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The Farnesyl-diphosphate/Geranylgeranyl-diphosphate Synthase of *Toxoplasma gondii* Is a Bifunctional Enzyme and a Molecular Target of Bisphosphonates*[S]

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Farnesyl-diphosphate synthase (FPPS) catalyzes the synthesis of farnesyl diphosphate, an important precursor of sterols, dolichols, ubiquinones, and prenylated proteins. We report the cloning and characterization of two *Toxoplasma gondii* farnesyl-diphosphate synthase (TgFPPS) homologs. A single genetic locus produces two transcripts, *TgFPPS* and *TgFPPSi*, by alternative splicing. Both isoforms were heterologously expressed in *Escherichia coli*, but only TgFPPS was active. The protein products predicted from the nucleotide sequences have 646 and 605 amino acids and apparent molecular masses of 69.5 and 64.5 kDa, respectively. Several conserved sequence motifs found in other prenyl-diphosphate synthases are present in both TgFPPSs. *TgFPPS* was also expressed in the baculovirus system and was biochemically characterized. In contrast to the FPPS of other eukaryotic organisms, TgFPPS is bifunctional, catalyzing the formation of both farnesyl diphosphate and geranylgeranyl diphosphate. TgFPPS localizes to the mitochondria, as determined by the co-localisation of the affinity-purified antibodies against the protein with MitoTracker, and in accord with the presence of an N-terminal mitochondria-targeting signal in the protein. This enzyme is an attractive target for drug development, because the order of inhibition of the enzyme by a number of bisphosphonates is the same as that for inhibition of parasite growth. In summary, we report the first bifunctional farnesyl-diphosphate/geranylgeranyl-diphosphate synthase identified in eukaryotes, which, together with previous results, establishes this enzyme as a valid target for the chemotherapy of toxoplasmosis.

Toxoplasma gondii is a pathogenic protozoan parasite that infects a wide range of vertebrate hosts, including humans. *T. gondii* has been recognized as a major opportunistic pathogen of fetuses from recently infected mothers and of immunocompromised patients, *i.e.* those with AIDS. Toxoplasmic encephalitis is associated with high mortality and morbidity and is one of the most common opportunistic infections of the central nervous system in human immunodeficiency virus-infected patients (1). The chemotherapy for toxoplasmosis is not ideal especially for the AIDS patient because there is relapse of the infection if the treatment is halted because of intolerance of the patient to the side effects. Almost all the drugs in use have toxicity associated with their continued use. In addition, most of the recommended treatments do not have an effect on the slow growing bradyzoite forms.

Isoprenoids are the most diverse and abundant compounds occurring in nature. Many types of isoprenoids (*e.g.* steroids, cholesterol, retinoids, carotenoids, ubiquinones, and prenyl groups bound to proteins) are essential components of the cellular machinery of all organisms because of their roles in a variety of biological processes. Despite their structural and functional variety, all isoprenoids derive from a common precursor, isopentenyl diphosphate (IPP),[2] and its isomer, dimethylallyl diphosphate (DMAPP). Farnesyl-diphosphate synthase (FPPS) catalyzes the consecutive condensation of IPP with DMAPP and geranyl diphosphate (GPP) to form the 15-carbon isoprenoid compound, farnesyl diphosphate (FPP). FPP is the substrate for enzymes catalyzing the first committed step for biosynthesis of sterols, ubiquinones, dolichols, heme a, and prenyl groups that bind to proteins. FPP can also be condensed with an additional molecule of IPP by geranylgeranyl-diphosphate synthase (GGPPS) to form the 20-carbon isoprenoid geranylgeranyl diphosphate (GGPP), also used in protein prenylation. Genes encoding FPPS and GGPPS, also referred as short chain prenyltransferases, have been cloned from various species, including rats (2), humans (3, 4), *Saccharomyces cerevisiae* (5), and plants (6, 7). In protist parasites, the FPPS gene has been

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY196327 and DQ630749.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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[2] The abbreviations used are: IPP, isopentenyl diphosphate; FPP, farnesyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPPS, geranylgeranyl diphosphate synthase; FPPS, farnesyl diphosphate synthase; TgFPPS, *T. gondii* farnesyl diphosphate/geranylgeranyl diphosphate synthase; PBS, Dulbecco's phosphate-buffered saline; RT, reverse transcription; ORF, open reading frame; RACE, rapid amplification of cDNA ends; Ni-NTA, nickel-nitrilotriacetic acid; FARM, first aspartic acid-rich motif; ACP, acyl carrier protein.

cloned from *Trypanosoma cruzi* (8) and *Trypanosoma brucei* (9). Both of these genes are single copy. RNA interference experiments in *T. brucei* show that the FPPS gene is essential (9), as it appears to be in many organisms (10, 11).

Recent work has shown that bisphosphonates, diphosphate analogs in which a carbon atom replaces the oxygen atom bridge between the two phosphorus atoms of the diphosphate, are potent inhibitors of FPPS (12). Several bisphosphonates are potent inhibitors of bone resorption and are in clinical use for the treatment and prevention of osteoporosis, Paget disease, hypercalcemia caused by malignancy, tumor metastases in bone, and other diseases (13). In early work, it was shown that nitrogen-containing bisphosphonates were effective in the inhibition of *T. cruzi*, *in vitro* and *in vivo*, without toxicity to the host cell (14). More recently, we tested a series of bisphosphonates for their effects on the growth of *T. gondii*, *T. brucei rhodesiense*, *Leishmania donovani*, and *Plasmodium falciparum in vitro*, finding that several bisphosphonates can effectively inhibit the growth of these parasites (15). Moreover, *in vivo* testing of bisphosphonates against *T. gondii* in mice has shown that one of the nitrogen-containing bisphosphonates, risedronate, significantly increases the survival of mice infected by *T. gondii* (16). All these results indicate that bisphosphonates are promising candidate drugs to treat infections caused by *T. gondii* as well as some other protozoan parasites.

In this study we report the cloning and characterization of a *T. gondii* FPPS (*TgFPPS*) homolog. Interestingly, we found the enzyme to be bifunctional, capable of forming both farnesyl diphosphate as well as geranylgeranyl diphosphate. This unique characteristic was previously found only in enzymes of the Archaea (17, 18). Bisphosphonates that inhibit the proliferation of *T. gondii in vitro* and *in vivo* are shown to be potent inhibitors of the enzyme, indicating that the isoprenoid biosynthesis pathway may constitute a novel target for the development of drugs to treat toxoplasmosis.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). *Taq*DNA polymerase, reverse transcriptase Superscript II, herring sperm DNA, TOPO TA cloning kit, Klenow fragment of DNA polymerase I, restriction enzymes, Sf9 insect cells, the Bac-to-Bac baculovirus expression kit, and MitoTracker™ Red CMXRos (M-7512) were from Invitrogen. BigDye® Terminator version 3.1 cycle sequencing kit was from Applied Biosystems (Foster City, CA). FastStart *Taq*DNA polymerase was from Roche Applied Science. Prime-a-Gene labeling system was from Promega (Madison, WI). DNA sequencing kit was from Applied Biosystems (Foster, CA). ECL chemiluminescence detection kit, [α-^{32}P]dCTP (3000 Ci/mmol), and nylon membranes were from Amersham Biosciences. *Escherichia coli* XL1-Blue MRF′ strain, ExAssist helper phage, silver staining kit, Bio-Rad protein assay, and nitrocellulose membranes were from Bio-Rad. Plasmid miniprep kit, gel extraction kit and plasmid maxiprep kit were from Qiagen Inc. (Chatsworth, CA). Chroma Spin+TE-100 columns were from Clontech. IPP, DMAPP, GPP, FPP, GGPP, and anti-FLAG column were from Sigma. [4-^{14}C]Isopentenyl diphosphate triammonium salt (55.0 mCi/mmol) was from PerkinElmer Life Sciences. All other reagents were analytical grade.

Cell Cultures—Tachyzoites of *T. gondii* RH strain were cultivated in human fibroblasts and purified as described before (19), whereas bradyzoites were obtained by differentiation of tachyzoites of the ME49 strain as described before (20). Host cells were cultivated in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum. Host cells were infected with tachyzoites at a ratio of 5:1 (parasites:host). Tachyzoites were collected 2–3 days post-infection. Cell cultures were maintained at 37 °C with 5% CO_2. *T. gondii* tachyzoites of the 2F1 clone expressing β-galactosidase were a gift from Dr. L. David Sibley (21) and were routinely maintained *in vitro* in the same media as used for the RH strain. *In vitro* growth inhibition was done using confluent human fibroblast cells in 96-well plates as described previously (22). Tachyzoites of the 2F1 clone at 10^4 per ml were used for infection, and the plates were incubated at 37 °C. At 48 h post-infection, the plates were processed for β-galactosidase activity as described previously (22).

Bioinformatics—Preliminary genomic and/or cDNA sequence data were accessed on line. Genomic data were provided by The Institute for Genomic Research and by the Sanger Center (Wellcome Trust). EST sequences were generated by Washington University, St. Louis. The N-terminal mitochondrial targeting sequence was predicted by use of the iPSORT prediction program.

RT-PCR and 5′-RACE—Total RNA was isolated from ~5×10^8 *T. gondii* tachyzoites using TRIzol reagent. About 2 μg of total RNA was reverse-transcribed to cDNA by SuperScript II reverse transcriptase using oligo(dT) primers. Gene-specific primers were designed and used to do standard PCR using the first strand cDNA as template. 5′-RACE was carried out using the kit from Invitrogen. The first cDNA strand was synthesized with a gene-specific primer 1 (5′-CTGAGAGCTGCAAGTGCATCTTCG-3′) by SuperScript II reverse transcriptase. cDNA was tailed with dCTP and then amplified using the abridged anchor primer and nested gene-specific primer 2 (5′-GGAGATCTTGGAGCTGCACATACG-3′). The primary PCR product was amplified using abridged universal amplification primer and nested gene-specific primer (5′-GTCCATCACATCGTCCATGACCAA-3′).

DNA Sequencing and Sequence Analysis—Sequencing reactions were done following the instructions of the DNA sequencing kit. An ABI 3730XL capillary sequencer was used.

Screening of the cDNA Library—A cDNA library of *T. gondii* tachyzoites was obtained from the Division of AIDS, NIAID, National Institutes of Health (catalog number1896). Membranes with λ phage plaques were hybridized with an [α-^{32}P]dCTP-labeled probe obtained by RT-PCR. The first screening of 5×10^5 plaques yielded five positive cDNA clones. After the second and third screenings, only one positive clone was obtained. The positive plaque was purified, and the phagemid was isolated by *in vivo* excision. The insert DNA from the phagemid was then sequenced.

Construction of TgFOi and TgFO—Both *TgFPPSi* (GenBank™ accession number AY196327) and *TgFPPS* (GenBank™ accession number DQ630749) open reading frames (ORFs) were

amplified by PCR using primers *TgFPPS*-BamHI, 5′-GGATCCATGGTGGATGCGGTG-3′ (sense) and *TgFPPS*-AvrII: 5′-CCCTAGGTTTCTGCCGTTTGT-3′ (antisense), and introduced into the expression vector ptubP30-FLAG/sag-CAT (a gift form Dr. William Sullivan, Indiana University, IN) in place of P30, to yield the plasmid ptub*TgFPPS*-FLAG/sag-CAT.

A basic electroporation protocol (23) was then used for transfection. Briefly, 10^7 freshly prepared parasites and 50 μg of sterilized plasmid ptub*TgFPPS-FLAG/sag-CAT* DNA in a 2-mm gap electroporation cuvette were mixed. After electroporation, parasites were allowed to recover for 15 min and then inoculated into human fibroblasts cells in 25-cm^2 T flasks. Stable transformants were selected in medium containing 20 μM chloramphenicol and cloned by limited dilution in 96-well plates.

The selected positive clone was denoted as TgFOi if expressing recombinant TgFPPSi or TgFO if expressing recombinant TgFPPS. The isolated positive clones were verified by PCR with chloramphenicol acetyltransferase-specific primers and Western blot analysis with specific antibodies.

Bac-to-Bac Baculovirus Expression—Gene specific primers with two restriction enzyme sites were designed as 5′-CGGATCCATGCGTGCTCCGCCT-3′ (sense primer with BamHI site, underlined) and 5′-CCTCGAGCTAGTGATGGTGATGGTGATGTTTCTGCCGTTTGTG-3′ (antisense primer with a His_6 tag and an XhoI site underlined). PCRs were performed using FastStart *Taq*DNA polymerase (Roche Applied Science) at 94 °C for 10 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1.5 min. The PCR product was cloned into a TA vector using the TOPO TA cloning kit and double digested with XhoI and BamHI. The digested fragment was separated by agarose-gel electrophoresis, purified with QIAquick gel extraction kit (Qiagen, Valencia, CA), and ligated with a linearized pFastBac 1 vector predigested with XhoI and BamHI. The correct construction of the expression cassette pFastBac1-*TgFPPS* was verified by DNA sequencing. The DH10Bac-competent cells (Invitrogen) were transformed with pFastBac1-*TgFPPS* plasmid, and blue/white selection was used to identify the colonies containing the recombinant bacmid. The incorporation of pFastBac1-*TgFPPS* into the baculovirus genome (bacmid DNA) with help of Tn7 site-specific transposition was verified by PCR with M13 forward (−40) and M13 reverse primers, according to the manufacturer's instructions (Invitrogen). The recombinant bacmid DNA was purified with plasmid miniprep kit (Qiagen), incubated with Cellfectin reagent (Invitrogen), and transfected to Sf9 cells. The resultant recombinant baculovirus was amplified, and viral titers were determined using a conventional plaque assay. Transfected Sf9 cells were collected after 24, 48, 72, and 96 h of infection, and the overexpression of the recombinant protein was detected by SDS-PAGE and Coomassie Blue staining.

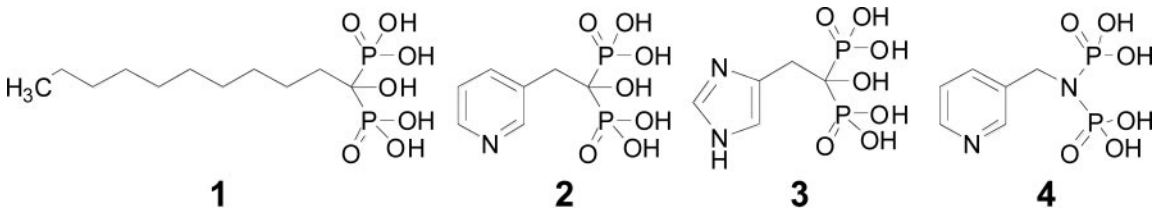
Purification of Recombinant Protein by Ni-NTA-Agarose—150 ml of baculovirus-infected H5 insect cells were washed with phosphate-buffered saline (PBS) and collected by centrifugation at 1000 × *g* for 5 min. The cell pellet was resuspended in 4 ml of lysis buffer (50 mM NaH_2PO_4, 600 mM NaCl, 10 mM imidazole, pH 8.0) and then broken by freezing and thawing six times. Supernatant and pellet fractions were separated by centrifugation at 12,000 × *g* for 10 min at 4 °C. 200 μl of 50% Ni-NTA-agarose (Qiagen) equilibrated with lysis buffer were added per 4 ml of the cleared lysate and then mixed gently on a rotary shaker at 4 °C for 2 h. After washing with 8 ml of lysis buffer and 8 ml of washing buffer (50 mM NaH_2PO_4, 600 mM NaCl, 50 mM imidazole, pH 8.0), the His tag protein was eluted with elution buffer (50 mM NaH_2PO_4, 600 mM NaCl, 300 mM imidazole, pH 8.0). The purified protein was dialyzed against storage buffer (10 mM Hepes, 100 mM NaCl, and 10 mM β-mercaptoethanol, pH 7.4) at 4 °C overnight. The purity of the protein was analyzed by SDS-PAGE, and the protein concentration was determined by using a Bio-Rad protein assay (Bio-Rad). The protein solution was mixed with 30% (v/v) glycerol and stored at −80 °C.

Expression and Purification of Recombinant TgFPPS in E. coli—The primers 5′-TCCATGGTTTCCGACGAACGGACTTCC-3′ and 5′-CCTCGAGTTTCTGCCGTTTGTGGAGCC-3′ were used to amplify the gene encoding the N-terminal truncated TgFPPSi. The PCR product was then subcloned into pCR 2.1-TOPO vector, and the sequence was verified by sequencing. The NcoI and XhoI fragment was then removed and subcloned into pET28a (Novagen) vector. The region corresponding to the C-terminal portion of TgFPPSi was replaced with the PstI-XhoI fragment of *TgFPPS* to generate pET28a-*TgFPPS*. Both expressing constructs were introduced into *E. coli* BL21(DE3) strain. The expression of recombinant TgFPPS and TgFPPSi was induced by 0.4 mM isopropyl β-thiogalactopyranoside at 20 °C overnight. The recombinant proteins were purified using HisBind 900 cartridges (Novagen) following the manufacturer's instructions.

Preparation of Anti-TgFPPS Antibody—PCR with two primers TgFPPSAntiF "5′-CATATGTATCGCTGCGACGGCATTGG-3′" (including an NdeI site, underlined) and TgFPPSExp4 "5′-CCTCGAGTTTCTGCCGTTTGTGGAGCC-3′" (including a XhoI site, underlined) was performed to amplify the region corresponding to the C-terminal portion of TgFPPS (amino acids 348–605). The PCR product was ligated with the *E. coli* expression vector pET-28a, and the recombinant peptide was overexpressed in *E. coli*. The resulting recombinant protein, Ag-TgFPPS, contained a His_6 tag at both the N terminus and C terminus and had a molecular mass of ~31.7 kDa, as shown by SDS-PAGE. The recombinant protein was found in the insoluble fraction. The Ag-TgFPPS was solubilized with 6 mM urea, purified using a Quick 900 cartridge nickel resin column (Novagen) under denaturing conditions according to the manufacturer's instructions, and then dialyzed to remove the urea. The purified protein was then sent to Cocalico Biologicals Inc. (Reamstown, PA) to generate antibodies in a guinea pig.

Affinity Purification of the Anti-TgFPPS Antibody—7 mg of Ag-TgFPPS was coupled to a CNBr-activated resin (Sigma C9142). Two ml of the final bleeding serum was applied to the resin and incubated at 4 °C overnight. The resin was washed with ~20 volumes of PBS and eluted with 0.1 M glycine (pH 2.5–3.0) at 4 °C. The eluted antibody was neutralized immediately after elution.

Purification of the Overexpressed TgFPPS with an Anti-FLAG Column—Approximately 8 × 10^9 of TgFO cells from 20 75-cm^2 flasks were filtered with a 5 μm pore-size membrane,



SCHEME 1

collected, and resuspended with lysis buffer (20 mM Hepes, 50 mM KCl, 125 mM sucrose, 0.5 mM EDTA, pH 7.2). A freeze and thaw method was used to break the parasites. The supernatant was collected by centrifugation at 12,000 × *g* for 10 min at 4 °C and then mixed with the anti-FLAG M2 affinity resin for 3 h at 4 °C. After washing with TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), the FLAG-tagged protein was eluted with 3× FLAG peptide in TBS buffer at 200 ng/μl.

SDS-Gel Electrophoresis and Western Blot Analysis—The electrophoresis system originally described by Laemmli (24) was used. The protein sample was mixed with an equal volume of 2× SDS-PAGE buffer (1 M Tris-HCl, pH 6.6, 20% SDS, 6% glycerol, 10% (v/v) β-mercaptoethanol, 0.4% bromphenol blue) and boiled for 5 min before loading into an SDS-polyacrylamide gel. After separation by electrophoresis, proteins were transferred to a nitrocellulose membrane with a Bio-Rad Transblot apparatus. 5% nonfat milk in PBS-T (0.1% Tween 20 in PBS) was used to block the nitrocellulose membrane at 4 °C overnight. The primary antibodies at the appropriate dilution in PBS-T were then incubated with the nitrocellulose membrane for 1 h at room temperature or 4 °C overnight. After three washes with PBS-T for 15 min each, the membrane was incubated with the horseradish peroxidase-conjugated secondary antibody at the appropriate dilution at room temperature for 1 h. After three washes with PBS-T for 15 min each, the immunoblots were visualized on blue-sensitive x-ray film by using ECL detection kit.

Immunofluorescence Microscopy—Freshly isolated tachyzoites from lysed HFB cells were stained with 50 nM MitoTracker™ Red CMXRos at 37 °C for 45 min, fixed in 4% formaldehyde at room temperature for 1 h, and then allowed to adhere to poly-L-lysine-coated coverslips. 0.3% Triton X-100 was used to permeabilize the parasites for 30 min. 3% bovine serum albumin, 1% fish gelatin, 50 mM NH_4Cl, and 5% goat serum in PBS were used to block at room temperature for 1 h. A 1:100 dilution of the purified anti-TgFPPS antibody and a 1:200 dilution of fluorescein isothiocyanate-coupled goat anti-guinea pig secondary antibody were incubated with the parasites for 1 h each. Alternatively, mouse monoclonal antibodies raised against *T. gondii* rhoptry protein 1 (ROP1) a gift from Dr. J. Dubremetz (25) and rabbit polyclonal antibodies raised against the apicoplast protein acyl carrier protein (ACP) a gift from Dr. B. Striepen (26) were used in combination with anti-TgFPPS antibody in co-localization studies. Cells were observed with a Delta Vision fluorescence microscope. Images were recorded with a Photometrics Coolsnap camera and deconvolved over 15 cycles using Softwarx deconvolution software.

Enzymatic Activity Assay and Kinetic Studies—The activity of the enzyme was determined by a radiometric assay. The assay was performed in a total volume of 100 μl and included a 10 mM Hepes buffer, pH 7.4, 1 mM $MgCl_2$, 2 mM dithiothreitol, 100 μM [4-^{14}C]IPP (10 μCi/μmol), an allylic substrate (100 μM DMAPP, 27 μM GPP, or 13 μM FPP), and 40–160 ng of protein. The reaction was carried out at 37 °C for 30 min and terminated by the addition of 10 μl of 6 M HCl. The reaction mixture was made alkaline by addition of 15 μl of 6 M NaOH, extracted with 1 ml of hexane, washed with water, then transferred to a scintillation vial for counting.

For kinetic studies, the concentration of DMAPP, GPP, FPP, or [4-^{14}C]IPP was varied, whereas the corresponding counter-substrate was kept at saturating concentration. A nonlinear regression analysis in Sigma Plot 7.0 was used to estimate the kinetic parameters.

Reverse Phase TLC—To analyze the products after enzymatic reaction, the radioactive prenyl products in the mixture were hydrolyzed to the corresponding alcohols by using alkaline phosphatase at room temperature, overnight (8). The resultant alcohols were extracted with hexane and separated on reverse phase Silica Gel 60 plates (Merck) with acetone:H_2O (6:1; v/v). The position of the standard prenyl alcohol was visualized using iodine vapor. Radioactivity was visualized by autoradiography.

Effects of Bisphosphonates on TgFPPS Activity—The activity of TgFPPS was assayed in the presence of bisphosphonates in mixtures containing 10 mM Hepes, pH 7.4, 1 mM $MgCl_2$, 2 mM dithiothreitol, 47 μM [4-^{14}C]IPP (10 μCi/μmol), 13 μM FPP, and 168 ng of protein in a final volume of 100 μl. Reactants were incubated for 30 min at 37 °C, and the prenyl products were extracted and quantitated by liquid scintillation counting. IC_{50} values were calculated using the same methods as described previously for *in vitro* testing (22).

Bisphosphonates—The structures of the bisphosphonates investigated are as shown in Scheme 1, and their synthesis followed standard methods (15).

RESULTS

Isolation of the TgFPPS Gene—The amino acid sequence of the *T. cruzi* FPPS was used to do a TBLASTN search of the *T. gondii* gDNA and EST data bases (ToxoDB Release 2.0). Two hits, TGG16412 and TGG12970, were found with high similarity to the *T. cruzi* FPPS. Using these sequences as template, primers were designed and used to amplify *T. gondii* cDNA by RT-PCR. A 574-bp (RT-574) RT-PCR product was obtained and used as a probe to screen a *T. gondii* cDNA library (AIDS Research and Reference Reagent Program, National Institutes of Health). A 1194-bp cDNA sequence (TgLibr) was obtained with 597 bp of ORF, a truncated 5′ end, and the complete 3′ end. Gene-specific primers according to the RT-574 sequence were designed and used to do 5′-RACE, which produced a 494-bp cDNA fragment (Tg 5′) without the start codon.

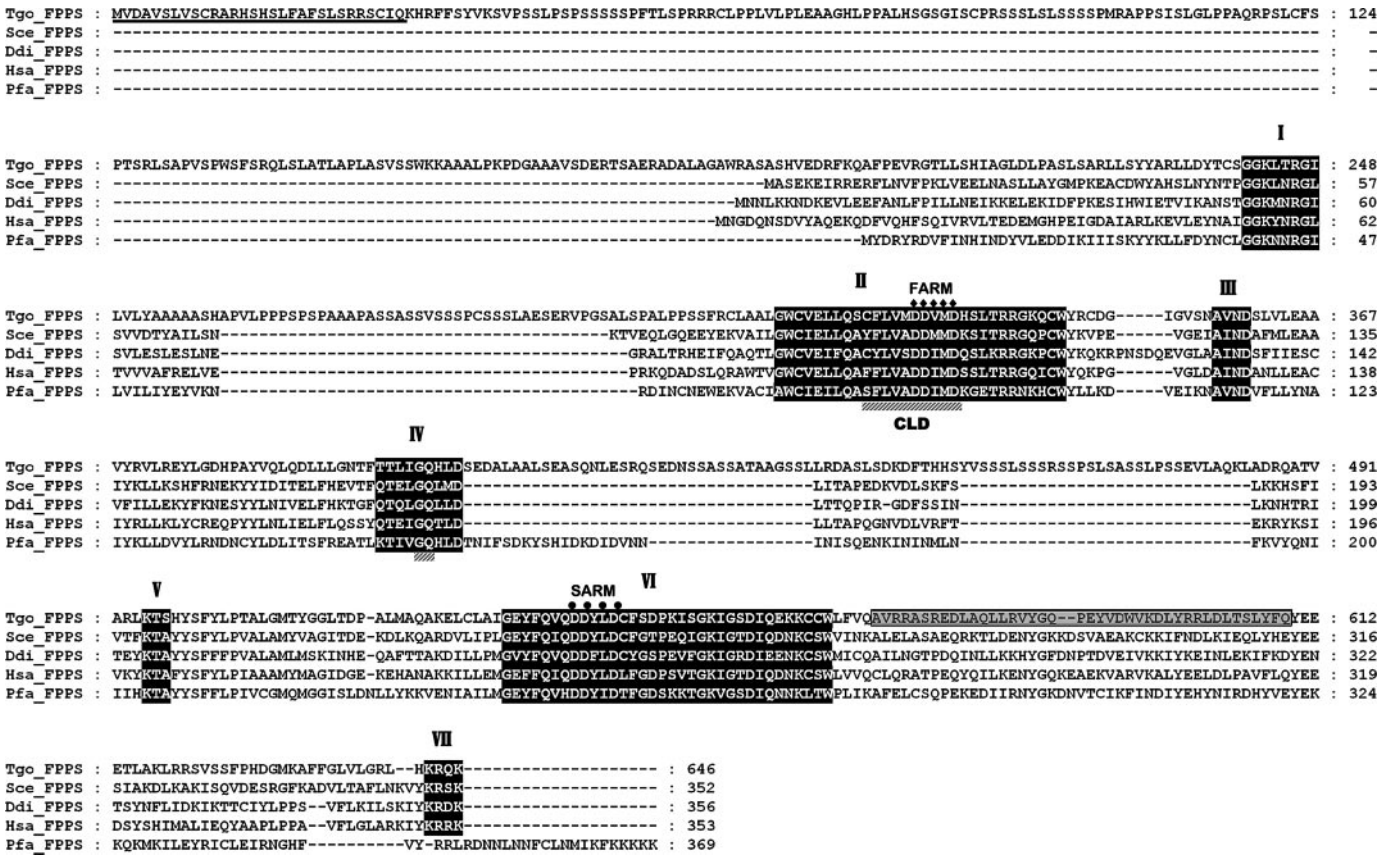


FIGURE 1. **Deduced amino acid sequence and transcription of TgFPPS.** Amino acids are numbered along the *right margin*. TgFPPS has 646 amino acids. A putative mitochondrial targeting sequence in the N terminus is *underlined*. The seven conserved amino acid motifs known to be characteristic of short chain prenyl-diphosphate synthases (*I–VII*) are in *black boxes*. The chain length determination (*CLD*) domain is marked by *hatched line* together with the FARM, which is marked with *diamonds* above the sequence. The second aspartate motif (*SARM*) is marked with *dots* above the sequence. The 41-amino acid insertion that is absent from TgFPPSi is in a *gray box*. *Tgo_FPPS*, *T. gondii* FPPS; *Sce_FPPS*, *S. cerevisiae* FPPS; *Ddi_FPPS*, *Dictyostelium discoideum* FPPS; *Hsa_FPPS*, *Homo sapiens* FPPS; *Pfa_FPPS*, *P. falciparum* FPPS.

The fragment Tg 5′ was used to search the ToxoDB 2.0 and a gDNA clone TGG_3342 was found. According to the sequence of TGG_3342, a primer F_6 (5′-GCGGCCACCGTCCATAATTG-3′) predicted to be upstream of the translation start codon ATG was designed. The other primer, R_6 (5′-TCTCTCTCACATACCGGCAG-3′), was designed according to the TgLibr sequence downstream to the stop codon TAG. Standard RT-PCR performed with these two primers produced a 2.4-kb RT-PCR product. After cloning this RT-PCR product into the 2.1 TOPO vector (Invitrogen) and sequencing it, two sequences were found. We named the ORF of the smaller one TgFPPSi (1815 bp) and the larger one TgFPPS (1938 bp). The only difference between TgFPPSi and TgFPPS is an in-frame 123-bp sequence insertion close to the 3′ terminus, which is present in TgFPPS but missing in TgFPPSi.

The deduced polypeptide sequences of TgFPPSi (605 amino acids) and TgFPPS (646 amino acids) are 93% identical. The only difference between the two protein sequences is a 41-amino acid insertion at the C terminus in TgFPPS (Fig. 1, *gray box*). The amino acid sequence of the predicted proteins showed that the seven conserved amino acid motifs known to be characteristic of short chain prenyl-diphosphate synthases are also conserved in TgFPPS (Fig. 1, *I–VII*, *black boxes*) and TgFPPSi (not shown). Regions II and VI, which contain the DD*XX*D*XXXX*RRG and G*XX*FQ*XX*DD*XX*D motifs, respectively, are the most conserved (Fig. 1). A long N-terminal extension and two insertions, one between the I and II conserved domains and the other between the IV and V conserved domains (Fig. 1) are not found in other prenyl-diphosphate synthases. The first aspartic acid-rich motif (FARM) is highly conserved and is indicated in Fig. 1 with *black diamonds*. The second aspartic-rich motif (SARM) is also highly conserved and is indicated in Fig. 1 with *black dots*.

Two Transcripts of TgFPPS Gene Originated by Alternative Splicing—To verify the existence of two transcripts, RT-PCR was performed using two primers, F_{10} (5′-CGCGTCTGAAGACCAGCCACTACT-3′) and R_{10} (5′-CCAAGCTTCTATTTCTGCCGTTTGTGGAGCC-3′) (see Fig. 2*A* for the position of F_{10} and R_{10}), upstream and downstream, respectively to the 123-bp insertion sequence. The results of RT-PCR showed that the transcription level of TgFPPS is much higher than that of TgFPPSi and that there is no apparent difference of the transcription level of these two isoforms between tachyzoites and bradyzoites (Fig. 2*B*).

Comparison of the ORFs of TgFPPSi and TgFPPS with the genomic sequence obtained from the *T. gondii* genome data base identified the presence of eight exons, separated by seven introns (Fig. 2*A*). The exons range in size from 30 to 913 bp. The size of the introns varies between 120 and 870 bp, with the 5′-(G/G)TRAGY and 3′-(Y)$_n$A(G/G) conserved splice-site sig-

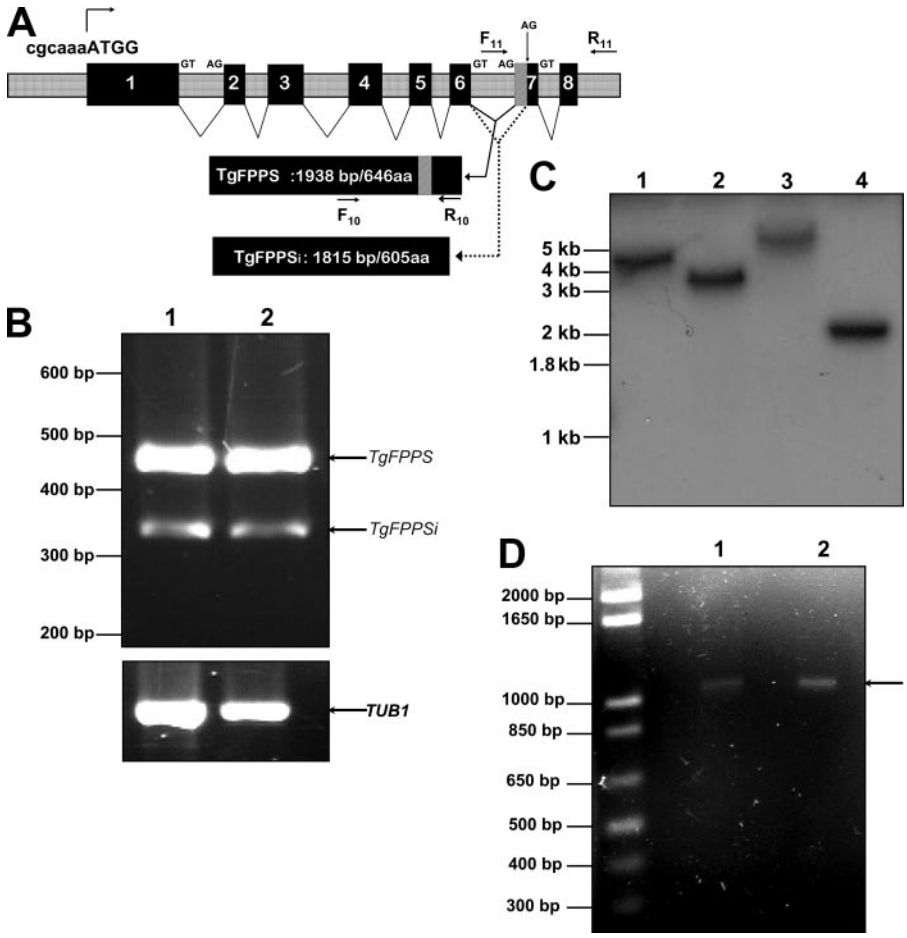


FIGURE 2. **Genomic structure (*A*), RT-PCR (*B*), and Southern blot (*C*) analyses and amplification of gDNA by PCR (*D*) of *TgFPPS*.** *A,* the relative position of introns and exons in the genomic DNA sequences are indicated by *gray* and *black boxes*, respectively. The nucleotide sequence around the initiation codon ATG was similar to the consensus translational initiation sequence "gNCAAaATGg" of other *T. gondii* genes. *B,* RT-PCR using primers (F_{10} and R_{10}) upstream and downstream to the 123-bp insertion sequence was performed to show the two transcripts of the *TgFPPS* gene. The RT-PCR product of the *TUB1* gene was used as loading control. The expression levels of both transcripts are similar in tachyzoite (*lane 1*) and bradyzoite (*lane 2*) stages obtained from the *T. gondii* ME49 strain. *C,* Southern blot analysis to demonstrate that the *TgFPPS* gene is a single copy gene in the *T. gondii* genome. Total genomic DNA was digested with different restriction endonucleases. *Lane 1,* EcoRI; *lane 2,* BamHI; *lane 3,* HindIII; *lane 4,* PstI. *D,* amplification of gDNA by PCR with primers F_{11} and R_{11} confirms that the *TgFPPS* gene is single copy. Ten μl and 20 μl of PCR product were loaded in *lane 1* and *lane 2,* respectively.

nal at the 5′ end and 3′ end, respectively, typical of eukaryotic nuclear introns (27, 28). Southern blot analysis with the RT-574 DNA fragment as a probe showed that digestion by the restriction enzymes gave a strong single band (Fig. 2*C*), suggesting the presence of a single copy gene.

To test whether *TgFPPSi* and *TgFPPS* represented two transcripts originating from only a single gene, or from two different loci in the genome, we did PCR with two gene-specific primers, F_{11} (5′-GCTGTCTCACATGCGTACATGCTT-3′) and R_{11} (5′-ACAGTGACGGAGAACGGCAAGTAA-3′) (Fig. 2*A*), to amplify the genomic sequence around the 123-bp insert. Only one band was identified in the PCR product (Fig. 2*D*), further confirming that the two transcripts originated from a single copy gene by alternative splicing.

Expression Levels of TgFPPS in T. gondii Tachyzoites—It was important to investigate whether both isoforms were expressed in tachyzoites. We generated two transgenic *T. gondii* tachyzoite strains by using the expression plasmid containing the target genes, as described under "Experimental Procedures." A clone overexpressing the FLAG-tagged TgFPPSi was isolated and named TgFOi, and another clone overexpressing the FLAG-tagged TgFPPS was isolated and named TgFO.

The level of expression of TgFPPS in the TgFOi and TgFO cells, as well as in wild-type cells, was studied by Western blot analysis (Fig. 3*A*) using an affinity purified polyclonal anti-TgFPPS antibody. Western blot analysis results showed that only TgFPPS is expressed at detectable levels in the wild-type cells, with an apparent molecular mass of ~61 kDa (Fig. 3*A*, *lane 3*). The difference between the observed and the calculated mass of 69.5 kDa (646 amino acids) could be due to intracellular cleavage of the mitochondrial targeting signal. Expression of TgFPPSi was below detectable levels. In TgFOi cells, the expression of both the larger endogenous TgFPPS protein and the smaller recombinant TgFPPSi could be detected by the anti-TgFPPS antibody, and the expression levels of both proteins were similar (Fig. 3*A*, *lane 2*). The apparent molecular weight of TgFPPSi was ~58 kDa. As expected, in TgFO cells, only TgFPPS was detected (Fig. 3*A*, *lane 1*). There was at least a 5-fold increase in the expression level of TgFPPS in the TgFO cells as compared with the wild-type cells. Fig. 3*B* shows a Western blot performed using the anti-FLAG antibody. This antibody recognized a 61-kDa protein in a lysate of TgFO cells that was not present in a lysate of wild-type cells, demonstrating the overexpression of the TgFPPS-FLAG protein in these cells.

Subcellular Localization of TgFPPS—By using the iPSORT prediction program, a mitochondrial targeting sequence "MVDAVSLVSCRARHSHSLFAFSLSRRSCIQ" was predicted at the N terminus of this putative TgFPPS protein, suggesting that it is a mitochondrial protein (Fig. 1, *underlined* at the N terminus). Immunofluorescence analysis with specific anti-TgFPPS antibody, as well as co-localization with the mitochondrial probe MitoTrackerTM Red CMXRos, showed that TgFPPS is partially localized in the mitochondria (Fig. 4, *A–D*). In experiments using antibodies raised against two other *T. gondii* organelle markers, ROP1 (for rhoptries) (Fig. 4, *E–H*) and ACP (for apicoplast) showed no co-localization with TgFPPS (Fig. 4, *I–L*).

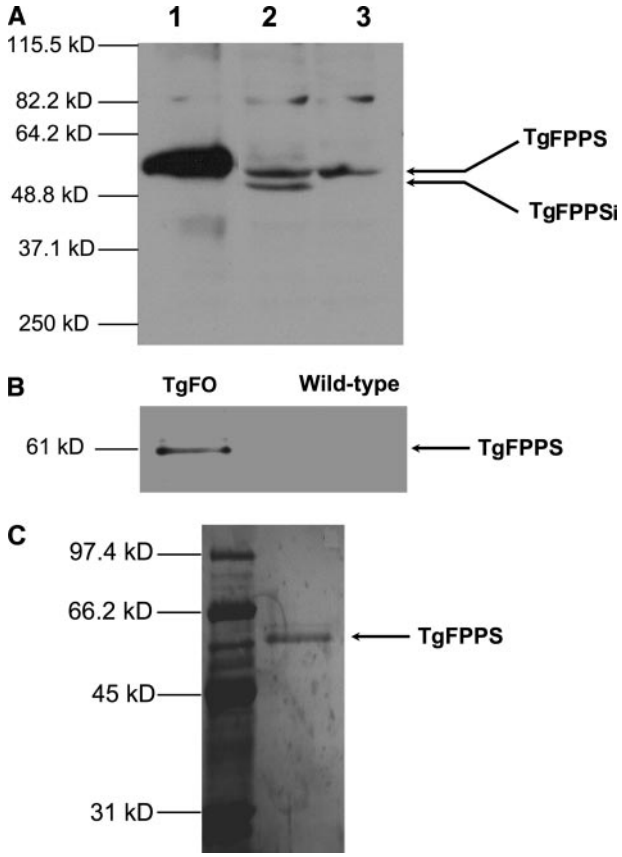


FIGURE 3. **Western blot analysis of TgFPPS in wild-type and overexpressing clones of *T. gondii*.** *A,* Western blot analysis shows that TgFPPS is the only isoform detected by the antibody in wild-type *T. gondii* tachyzoites. Wild-type 2F cells in *lane 3* only show TgFPPS. The TgFO cells in *lane 1* show overexpression of the TgFPPS protein as compared with the wild-type cells (*lane 3*). TgFOi cells in *lane 2* express the endogenous TgFPPS (~61 kDa) and the recombinant TgFPPSi protein (~58 kDa). 20 μg of total lysate was loaded to each lane, and affinity-purified anti-TgFPPS antibody was used to detect both TgFPPS and TgFPPSi. *B,* Western blot analysis with anti-FLAG antibody to verify the overexpression of TgFPPS-FLAG in TgFO cells. *C,* SDS-PAGE of the affinity purified TgFPPS-FLAG from TgFO cells. The TgFPPS-FLAG protein was purified using an anti-FLAG column as described under "Experimental Procedures." The purified protein was applied to an SDS-PAGE protein gel and visualized by silver staining.

Heterologous Expression and Activity of TgFPPSi and TgFPPS—We expressed the recombinant TgFPPSi and TgFPPS in *E. coli*. The N-terminal 171 amino acids, which are not present in FPPS from other organisms, were removed from both TgFPPSi and TgFPPS. This approach improved the solubility of the recombinant proteins because the expression of the proteins with the complete N-terminal yields insoluble proteins. The truncated TgFPPSi and TgFPPS (with C-terminal hexahistidine tags) were successfully expressed and purified from *E. coli*. The activity of the two recombinant proteins was tested using a standard activity assay. Prenyltransferase activity assay was carried out with DMAPP, GPP, FPP (Table 1), and GGPP (data not shown) as substrates. The activity found for GGPP as substrate was negligible. The results clearly indicated that the activity of recombinant TgFPPSi is negligible (Table 1).

To increase the solubility of TgFPPS with the longer N-terminal sequence, we turned to a baculovirus expression system. Both TgFPPSi and TgFPPS with a hexahistidine tag at the C terminus were expressed in the Bac-to-Bac baculovirus expression system. The activity of TgFPPSi measured in cell lysates or purified from baculovirus-infected H5 insect cells was negligible (data not shown).

The recombinant TgFPPS expressed in the baculovirus expression system was purified by one-step affinity chromatography as described under "Experimental Procedures." SDS-PAGE with Coomassie Blue staining showed that the purified TgFPPS protein has an apparent molecular mass of ~61 kDa (supplemental Fig. 1*A*), consistent with the protein size detected in the Western blot analysis of a lysate of wild-type *T. gondii* tachyzoites with anti-TgFPPS antibody (Fig. 3*A*, *lane 3*).

The supernatant fraction of wild-type insect cells was submitted to the same purification procedure. In this case, no protein could be detected in the eluted fraction as measured by SDS-PAGE with Coomassie Blue staining, and no detectable enzymatic activity could be found in the eluted buffer (data not shown). This means that the measured enzymatic activity in the elution fraction of the insect cells infected with recombinant Bac-to-Bac baculovirus was not because of contamination from the host insect cell enzyme.

TgFPPS with a C-terminal FLAG-tag was also purified from TgFO cells by using an anti-FLAG column (Sigma) (Fig. 3*C*). The size of the purified FLAG-tagged protein was ~61 kDa, identical to the one purified from insect cells (supplemental Fig. 1*A*). The purified protein also had enzymatic activity (not shown). Because of the limited amount of purified protein, we were not able to characterize this enzyme further.

Biochemical Characterization of TgFPPS—The purified TgFPPS from insect cells could be activated by either Mg^{2+} or Mn^{2+}, had no activity without these cofactors, and preferred Mg^{2+} to Mn^{2+}. At 1 mM Mg^{2+}, TgFPPS had the highest specific activity (data not shown). The optimum pH for this enzyme was around 7.38 in Hepes buffer (data not shown).

Kinetic parameters, K_m and V_{max}, were obtained by a nonlinear regression (SigmaPlot 7.0) fit of the data to the Michaelis-Menten equation, and the values obtained are shown in Table 2 and Fig. 5.

By doing reverse phase TLC to separate the products, it was found that the major products of the enzyme are FPP and GGPP (Fig. 6*B*). In contrast, *T. brucei* FPPS only generated FPP (Fig. 6*A*). The *T. gondii* enzyme (TgFPPS) is therefore actually a farnesyl diphosphate/geranylgeranyl-diphosphate synthase.

TgFPPS Is a Molecular Target for Bisphosphonates—The inhibition test with the purified enzyme showed that 3 of the 10 best *T. gondii* growth inhibitors (22) were also potent inhibitors of TgFPPS activity (Table 3). The best *T. gondii* inhibitor, compound **1** (an alkyl bisphosphonate), has an IC_{50} of 0.022 ± 0.003 μM, for TgFPPS. The IC_{50} of compound **2** (risedronate) for TgFPPS was 0.074 ± 0.017 μM and that for compound **3** (zoledronate) was 0.61 ± 0.29 μM (supplemental Fig. 2*B*). This order for the inhibition of the enzymatic activity correlates with the order for inhibition of parasite growth (**1** > **2** > **3**, see Table 3), which strongly supports the idea that TgFPPS is the main target for these more active bisphosphonates. The IC_{50} values obtained with these compounds against the human enzyme GGPPS were considerably higher (Table 3). Compounds **2** and **3** were both active against an *L. major* FPPS and in activating

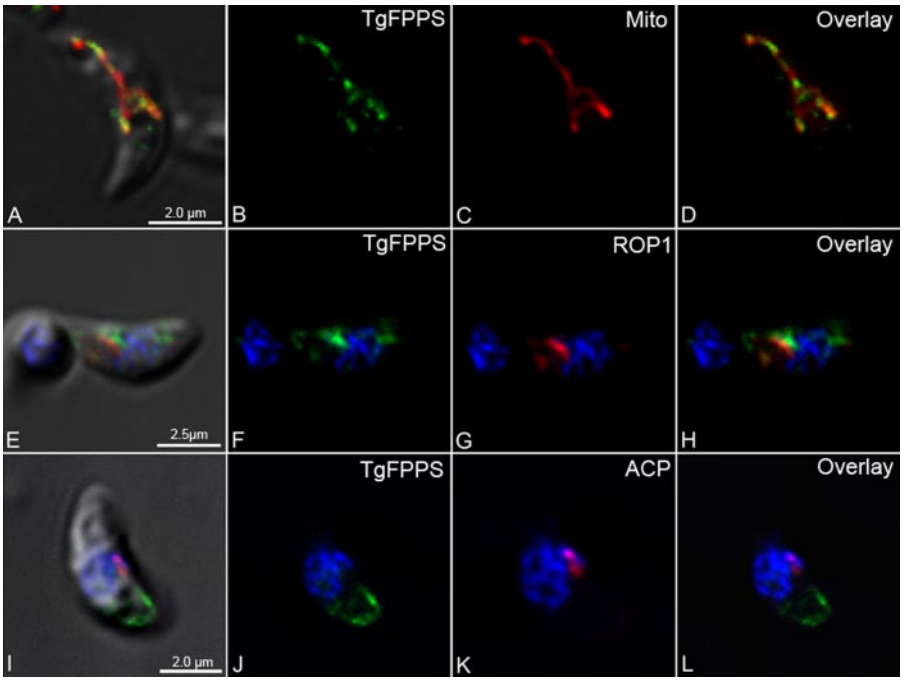


FIGURE 4. **TgFPPS partially localizes in the mitochondrion of *T. gondii* and does not co-localize with rhoptries (ROP1) and apicoplast (ACP) markers.** *T. gondii* mutants overexpressing FPPS were fixed and stained with antibodies raised against TgFPPS (*B, F,* and *J*), MitoTracker (*Mito, C*), or antibodies against rhoptries (*ROP1, G*), or apicoplast proteins (*K*). Differential interference contrast + overlay (*A, E,* and *I*) and overlay images (*D, H,* and *L*) are also shown. *Scale bars* are indicated in *A, E,* and *I*.

TABLE 1

Activity of recombinant TgFPPS and TgFPPSi in the presence of different substrates

Isoform[a]	Substrate		
	DMAPP	GPP	FPP
	nmol/min/mg	*nmol/min/mg*	*nmol/min/mg*
TgFPPSi	0.089 ± 0.178	0.356 ± 0.045	−0.178 ± 0.356
TgFPPS	28.56 ± 0.72	18.5 ± 0.54	8.45 ± 0.98

[a] The recombinant enzymes were expressed in *E. coli* and their activities measured as described under "Experimental Procedures." These isoforms lack 171 amino acids from the N terminus. Values shown are means ± S.D. of three independent experiments made in duplicate.

TABLE 2

Kinetic parameters of TgFPPS

TgFPPS was purified from baculovirus-infected H5 insect cells as is described under "Experimental Procedures." Values shown are means ± S.D. of two independent experiments made in duplicate. The enzymatic activity was measured as described under "Experimental Procedures" in the presence of different concentration of substrates and saturating concentration of counter-substrates. The kinetic parameters of TgFPPS, K_m *and* V_{max} were calculated using the non-linear regression method as described under "Experimental Procedures." Graphical data supporting these results is shown in Fig. 5.

Substrate	Counter-substrate	K_m	V_{max}
		μM	*nmol/min/mg*
DMAPP	100 μM IPP	6.2 ± 1.1	41.3 ± 1.4
GPP	100 μM IPP	1.0 ± 0.1	27.1 ± 0.9
FPP	100 μM IPP	1.9 ± 0.6	11.7 ± 1.2
IPP	100 μM DMAPP	23.5 ± 4.1	65.0 ± 4.4
IPP	27 μM GPP	17.4 ± 3.7	24.4 ± 1.8
IPP	13 μM FPP	38.1 ± 9.8	17.1 ± 2

human γδ T cells (which involves FPPS inhibition) (Table 3), whereas **1** was not. Compound **4** was inactive in all assays.

In a previous work (22), we correlated *T. gondii* growth inhibition by alkyl- and nitrogen-containing bisphosphonates with inhibition of human and *L. major* FPPS because TgFPPS had not yet been isolated. We now could do this correlation using the *T. gondii* enzyme, and the results are shown in Fig. 7. The correlation between *T. gondii* growth inhibition and TgFPPS inhibition is high for alkyl bisphosphonates ($R^2 = 0.949$) but low for nitrogen-containing bisphosphonates ($R^2 = 0.32$). This potent inhibition of TgFPPS by alkyl bisphosphonates resembles GGPPS inhibition more than FPPS inhibition, because alkyl bisphosphonates are among the most potent inhibitors of human GGPPS (29) and indicate a higher susceptibility of TgFPPS to alkyl bisphosphonates than other FPPS.

DISCUSSION

We report that a gene, *TgFPPS*, encoding a bifunctional FPPS/GGPPS is present in the *T. gondii* genome. Two transcripts were identified by RT-PCR. Southern blot analysis combined with the presence of a single band after PCR amplification of gDNA indicated that *TgFPPS* gene is a single copy gene and that the two transcripts result from alternative splicing. *TgFPPSi* mRNA is derived from a transcript that results from using a 3′ splice site signal inside exon 7. Alternative splicing is common in the *T. gondii* transcription process. For example, *T. gondii* myosins B and C are encoded by alternatively spliced mRNA and differ only in their C-terminal tails (30). Two cDNAs of *T. gondii* heat shock protein HSP60 corresponding to a single gene have also been isolated and characterized (31). In addition, two isoforms of the *T. gondii* hypoxanthine-xanthine-guanine phosphoribosyltransferase encoded by a single genetic locus, differing by only a 49-amino acid insertion, have recently been demonstrated (32). A special characteristic of the alternative splicing process in *TgFPPS* is that splicing occurs inside an exon, which is not the case for the *T. gondii* myosin, *HSP60,* or hypoxanthine-xanthine-guanine phosphoribosyltransferase genes. Alternative splicing diversifies the transcriptome and contributes significantly to proteome complexity. The reason for the presence of two transcripts of the *TgFPPS* gene is unknown. The deduced polypeptide sequences of TgFPPSi and TgFPPS are 93% identical. The only difference between the two protein sequences is the 41-amino acid insertion close to the C terminus of TgFPPS. Concerning the conserved domains, the deduced polypeptide sequence of TgFPPS shares about 60–76% similarity and 41–52% identity with human FPPS. With human GGPPS, the similarity of the conserved domains is 44–46% and the identity 27–33%. TgFPPS shares about 47–57% similarity and 30–38% identity with the conserved domains of *S. cerevisiae* FPPS and 45–52% similarity and 25–29% identity with the conserved domains of *S. cerevisiae* GGPPS.

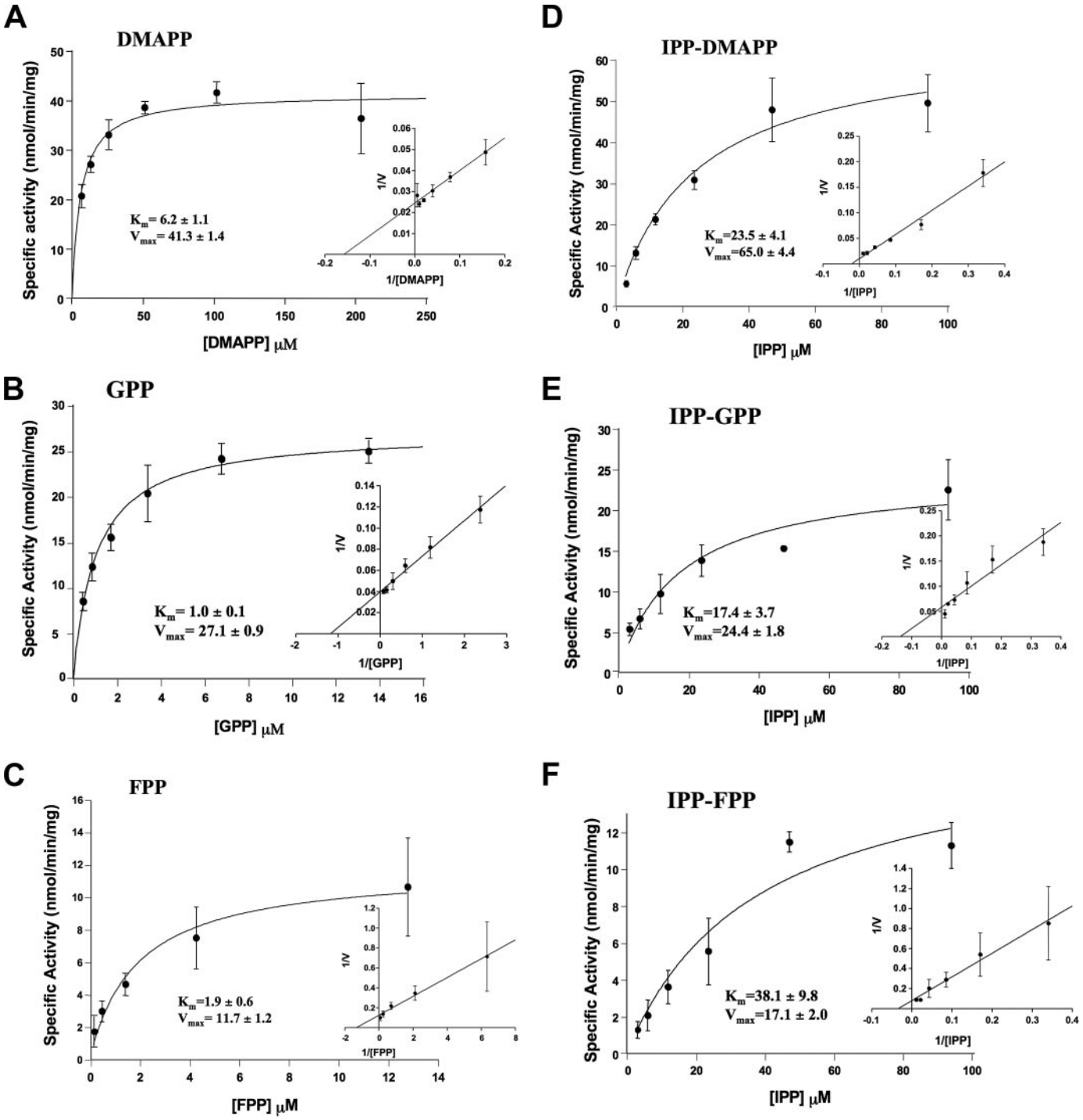


FIGURE 5. **TgFPPS activity in the presence of different substrates.** TgFPPS was purified from baculovirus-infected H5 insect cells. *A–C* show the effect of allylic substrates, DMAPP, GPP, and FPP concentrations on TgFPPS activity when the concentration of their counter-substrate IPP is 100 μM. *D–F* show the effect of IPP concentration on TgFPPS activity when the concentrations of its counter-substrates DMAPP, GPP, and FPP are 100, 27, and 13 μM, respectively. The activity of the enzyme was determined by a radiometric assay detailed under "Experimental Procedures." For kinetic studies, the concentration of DMAPP, GPP, FPP, or [4-^{14}C] IPP was varied, whereas the corresponding counter-substrate was kept at saturating concentrations. The *insets* show the Lineweaver-Burke plots used to calculate the K_m and V_{max} values. The nonlinear regression analysis in Sigma Plot 7.0 was used to calculate the kinetic parameters.

Comparative alignment of the amino acid sequences of TgFPPSi and TgFPPS with other short chain prenyl-diphosphate synthase shows that seven conserved amino acid motifs known to be characteristic of short chain prenyl-diphosphate synthase are also conserved in TgFPPSi and TgFPPS. Regions II and VI, which contain the DD*XX*-D*XXXX*RRG and G*XX*FQ*XX*DD*XX*D motifs, respectively, are the most conserved. When compared with other short chain prenyltransferases, the primary sequence of TgFPPS has some unique features. A long N-terminal extension and two insertions, one between the I and II conserved domains and the other one between the IV and V conserved domains, are only found in this enzyme. The N-terminal extension and the two insertions are serine-rich domains. The N-terminal

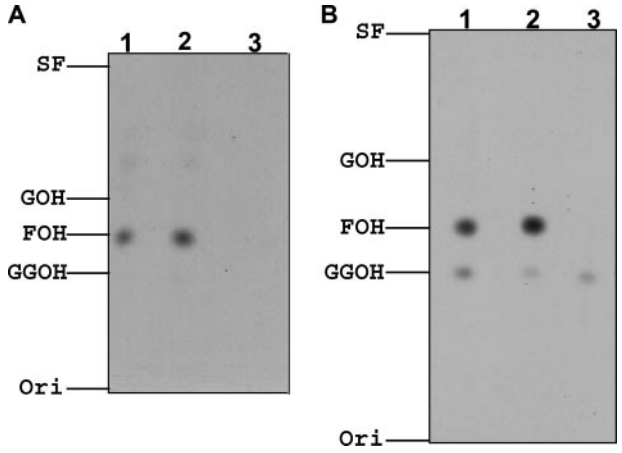


FIGURE 6. **Reverse phase thin layer chromatography of the products of TbFPPS (*A*) and TgFPPS (*B*) with different substrates.** TgFPPS was purified from baculovirus-infected H5 insect cells. *Lane 1*, DMAPP + IPP; *lane 2*, GPP + IPP; *lane 3*, FPP + IPP. *Ori,* origin; *SF,* solvent front. *GOH,* geraniol; *FOH,* farnesol; *GGOH,* geranylgeraniol. Other conditions are as described under "Experimental Procedures."

TABLE 3

Comparison of IC_{50}s for *T. gondii* growth inhibition, TgFPPS, *L. major* FPPS and human GGPPS activity inhibition, and γδT cell activation

Inhibitors	IC_{50} (μM)				
	*Tg*FPPS[a]	*T. gondii* growth	Human GGPPS	*L. major*[b] FPPS	γδ T cell[c] activation
1	0.022 ± 0.003	0.28 ± 0.02[d]	1.4 ± 0.2[c]	3.4	>100
2	0.074 ± 0.017	2.4 ± 0.7[d]	350 ± 50[c]	0.17	6.2
3	0.61 ± 0.29	6.4 ± 1.11[d]	220 ± 40[c]	0.33	12.0
4	>50	>200		>100	>100

[a] TgFPPS was purified from baculovirus-infected H5 insect cells as described under "Experimental Procedures." Both *in vitro* growth inhibition tests and TgFPPS inhibition tests were carried out as described under "Experimental Procedures."
[b] Data are from Ref. 29.
[c] Data are from Ref. 51.
[d] Data are from Ref. 22.

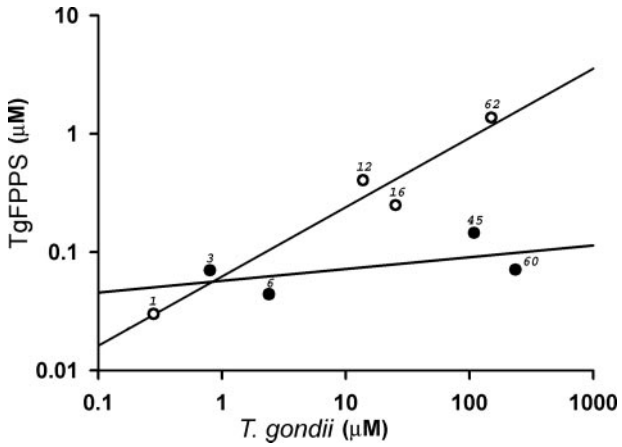


FIGURE 7. ***T. gondii* growth inhibition correlation.** Correlation between *T. gondii* IC_{50} values and TgFPPS inhibition for nitrogen-containing bisphosphonates (*closed circles*) and alkyl bisphosphonates (*open circles*). The compounds tested correspond to the compounds from Table 1 of Ref. 22. TgFPPS was purified from *E. coli*.

extension has about 164 amino acids, 42 of which (26%) are serine. Insertion 1 contains 37.5% (15:40) serine, and insertion 2 contains 40% (26:66) serine. The biological function of those serine-rich domains is not known. The translation of the two transcripts, TgFPPSi and TgFPPS, was tested by Western blot analysis. Only TgFPPS was expressed in wild-type cells by Western blot analysis. Although the transcript of TgFPPSi could be detected, it appears that either no protein was produced from this transcript or the amount of protein produced was too low to be detected.

By using the iPSORT prediction program, a mitochondrial targeting sequence "MVDAVSLVSCRARHSHSLFAFSLSRRSCIQ" is predicted at the N terminus of this protein, suggesting mitochondrial localization (Fig. 1). Immunofluorescence microscopy confirmed that TgFPPS is indeed localized in the mitochondrion (Fig. 4). The actual sizes of both TgFPPSi and TgFPPS are smaller than the calculated protein size, and this may be the result of the cleavage of the N-terminal targeting sequence of the two isoforms. When the N-terminal sequence of the TgFPPS was analyzed by MitoProp II (33), the program predicted a cleavage site at 150 amino acids from the N terminus. This would be in accord with the difference between the predicted molecular weight of TgFPPS and the results of the Western blot analyses.

TgFPPS catalyzes the condensation reaction between IPP and three allylic substrates: DMAPP, GPP, and FPP. In general, when a reaction is catalyzed by an FPPS starting from DMAPP, the products would be a mixture of GPP and FPP. However, a GGPPS would produce only GGPP, independent of the allylic substrate used. In the reaction catalyzed by TgFPPS, when DMAPP or GPP are substrates, both FPP and GGPP can be detected as products. But, when FPP was the substrate, the only product was GGPP. Hence, TgFPPS is actually an FPP/GGPP synthase, a bifunctional enzyme. The same behavior has been reported previously for an FPP/GGPP synthase isolated from a hyperthermophilic Archaea (17, 18). In general, FPPS require Mg^{2+} or Mn^{2+} ions as activators and prefer the former. However, GGPPS are activated by Mn^{2+} ions more effectively than by Mg^{2+}. For TgFPPS, both Mg^{2+} and Mn^{2+} acted as activators, but Mg^{2+} was slightly more effective than Mn^{2+}. The K_m values for GPP and FPP were comparable but that for DMAPP was much higher, probably because GPP and FPP have longer hydrocarbon chains, which would increase their hydrophobic interactions with the enzyme. FPP accumulates as an intermediate, as shown by TLC (Fig. 6*B*), probably because the turnover rate from FPP to GGPP is slower than that from GPP to FPP because the V_{max} for FPP is low when compared with those of DMAPP and GPP. Based on the heterologous expression results in *E. coli* (Table 1), only TgFPPS is active.

Both FPPS and GGPPS have been found in different cellular compartments, namely the cytosol (34, 35), mitochondria (35, 36), plastids in plants (35, 37), and the peroxisome in animals (38). The presence of ubiquinol-cytochrome *c* oxidoreductase, which couples the transfer of electrons from ubiquinol (QH_2) to cytochrome *c*, in *T. gondii* mitochondria, and the inhibition of respiration and collapse of mitochondrial membrane potential by antimycin A and cyanide (39) support the presence of ubiquinone in the *T. gondii* mitochondrion. It is reasonable to think that the FPP produced by TgFPPS may serve as the precursor for ubiquinone in mitochondria. Prenylated proteins were found in rat mitochondria (40). Protein isoprenylation (farnesylation and geranylgeranylation) was also found to take place in mitochondria and microsomes, in addition to the cytosol (41–43). Hence, FPP and GGPP produced by TgFPPS may be able to be transferred to some proteins inside the mito-

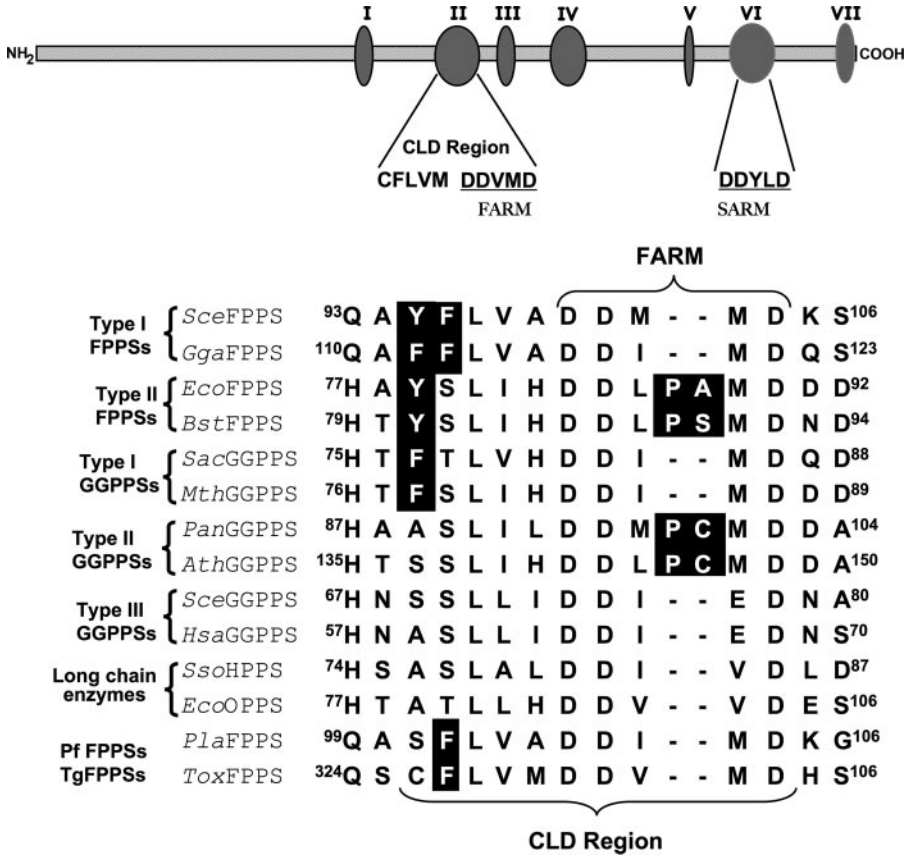


FIGURE 8. **Comparison of CLD regions and FARM domains of predicted amino acid sequences of several short chain prenyltransferases.** *SceFPPS*, *S. cerevisiae* FPPS; *GgaFPPS*, *Gallus gallus* (avian) FPPS; *EcoFPPS*, *E. coli* FPPS; *BstFPPS*, *Bacillus stearothermophilus* FPPS; *SacGGPPS*, *Sulfolobus acidocaldarius* GGPPS; *MthGGPPS*, *Methanobacterium thermoautotrophicum* GGPPS; *PanGGPPS*, *Pantoea ananatis* (*Erwinia uredovora*) GGPPS; *AthGGPPS*, *Arabidopsis thaliana* GGPPS; *SceGGPPS*, *S. cerevisiae* GGPPS; *HsaGGPPS*, *Homo sapiens* GGPPS; *EcoOPPS*, *E. coli* octaprenyl-diphosphate (C40) synthase; *TgFPPS*, *T. gondii* FPPS; *PlaFPPS*, *P. falciparum* FPPS. The aromatic amino acid residues and the two amino acids inserted within the FARM are in *black boxes*.

chondrion. However, it is also possible that FPP and GGPP can be transferred outside the mitochondrion where they could act as substrates for a protein farnesyltransferase or become precursors for other isoprenoid compounds, such as dolichols in the endoplasmic reticulum. The presence of isoprenylated proteins has been demonstrated in *T. gondii* by metabolic labeling (44).

Comparison with other short chain prenyltransferases at the primary amino acid level shows that this bifunctional TgFPPS is more similar to other FPPS than it is to other GGPPS. GGPPS have been divided into three types (45, 46). Type I GGPPS includes archaeal GGPPS, type II GGPPS from eubacteria and plants, and yeast and mammalian GGPPS that belong to the type III group. FPPS can be divided into two types: type I (eukaryotic) and type II (eubacterial) (45, 46). The CLD region, especially the fourth and fifth position N-terminal to the FARM as well as the two amino acid insertions within the FARM region, were reported to determine the final products of the reaction (45) (Fig. 8). It has been hypothesized that the bulky side chain of the amino acid at the fourth and fifth position can prevent product chain elongation (45). When comparing the CLD region of TgFPPS with other GGPPS and FPPS, we find that TgFPPS is different from all other FPPS or GGPPS found so far (Fig. 8). TgFPPS does not have the two-amino acid insert within the FARM region, as in most type I FPPS, type I GGPPS, and type III GGPPS (45). Also, in the TgFPPS sequence, the fourth amino acid upstream to the FARM region is phenylalanine, an amino acid with a bulky aromatic side chain, whereas the fifth amino acid upstream to the FARM region is cysteine, having a small side chain (Fig. 8). There are three possible patterns for the fourth and fifth amino acids upstream to the FARM in other GGPPS and FPPS (45) as follows: 1) both are aromatic amino acids (type I FPPS); 2) neither of them is an aromatic amino acid (type II and type III GGPPS); 3) only the fifth one is an aromatic amino acid (type II FPPS and type I GGPPs and long chain prenyl synthases). In contrast, TgFPPS has just one aromatic amino acid, and this occurs at the fourth position of the FARM region, instead of the fifth position. This unusual combination of fourth and fifth amino acids may provide the enzyme with the unusual characteristic of being a bifunctional enzyme producing both FPP and GGPP as products. Interestingly, there is no evidence for the presence of a gene with homology to GGPPS of other organisms in the genome of *T. gondii*.

From a cell growth and enzyme inhibition perspective, the results shown in Table 3 are of interest because they show that compound **1**, the most potent TgFPPS inhibitor, is also the most effective in *T. gondii* growth inhibition. This pattern of activity may at first appear surprising because the compound is an *n*-alkyl bisphosphonate lacking the key positively charged nitrogen site present in the potent bone resorption drugs (such as risedronate, **2**) which inhibit human FPPS, and there is only low activity of this compound against an *L. major* FPPS and in human γδ T cell activation (which involves FPPS inhibition) (Table 3). However, the long alkyl chain bisphosphonates are actually among the most potent inhibitors of human GGPPS (29) (Table 3) in addition to being among the more active inhibitors of *P. falciparum* cell growth (15), correlating perhaps with the presence of the serine/phenylalanine sequence upstream of the FARM region (which is cysteine/phenylalanine in TgFPPS, Fig. 8). Compound **2** (risedronate) inhibits TgFPPS as well as *T. gondii* cell growth, and compound **2** is a potent inhibitor of human (and *L. major*) FPPS but not human GGPPS (Table 3). Similar results are found with compound **3** (Table 3). Compound **4** has no activity in any assay, and although a bisphosphonate having a structure that is chemically quite close to **2**, it apparently does not bind well to the Mg^{2+} in the DMAPP site, perhaps because of a change in the phosphate pK_a values. In any

case, the potent inhibition of the TgFPPS by the alkyl bisphosphonate resembles GGPPS inhibition more than FPPS inhibition, is similar to that seen with *P. falciparum*, and may be related to subtle FPPS/GGPPS structural differences in and near the active site generated by the unique Cys-Phe/Ser-Phe "gate," which appears to be related to bifunctionality, at least in the *T. gondii* enzyme. This is supported by the results shown in Fig. 7 demonstrating a better correlation between inhibition of TgFPPS and *T. gondii* growth when using alkyl bisphosphonates, and different to what occurs with other FPPS, in which there is a high correlation between inhibition of the enzyme and inhibition of growth (47), or processes depending on the enzyme (48, 49), by amino bisphosphonates.

The correlation between enzyme and growth inhibition by the different bisphosphonates used here (Table 3 and Fig. 7) and our previous results showing that *T. gondii* engineered to overexpress FPPS required considerably higher levels of bisphosphonates to achieve 50% growth inhibition (22) support the conclusion that TgFPPS is a target of bisphosphonates. In this regard, Ortiz-Gomez *et al.* (50) recently reported that overexpression of *L. major* FPPS also resulted in resistance to risedronate.

In summary, we have cloned, sequenced, and expressed a bifunctional FPPS enzyme from *T. gondii*. The enzyme produces both farnesyl diphosphate as well as geranylgeranyl diphosphate, due most likely to the presence of both one large and one small amino acid residue (Cys-Phe) in the active site region, which control chain elongation. The enzyme is active and is inhibited by various bisphosphonates; the most potent bisphosphonate contains a long alkyl chain and is a potent inhibitor of *T. gondii* growth. A similar (Ser-Phe) motif is found in the *P. falciparum* FPPS, and the growth of *P. falciparum* is likewise inhibited by alkyl bisphosphonates, suggesting that FPPS of both apicomplexan parasites may be bifunctional.

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