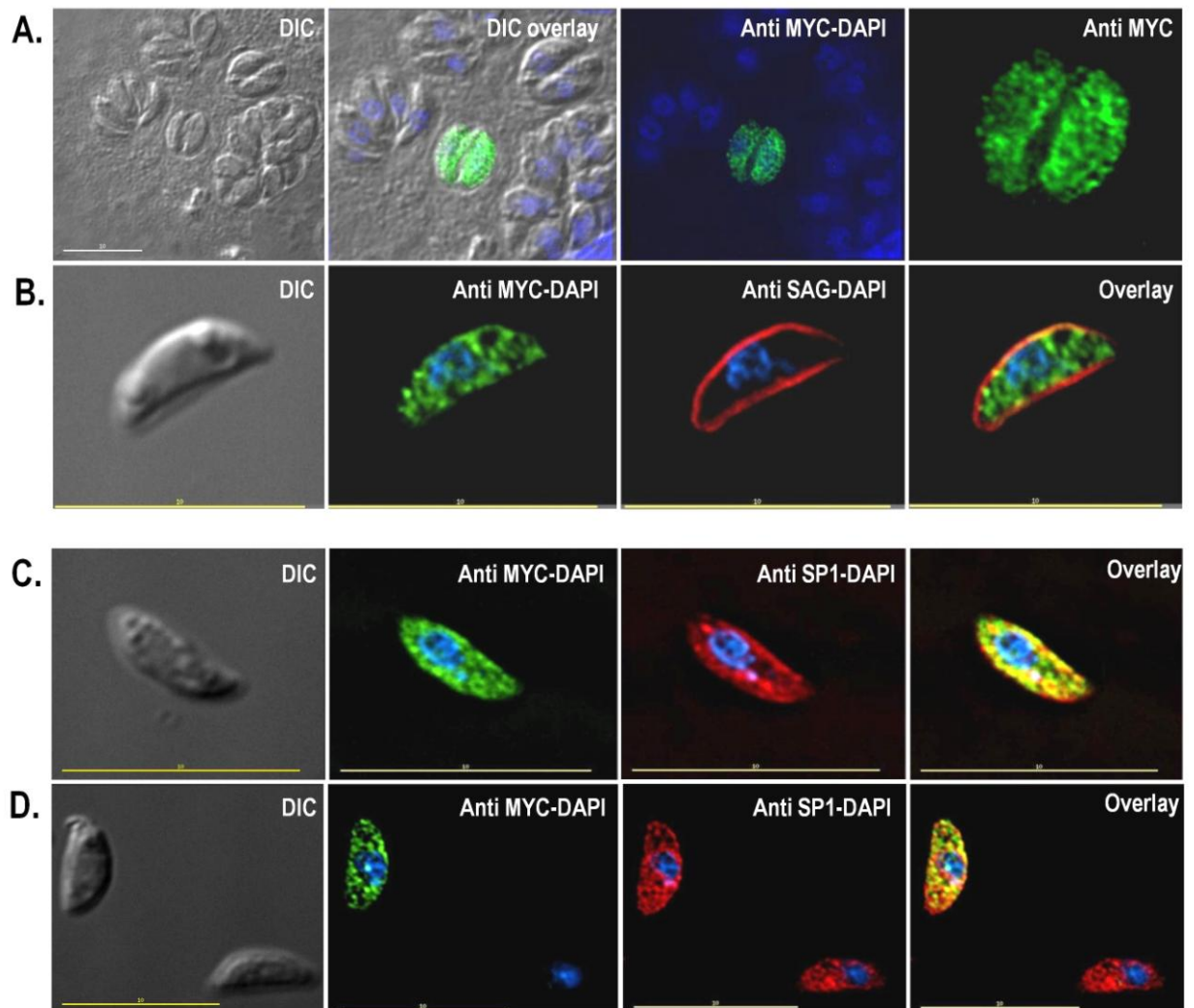
Figure 2.7: A. Western blot analysis of TgPPS. The Western blot using polyclonal antibodies against recombinant-TgPPS. *T gondii cells* were disrupted by freeze/thaw method and nearly 20µgm of both the pellet (P) and the supernatant(S) were loaded into SDS-PAGE for western blot. **B. TgPPS activity assay.** Nearly 36µgm of total protein from the pallet (P) and the supernatant (S) fractions were used for enzymatic assay following the standard protocol. RH is the wild type *T. gondii* cells where as OE means *T. gondii* cells over-expressing TgPPS. Both RH and OE cells were used for fractionation. The error bars represents the standard error from two independent experiments performed in duplicate for determination of enzymatic activity.
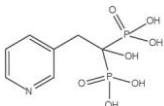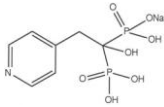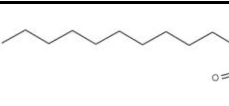
Figure 2.7:
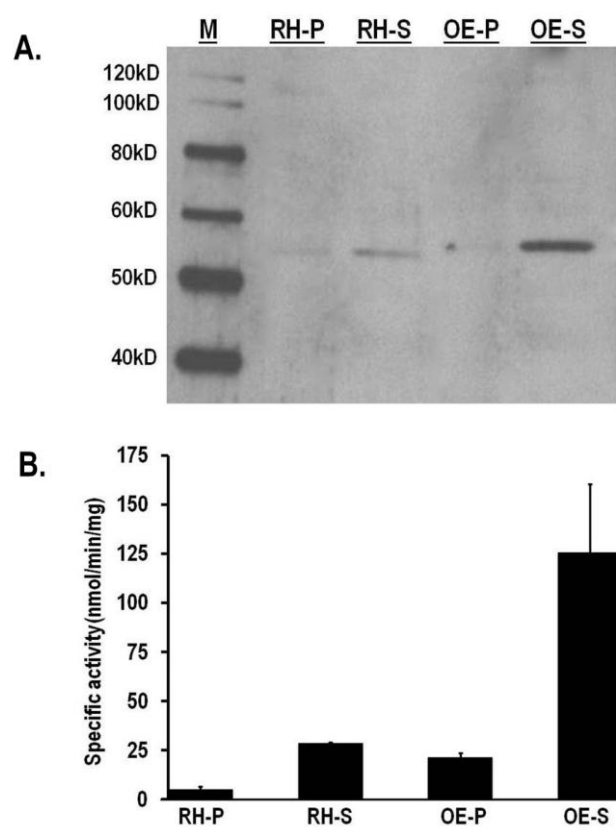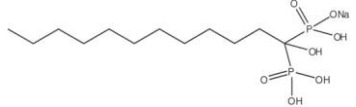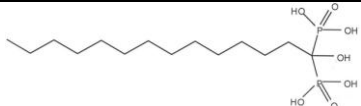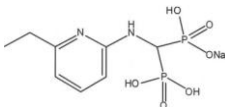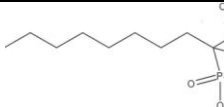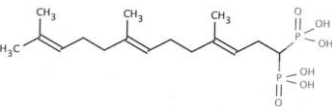
Table 2: Comparison of IC-50s for TgPPS inhibition and *T. gondii* growth inhibition, *in vitro* with Bisphosphonates. The identification numbers, names, and structures of the com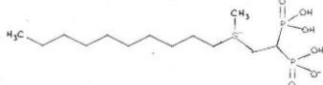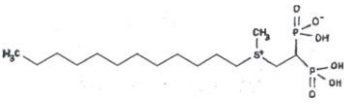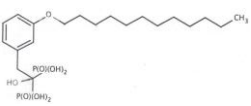pounds have been shown in the table. TgPPS enzyme inhibitions were carried out as described in the experimental procedure for calculation of IC-50s (Column 4). The corresponding IC-50s for the *T. gondii* tachyzoites growth inhibition data have been presented in column-5 for comparison. TgPPS inhibition assays were obtained from two independent experiments performed in duplicates, except for compound 249A. NA denotes the data is not available. In some cases, two independent sets of data for IC-50s for the *T. gondii* tachyzoites growth inhibition have been shown.

Table 2:

Compound	Name	Structure	*TgPPS* Inhibition IC-50s(μM)	*T. gondii growth* Inhibition IC-50s(μM)
2E	2-(3-pyridyl)-1-hydroxy-ethane-1,1-bisphosphonate		7.78/4.13	2.40
3B	2-(4-pyridyl)-1-hydroxy-ethane-1,1-bisphosphonate		23.78/9.04	3.5
19	Br-43	NA	0.01/0.02	NA
22A	Undecane-1-hydroxy-1,1-bisphosphonate		0.75/0.49	0.28/0.45
23A	Dodecane-1-hydroxy-1,1-bisphosphonate		0.72/0.40	0.55
203A	Tetradecyl1-1-hydroxy-1,1-bisphosphonate		0.55/0.21	NA
249A	[(6-Ethyl-pyridin-2yl)-aminomethylene]-1,1-bisphosphonate		540.85/NA	2.8
252A	1-hydroxy-nonane-1,1-bisphosphonate		42.64/11.05	2.38/3.72
687A	2-(methyldecylsul-fonium-1-yl)ethylidene-1,1-bisphosphonic acid		0.16/0.09	6.22
688A	2-(methyldodecyl sulfonium-1-l)ethylidene-1,1-bisphosphonic acid		0.08/0.04	6.79/4.26
764A	1-Hydroxy-2-(3-dodecyloxylbenzyl)-1,1-bisphosphonic acid		0.59/0.27	NA
850A	1-farnesyl-1,1-bisphosphonic acid		1.48/0.59	NA

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CHAPTER III

CONCLUSION

Toxoplasma gondii is an opportunistic human pathogen. It infects a variety of vertebrate hosts and causes a disease called toxoplasmosis. The disease may lead to fatal consequences in case of immunocompromised individuals like AIDS patients[1]. Also, developing fetuses from infected mothers and organ transplant or blood transfusion recipients from infected donors are at higher risk of developing a severe disease[2,3]. The existing medications against *T. gondii* suffer from many drawbacks. Therefore, identification and validation of novel drug targets in this parasite is crucial for development of potential therapeutic drugs. Metabolic pathways which are unique and essential to the parasite have been considered useful for this purpose. Isoprenoid derivatives serve many important biological functions. Therefore, enzymes which catalyze the synthesis of isoprenoids are indispensable and could be potential targets. Isoprenoid pathway of *T. gondii* has been asserted as a promising drug target in recent years[4]. A clear-cut understanding of the different enzymes of this pathway will facilitate the discovery of more effective drugs against *T. gondii*.

In the present study, we describe the identification, molecular cloning, recombinant expression, and biochemical characterization of a polyprenyl diphosphate synthase homolog from *Toxoplasma gondii* (TgPPS). According to our finding, TgPPS is a soluble protein and localizes to the cytoplasm of the parasite. The recombinant TgPPS was expressed using an *E.coli* expression system. The purified recombinant TgPPS was used for biochemical and kinetic characterization of the enzyme.

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The enzyme requires divalent cation like Mg^{+2} for enzymatic activity. It has a higher preference for FPP as the allylic substrate when compared to GPP and GGPP. Also, the efficiency of TgPPS is highest when the substrate is FPP. Analysis of the enzymatic hydrolyzed product of TgPPS was carried out by thin layer chromatography with authentic standards. TgPPS synthesizes an isoprenoid chain of seven isoprene units and could be a medium chain heptaprenyl diphosphate synthase. Polyprenyl diphosphate synthases make the isoprenoid side chain of ubiquinone (CoQ) and determine the ubiquinone species in an organism[5]. Ubiquinone analysis from *T. gondii* revealed that the parasite makes CoQ-7. But, TgPPS could not complement the *Escherichia coli (KO229)* octaprenyl diphosphate synthase, which synthesizes CoQ-8[7]. This indicates that TgPPS lost its functional activity in *E.coli* which could be due to improper folding or mistargeting of the TgPPS protein. Also, TgPPS might be fully functional only when associated with other protein subunits which are present in *T. gondii* but not in *E.coli*.

 Bisphosphonates are modified pyrophosphate analogs in which the central oxygen atom is replaced by a carbon atom. Many bisphosphonates are now in clinical use for treatment of bone resorption disorders[6]. Our *in vitro* drug inhibition studies show that TgPPS is inhibited by many bisphosphonates at lower concentrations. For some bisphosphonates, the IC-50s of TgPPS inhibition is very similar to the *T. gondii* growth inhibition. This finding suggests that these compounds might be targeting the TgPPS in the parasite. Based on the preliminary inhibition data, we hypothesize that TgPPS could be potential target of bisphosphonates and other drugs.

These results suggest that TgPPS is an important functional enzyme in *T. gondii* and could be exploited for therapeutic intervention with suitable drugs. Future studies

could be directed towards validation of TgPPS as a potential drug target by gene disruption strategy. Before that, it may be helpful to substantiate the biological role of TgPPS in the parasite. Along with this, the three dimensional structure of TgPPS will provide valuable information for designing better inhibitors and discovery of potential drugs.

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