Kinetic Characterization and Phosphoregulation of the Francisella tularensis 1-Deoxy-D-Xylulose 5-Phosphate Reductoisomerase (MEP Synthase)

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Abstract

Deliberate and natural outbreaks of infectious disease underscore the necessity of effective vaccines and antimicrobial/antiviral therapeutics. The prevalence of antibiotic resistant strains and the ease by which antibiotic resistant bacteria can be intentionally engineered further highlights the need for continued development of novel antibiotics against new bacterial targets. Isoprenoids are a class of molecules fundamentally involved in a variety of crucial biological functions. Mammalian cells utilize the mevalonic acid pathway for isoprene biosynthesis, whereas many bacteria utilize the methylerythritol phosphate (MEP) pathway, making the latter an attractive target for antibiotic development. In this report we describe the cloning and characterization of Francisella tularensis MEP synthase, a MEP pathway enzyme and potential target for antibiotic development. In vitro growth-inhibition assays using fosmidomycin, an inhibitor of MEP synthase, illustrates the effectiveness of MEP pathway inhibition with F. tularensis. To facilitate drug development, F. tularensis MEP synthase was cloned, expressed, purified, and characterized. Enzyme assays produced apparent kinetic constants (K_m = 104 μM, k_cat/DXP = 2 s^-1, k_cat/NADPH = 1.3 s^-1), an IC_50 for fosmidomycin of 247 nM, and a K_i for fosmidomycin of 99 nM. The enzyme exhibits a preference for Mg^2+ as a divalent cation. Titanium dioxide chromatography-tandem mass spectrometry identified Ser177 as a site of phosphorylation. S177D and S177E site-directed mutants are inactive, suggesting a mechanism for post-translational control of metabolic flux through the F. tularensis MEP pathway. Overall, our study suggests that MEP synthase is an excellent target for the development of novel antibiotics against F. tularensis.

Introduction

The US Centers for Disease Control and Prevention (CDC) classify biothreat agents based upon their ease of dissemination, associated morbidity/mortality rates, projected social impact, and emergency response procedures. Category A agents (i.e. those of highest concern) include Bacillus anthracis (the causative agent of anthrax), Yersinia pestis (plague), and Francisella tularensis (tularemia), while category B agents (exhibiting lower morbidity/mortality rates) include Brucella species (brucellosis), Burkholderia mallei (glanders), and Burkholderia pseudomallei (melioidosis). The 1984 Rajneeshee Salmonella attack, 2001 anthrax letter attacks, 2003 SARS outbreak, and 2009 H1N1 swine flu pandemic illustrate our vulnerability to both deliberate and natural outbreaks of infectious disease and underscore the necessity of effective vaccines and antimicrobial/antiviral therapeutics. The prevalence of antibiotic resistant strains and the ease by which antibiotic resistance can be engineered into bacteria further highlights the need for continued development of novel antibiotics against new bacterial targets.

Isoprenoids are a class of molecules fundamentally involved in a variety of crucial biological functions including electron transport (quinones), cell wall biosynthesis (dolichols), signal transduction (prenylated proteins), and the regulation of membrane fluidity (hopanoids and cholesterol). Despite their structural and functional diversity, all isoprenoids are derived from two building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which originate from either the mevalonic acid (MVA) or methylerythritol phosphate (MEP) pathway (Fig. 1). Because mammalian cells exclusively utilize the MVA pathway, enzymes within the MEP pathway make an attractive target for the development of novel antimicrobials (reviewed in [1–3]).

Genome sequences reveal that Francisella, Brucella, Bacillus, Burkholderia, and Yersinia each harbor MEP pathway genes, but little else is known about isoprene biosynthesis in these biothreat agents. In this report we describe the characterization of F. tularensis MEP synthase, a MEP pathway enzyme and potential target for drug development. The F. tularensis MEP synthase gene was cloned, expressed in Escherichia coli, and the recombinant
protein was purified and enzymatically characterized. Post-translational modification (phosphorylation) was revealed by protein mass spectrometry and correlated with the loss of enzyme activity, suggesting a possible regulatory mechanism. The stability of *F. tularensis* MEP synthase in high-throughput type assays amenable to drug development was also evaluated. Overall, our results suggest that MEP synthase is an excellent target for the development of novel antibiotics against *F. tularensis*.

**Materials and Methods**

**Bacterial strains and growth conditions**

The following reagents were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: *Francisella tularensis* subsp. *holantarctica* Strain LVS (FSC 155) and *Francisella tularensis* subsp. *novicida* Strain Utah 112. Each was cultured at 37°C in tryptic soy broth supplemented with 0.1% cysteine or in modified Chamberlain’s Defined Medium (0.4 g/L L-Arginine, 0.4 g/L L-Aspartic acid, 0.2 g/L L-Cysteine, 0.2 g/L L-Histidine, 0.4 g/L L-Isoleucine, 0.4 g/L L-Leucine, 0.4 g/L L-Lysine, 0.4 g/L L-Methionine, 2.0 g/L L-Proline, 0.4 g/L L-Phenylalanine, 0.4 g/L L-Threonine, 0.4 g/L L-Valine, 0.04 g/L Thiamine HCl, 0.002 g/L L-Calcium pantothenate, 4.0 g/L Glucose, 10.0 g/L NaCl, 0.135 g/L MgSO4.7H2O, 1.0 g/L KH2PO4, 1.0 g/L K2HPO4, 1.92 g/L sodium citrate, 0.02 g/L FeSO4.7H2O, pH 6.2). *Escherichia coli* BL21 CodonPlus (DE3)-RIL cells (Stratagene, La Jolla, CA) were used for recombinant protein expression whereas *E. coli* TOP10 (Invitrogen, Carlsbad, CA) or XL1Blue (Stratagene) cells were used for general cloning procedures. *E. coli* was grown at 37°C in Luria-Bertani (LB) media with constant shaking (250 rpm). Protein expression was performed in LB media containing ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml). Solid media were prepared by addition of 1.5% (wt/vol) agar.

**Fosmidomycin IC50 determination**

The half-maximal inhibitory concentration (IC50) was determined via a dose-response plot of fractional growth (OD595 in the presence of inhibitor/OD595 in the absence of the inhibitor) as a function of fosmidomycin concentration. An overnight culture of *F. tularensis* subsp. *novicida* was harvested by centrifugation, diluted to an OD595 = 1.0, then used to inoculate modified Chamberlain’s Defined Medium (using a 96-well plate; 17 μL of inoculum was...
added to 158 µL media containing varying concentrations of fosmidomycin. All conditions were evaluated in duplicate. Bacterial growth (OD\textsubscript{595} at 37°C) was determined using a Beckman Coulter DTX800 plate reader. A nonlinear regression analysis was carried out on the data obtained using GraphPad PRISM version 4.00 for Windows (GraphPad Software Inc., San Diego, CA) and the equation F = 1/(1+[I]/IC\textsubscript{50}) where F = fractional growth and [I] = inhibitor concentration.

**Genomic and Plasmid DNA isolation**

*F. tularensis* subsp. *holarctica* genomic DNA was isolated using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI), per the manufacturer’s instructions. Plasmid DNA was isolated from *E. coli* using a GenElute Plasmid miniprep kit (Sigma-Aldrich, St. Louis, MO).

**Construction of the *F. tularensis* MEP synthase expression plasmid**

The MEP synthase coding region (ispC) was identified in the *F. tularensis* subsp. *holarctica* LVS genome (accession number NC_007880) via a BLAST search using the *E. coli* K12 homologous sequence as the query. Polymerase chain reaction (PCR) primer pairs, designed to flank ispC (FtispC-f; 5'-CAGCTATTTTAAAAACAAATGGATAC-3' and FtispC-r; 5'-CCCCAAACAGATACTGACCATTTGC-3'), were purchased from Sigma-Genosys (The Woodlands, TX) and used to amplify the gene from *F. tularensis* subsp. *holarctica* LVS genomic DNA. FtispC-f contains four additional 5' residues (CACG) to facilitate the unidirectional insertion of the PCR product into plasmid pET101/D-TOPO ([Invitrogen](https://www.invitrogen.com/)). FtispC-r is designed to eliminate the stop codon in the PCR product to permit the expression of a C-terminal His-tagged MEP synthase protein. PCR was performed with Platinum Pfx polymerase ([Invitrogen](https://www.invitrogen.com/)) and the following parameters: 2 minutes at 94°C followed by 22 cycles of 13 sec at 94°C, 30 sec at 54°C, 1.5 min at 68°C, and a final elongation of 10 min at 68°C. The PCR product was purified using the Qiagen PCR Purification Kit ([Qiagen](https://www.qiagen.com/)) and cloned into pET101/D-TOPO to create pFtispC. Restriction mapping and DNA sequencing were used to confirm the fidelity of the PCR and the correct construction of the plasmid. pFtispC was transfected into chemically competent *E. coli* BL21 CodonPlus (DE3)-RIL cells to express the protein.

**Expression and purification of *F. tularensis* MEP synthase**

A one liter shake flask was used for protein expression (37°C, 250 rpm). The flask was inoculated with a 10 mL overnight culture of *E. coli* BL21 CodonPlus (DE3)-RIL containing pFtispC and upon reaching an OD\textsubscript{600} of 1.1, protein expression was induced by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside ([IPTG](https://en.wikipedia.org/wiki/Isopropyl_β-D-thiogalactopyranoside)). After incubation for an additional 18 hours, the cell pellet was thawed then cells were harvested by centrifugation and stored at -80°C. To purify the His-tagged protein, the cell pellet was thawed then cells were lysed using Lysis Buffer A (100 mM Tris pH 8.0, 0.5% lysozyme; 3 mL per mg cell pellet), followed by Lysis Buffer B (0.1 M CaCl\textsubscript{2}, 0.1 M MgCl\textsubscript{2}, 0.1 M NaCl, 0.2% DNase; 0.3 mL per mg cell pellet). Clarified cell lysate was obtained by centrifugation (40,000 x g, 20 min) then passed through a TALON immobilized metal affinity chromatography column (Clontech Laboratories, Mountain View, CA). The column was washed with 15 column volumes of 1X equilibration buffer (50 mM HEPES pH 7.5, 300 mM NaCl), 10 column volumes of 1X wash buffer (30 mM HEPES pH 7.5, 300 mM NaCl, 20 mM imidazole), and the His-tagged protein was then eluted with 5 column volumes of 1X elution buffer (150 mM imidazole pH 7.0, 300 mM NaCl). Buffer was exchanged by addition of 0.1 M Tris pH 7.5, 1 mM NaCl, 5 mM DTT while concentrating the protein by ultrafiltration. Protein concentration was determined using the Advanced Protein Assay Reagent ([Cytoskeleton](https://www.cytoskeleton.com/), Denver, CO) with γ-globulins ([Sigma-Aldrich](https://www.sigma-aldrich.com/)) as the standard. The protein was visualized via Coomassie stained SDS-PAGE and a Western blot with an anti-His antibody ([Qiagen](https://www.qiagen.com/)). The yield of purified MEP synthase averaged 5–10 mg per 1 L LB shake flask.

**Size-Exclusion Chromatography**

The molecular mass of MEP synthase and the mutant derivatives were estimated by loading 1 mg of protein onto a Sephacryl 200HR ([Sigma-Aldrich](https://www.sigma-aldrich.com/), St. Louis, MO) size-exclusion chromatography column equilibrated with 0.1 M Tris pH 7.5, 1 mM NaCl, 5 mM DTT (flow rate of 2 mL/min) and calibrated with a gel filtration standard kit purchased from Bio-Rad ([Hercules, CA](https://www.bio-rad.com/)). Blue dextran was used to determine the void volume of the column.

**Mutagenesis**

The Ser\textsubscript{177} to Asp\textsubscript{177} mutant of MEP synthase was created via PCR based site directed mutagenesis using primers FT-IspC-SD177-FP (5’ TTA ACA GCA GCA TGA GGT CCT TTG AG 3’; lowercase residues indicate site of mutation) and FT-IspC-SD177-RP (5’ AGG ACC TCC ATC TGC TGT TAA TAT TAT C 3’), whereas the Ser\textsubscript{177} to Glu\textsubscript{177} mutant was created using primer pairs FT-IspC-SE177-FP (5’ TTA ACA GCA GCA GGT CCT TTG AG 3’) and FT-IspC-SE177-RP (5’ AGG ACC TCC ATC TGC TGT TAA TAT TAT C 3’). Each primer pair is oriented outward from a central overlapping region. In the first step of the mutagenesis, four unidirectional PCRs were performed with each of the four individual PCR primers (FT-IspC-SD177-FP, FT-IspC-SD177-RP, FT-IspC-SE177-FP, or FT-IspC-SE177-RP) using the following parameters: 5 minutes at 95°C followed by 30 cycles of 30 sec at 95°C, 30 sec at 60°C, 40 sec at 72°C, and a final elongation of 7 min at 72°C. In the second step, 5 µL of each PCR product was appropriately combined with the product obtained from the companion primer (i.e. the product using FT-IspC-SD177-FP was combined with the FT-IspC-SD177-RP product, whereas the products obtained from the PCRs with FT-IspC-SE177-FP and FT-IspC-SE177-RP were combined) and each mixture served as template for a subsequent PCR using T7 forward and reverse primers. PCR conditions were as follows: 5 minutes at 95°C followed by 5 cycles of 30 sec at 95°C, 30 sec at 60°C, 40 sec at 72°C, then 25 cycles of 30 sec at 95°C, 30 sec at 52°C, 40 sec at 72°C and a final elongation of 10 min at 72°C. In the third step, the PCR products from step 2 were amplified using primers FtispC-f and FtispC-r (described above), and the resulting products were cloned into pET101/D-TOPO, transformed into XL1Blue cells, and sequence confirmed by restriction digestion and nucleotide sequencing. Each expression vector was introduced into chemically competent *E. coli* BL21 CodonPlus (DE3)-RIL cells to express the C-terminal His-tagged protein. The S177D and S177E mutants were purified as described for the *F. tularensis* MEP synthase.

**Fluorescence spectroscopy**

Fluorescence spectra of MEP synthase and the mutant derivatives were measured using a Fluoromax-3 fluorometer ([Horiba Jobin Yvon](https://www.horiba.com/)) at an excitation wavelength of 290 nm using cuvettes with an optical path length of 1 cm. The emission spectra of protein samples with a concentration of 5 µM in 0.1 M Tris
pH 7.5, 1 mM NaCl, 5 mM DTT were measured from 310 to 400 nm (excitation and emission slit width = 5 nm). The temperature was maintained at 30°C. All fluorescence spectra were corrected for background with pure buffer.

Enzyme Assays
MEP synthase activity was evaluated at 22°C by spectrophotometrically monitoring the enzyme catalyzed oxidation of NADPH using an assay derived from that described by Takahashi et al [4]. All assays were performed in triplicate. To determine the apparent $K_{M}$ for 1-deoxy-D-xylulose 5-phosphate (DXP), assay mixtures (200 µL) contained 100 mM Tris pH 7.8, 25 mM MgCl₂, 0.15 mM NADPH, 7 µM MEP synthase, and a variable concentration of DXP (Echelon Biosciences, Salt Lake City, UT). To determine cation specificity, assays were performed with 25 mM MgCl₂, CaCl₂, CoCl₂, CuCl₂, MnCl₂, ZnCl₂, or NiCl₂. To determine the apparent $K_{M}$ for NADPH, assays were performed with fixed DXP concentration (0.4 mM) and variable NADPH concentration. Nonlinear regression fitting to the Michaelis-Menten equation was used to determine the kinetic constants. Half-maximal inhibition ($IC_{50}$) by fosmidomycin was determined using a plot of enzyme fractional activity as a function of inhibitor concentration. A plot of $K_{I}^{app}$ as a function of inhibitor concentration was used to determine the $K_{I}$ for fosmidomycin (negative value of the x intercept). Because fosmidomycin is a slow, tight binding inhibitor [5], the enzyme was preincubated with fosmidomycin for 10 minutes prior to addition of substrate. High-throughput assays were performed using 96-well plates with assay volumes adjusted to 100 µL. The Z-factor was calculated as described by Zhang et al [6] with fosmidomycin as the inhibitor control.

Mass spectrometry method for phosphopeptide identification
To obtain MEP synthase for phosphopeptide analysis, protein expression and purification was essentially as described above, with the exception of using 0.01 mM IPTG for induction. Purified MEP synthase was reduced with 10 mM dithiothreitol (DTT), alkylated by iodoacetamide (50 mM), and then digested by trypsin (Promega) in buffer containing ammonium bicarbonate (50 mM, pH 9) and urea (2 M). The digestion mixture was then desalted by a SepPak column (Waters, Milford, MA). Phosphopeptides were enriched from the tryptic peptides by a TiO₂ column as described by Thingholm et al [7] with modification. In brief, 30 cm fused silica capillary tubing (360 µm OD, 200 µm ID, Polymicro Technologies, Phoenix, AZ) was attached to the frit end of Inline MicroFilter Assembly (Upchurch Scientific), and TiO₂ loose media (GL Sciences, Inc) was slurry-packed into the tubing using a Pressure Cell (Brechbuhler Inc.) to form a 200 µm ID, 200 µm OD, 200 µm length of tubing using an LTQ-Orbitrap mass spectrometer (ThermoFisher) using previously described methods [8]. Tandem mass spectra were searched using the program SEQUEST (Bioworks software, Thermo) with full tryptic cleavage constraints, static cysteine alkylation by iodoacetamide, and variable phosphorylation of Ser/Thr/Tyr. Phosphopeptide identification was determined using database match scoring criteria filters followed by manual evaluation of the raw data, as described [8].

Molecular Modeling
F. tularensis subsp. holarctica LVS MEP synthase (protein accession number CAJ70974) was homology-modeled using SWISS-MODEL [9] (http://swissmodel.expasy.org/) in automated modeling mode. The template used for modeling was identified via the template identification tool (with default parameters), which performs a primary sequence comparison of the query sequence with those in a structural database. The resolved structure that exhibited the greatest sequence homology with F. tularensis MEP synthase was chosen as the template for modeling. (E. coli MEP synthase; PDB ID# 1T1R, BLAST e value = 3 × 10⁻⁹¹, 48% identity and 66% homology with the F. tularensis sequence). The quality of the F. tularensis MEP synthase model was evaluated using ProQRes[10], which uses atom-atom contacts, residue-residue contacts, solvent accessibility, and secondary structure information to assign an accuracy score from 0 (unreliable) to 1 (reliable). Swiss-PdbViewer 4.0 (http://spdbv.vital-it.ch/) was used to visualize and annotate the model.

Results and Discussion
The F. tularensis MEP pathway as an antimicrobial target
Since MEP pathway orthologs are not found in the human genome, the pathway makes an attractive target for the development of novel antibiotics. To assess if MEP pathway inhibition would restrict the in vitro proliferation of F. tularensis subsp. novicida, we monitored bacterial growth in media supplemented with fosmidomycin[11], a strong and specific inhibitor of MEP synthase (E. coli genetically engineered to use mevalonate for IPP biosynthesis (Fig. 1) is unaffected by fosmidomycin when the culture medium is supplemented with mevalonate, but growth is inhibited by fosmidomycin when mevalonate is excluded[12], illustrating the specificity of fosmidomycin for the MEP pathway.

![Figure 2. Dose-response plot of F. tularensis subsp. novicida growth as a function of fosmidomycin concentration. Fractional growth was calculated as the ratio of cell density (OD₅₇₀) in the presence of inhibitor to cell density in the absence of inhibitor. Nonlinear regression fitting indicates half maximal activity at 12 µM. The goodness-of-fit (R²) value is indicated. Growth curves are presented in supportive Fig. S1.](image-url)
Dose-dependent inhibition of purified *E. coli* MEP synthase[11] and a resolved crystal structure of *E. coli* MEP synthase bound to the inhibitor[13] further illustrate this specificity. As illustrated in the dose-response plot shown in Fig. 2, fosmidomycin inhibits *F. tularensis* growth, with half-maximal inhibition at 12 μM (2.2 μg/mL). A transposon mutant library of *F. tularensis* [14] further confirms the essentiality of the pathway, as MEP pathway knockouts are lethal, further validating the pathway as an attractive target in *F. tularensis*. To facilitate drug development, we next set out to clone and evaluate MEP synthase.

Cloning, expression, and purification of *F. tularensis* MEP synthase

The *F. tularensis* subsp. *holarctica* LVS *ispC* gene, identified via a BLAST search of the genome using the *E. coli* K12 homologous sequence, is 1158 bp in length and encodes a polypeptide of 385 amino acids with a calculated molecular mass of 42.7 kDa. The subsp. *holarctica* MEP synthase amino acid sequence shares 99.7, 99.0, and 99.7% identity with the MEP synthase sequence from subsp. *tularensis*, subsp. *novicida*, and subsp. *mediasiatica*, respectively. PCR primer pairs, designed to flank the subsp. *holarctica ispC*, were used to amplify the gene. The PCR product was cloned into an expression plasmid engineered to express a C-terminal His-tagged protein in *E. coli*. Purified protein was visualized by SDS-PAGE and Western blot hybridization using an anti-His antibody (Fig. 3).

![Figure 3. Purification of recombinant *F. tularensis* MEP synthase.](image)

**A** Coomassie stained SDS-PAGE showing a molecular weight marker (MW) and purified His-tagged MEP synthase. His-tagged MEP synthase has a predicted molecular weight of 46.4 kDa. **B** Western blot hybridization analysis of purified MEP synthase using an anti-His antibody results in an intense band of the expected size. The appearance of a weak, smaller molecular weight band suggests that some degradation may have occurred.

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### Figure 4. The substrate dependent activity of *F. tularensis* MEP synthase.
Michaelis-Menten plots of reaction velocity as a function of A) DXP concentration and B) NADPH concentration were used to derive the kinetic parameters listed in Table 1. The solid line represents the nonlinear least-squares best fit of the data to the Michaelis-Menten equation. The $R^2$ value for each plot is indicated.

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### Table 1. MEP synthase Apparent Kinetic Parameters.

| MEP synthase     | $K_{\text{M, DXP}}$ (μM) | $K_{\text{M, NADPH}}$ (μM) | $k_{\text{cat, DXP}}$ (s$^{-1}$) | $k_{\text{cat, NADPH}}$ (s$^{-1}$) | $k_{\text{cat, DXP}}/K_{\text{M, DXP}}$ (M$^{-1}$min$^{-1}$) | $K_{i}$ (nM) | Refer. |
|------------------|--------------------------|----------------------------|-------------------------------|-------------------------------|-------------------------------------------------|--------------|--------|
| *F. tularensis*  | 103.7 +/− 12.1           | 133 +/− 1.5                | 2.0 +/− 0.09                  | 1.3 +/− 0.04                 | 1.2 × 10$^{3}$/+/− 9 × 10$^{3}$ 247              | 98.9 +/− 4.5 | This study |
| *E. coli*        | 81–175                   | 0.5–18                     | 33                            | -                            | 2.4 × 10$^{7}$                                  | 35           | 21–215  [5,33,49,50] |
| *M. tuberculosis*| 47                       | 29.7                       | 1.2                           | -                            | 1.5 × 10$^{6}$                                  | 310          | -      [51] |
| Synechocystis sp. PCC6803 | 170                    | 3.5                        | 17                            | -                            | 6 × 10$^{6}$                                    | -            | 4      [16,52] |

*The values were calculated from data obtained in triplicate.

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Size-exclusion chromatography using a calibrated column revealed that *F. tularensis* MEP synthase exists in solution as a dimer of $94$ kDa (Fig. S2), similar to MEP synthase from *Mycobacterium tuberculosis* [15] and *Synechocystis* sp. PCC6803 [16].

Kinetic characterization of *F. tularensis* MEP synthase

The kinetic activity of purified MEP synthase was spectrophotometrically evaluated by monitoring the substrate dependent enzyme catalyzed oxidation of NADPH (Fig. 1C). Nonlinear regression fitting of enzyme velocity versus substrate concentration was used to determine the apparent kinetic constants (Fig. 4 and Table 1). The $K_M^{app}$ for 1-deoxy-D-xylulose 5-phosphate (DXP) was obtained using assays performed with a saturating concentration of NADPH (150 μM), whereas the $K_M^{app}$ for NADPH was determined using assays with saturating levels of DXP (400 μM). The $K_M^{app, DXP}$ and $K_M^{app, NADPH}$ for recombinant *F. tularensis* MEP synthase are consistent with values reported for the enzyme from *E. coli*, *M. tuberculosis*, and *Synechocystis* sp. PCC6803 (Table 1). The apparent specificity constant of *F. tularensis* MEP synthase is also comparable to the *Mycobacterium* and *Synechocystis* enzymes, although it is 20 fold less than that reported for the *E. coli* enzyme (due to the difference in $k_{cat}^{DXP}$). Assays performed with various divalent cations revealed that recombinant *F. tularensis* MEP synthase prefers MgCl$_2$, although 60% of the enzyme activity is retained with MnCl$_2$ (Fig. 5).

Having established assay conditions for *F. tularensis* MEP synthase, we set out to evaluate the protein sensitivity to fosmidomycin. Half maximal activity (IC$_{50}$) was observed at 247 nM, similar to that reported for the *Mycobacterium* homolog (Table 1). The $K_i$ for fosmidomycin (99 nM), obtained from a plot of $K_M^{app, DXP}$ as a function of inhibitor concentration (Fig. 6), is greater than that reported for *E. coli* and *Synechocystis* sp. PCC6803 (Table 1). This may reflect structural differences in the MEP synthase homologs, although this remains to be determined.

*F. tularensis* MEP synthase in a high-throughput assay

One method of identifying lead molecules in the drug development process involves the screening of large molecular libraries for inhibitors of an assay. A crucial issue for reliable high-throughput screening is the quality and robustness of the assay, often described in terms of the Z-factor [6]. An assay with a Z-factor score between 0.5 and 1.0 is considered excellent for high-throughput screening. To determine the Z-factor for the assay using *F. tularensis* MEP synthase, we adjusted the assay volume to accommodate a 96-well plate, fixed the substrate concentration at the $K_M$ (104 μM), used a saturating concentration of NADPH (150 μM), and evaluated three separate lots of purified MEP synthase in a series of assays performed over three consecutive days. Fosmidomycin was used as a positive control for inhibition. The Z-factor with *F. tularensis* MEP synthase was found to be 0.8, indicative of an assay (and enzyme) well suited for use in a high-throughput screen.

![Figure 5. The divalent cation specificity of *F. tularensis* MEP synthase.](image-url) Enzyme assays were performed with several different divalent cations at a fixed DXP (150 μM) and NADPH (100 μM) concentration. Relative enzyme activity reveals the preference of the enzyme for Mg$^{2+}$. doi:10.1371/journal.pone.0008288.g005

![Figure 6. Graphical determination of the inhibition constant.](image-url) Because fosmidomycin is a slow, tight binding inhibitor [5], the enzyme was preincubated with fosmidomycin for 10 minutes prior to addition of substrate. The absolute value of the x intercept of the line produced from linear regression fitting the plot of $K_M^{app, DXP}$ as a function of fosmidomycin concentration defined the $K_i$ as 98.9 +/- 4.5 nM. The $R^2$ value is indicated. doi:10.1371/journal.pone.0008288.g006
Phosphorylation of *F. tularensis* MEP synthase

The first committed and principle regulatory step in the MVA pathway is catalyzed by HMG-CoA reductase (reviewed in [17]). Multifaceted control mechanisms regulate HMG-CoA reductase activity, including the modulation of enzyme concentration, the modulation of membrane composition/fluidity, and the regulation of enzyme activity via reversible phosphorylation (specifically, enzyme inhibition by phosphorylation of a serine residue in the catalytic domain [18]). In comparison to the MVA pathway, much less is known about the regulatory mechanisms that control metabolic flux through the MEP pathway. Engineered alterations of MEP pathway gene expression suggest that several MEP pathway enzymes may share control over metabolic flux (reviewed in [19]). The role of posttranslational modification, such as reversible phosphorylation, remains unknown. Thus, we sought to evaluate if a phosphorylation site on *F. tularensis* MEP synthase could be identified.

Recombinant *F. tularensis* MEP synthase was purified from *E. coli* (induced with 10 μM IPTG), subjected to trypsinization, and phosphopeptides were isolated and identified via titanium dioxide chromatography-tandem mass spectrometry. Phosphoserine 177 (equivalent to Ser186 in the *E. coli* enzyme) was identified (Fig. 7).

Figure 7. Predicted tertiary structure of *F. tularensis* MEP synthase, homology-modeled using SWISS-MODEL. A crystal structure of *F. tularensis* MEP synthase has not been reported. To permit the visualization of phosphoserine177 within the context of the tertiary structure, the *F. tularensis* MEP synthase was modeled based upon the resolved structure of the *E. coli* homolog[48] (48% identity, 66% homology). A cartoon representation of the model is shown, with alpha helices colored red, beta sheets colored yellow, and coiled regions colored gray. The beta sheets comprise the dimer interface in the *E. coli* structure. The quality of the model was evaluated with ProQRes (Fig. S3) which provides scores ranging from 0 (unreliable) to 1 (reliable). Regions of the model scoring <0.5 are colored light blue. Primary sequence alignment and the structure of *E. coli* MEP synthase were used to identify residues comprising the substrate binding site (colored dark blue with backbone and sidechain residues shown). Serine177 (colored green with backbone and sidechain residues shown) is equivalent to *E. coli* Ser186, which contributes to the substrate binding site and has been shown to participate in conformational changes that occur upon substrate binding [13]. Two tryptophan residues present in the *F. tularensis* MEP synthase model are colored pink.

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synthase, a blue shift in the intrinsic fluorescence maximum of S177D suggests changes in the protein globular fold are brought about by the introduction of the Asp residue (Fig. 8A). The slight red shift in fluorescence emission spectrum of S177E is also indicative of a conformational change, although the change in S177E suggests exposure of a tryptophan residue to a hydrophilic environment whereas the blue shift observed with S177D is indicative of a conformational change sequestering tryptophan residues into a hydrophobic environment. The increased quantum yield observed with both S177D and S177E is also indicative of a structural change in MEP synthase.

Conclusions
In conclusion, we have shown that \textit{F. tularensis} MEP synthase is a valid target for the development of novel therapeutics. Inhibition of MEP synthase is sufficient to inhibit the growth of \textit{F. tularensis} \textit{in vitro}. Purified MEP synthase is kinetically active and readily lends itself to use in high-throughput screens. Furthermore, our investigation is the first to show that metabolic flux through the \textit{F. tularensis} MEP pathway may be regulated by phosphorylation of MEP synthase, similar to the regulatory control observed in the MVA pathway.

Supporting Information

Figure S1 Growth curves of \textit{F. tularensis} subs. novicida in minimal media supplemented with the indicated concentration of fosmidomycin (FoS). All conditions were evaluated in duplicate.

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