

Expression and Characterization of Soluble 4-Diphosphocytidyl-2-C-methyl-D-erythritol Kinase from Bacterial Pathogens

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SUMMARY

Many bacterial pathogens utilize the 2-C-methyl-D-erythritol 4-phosphate pathway for biosynthesizing isoprenoid precursors, a pathway that is vital for bacterial survival and absent from human cells, providing a potential source of drug targets. However, the characterization of 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) kinase (IspE) has been hindered due to a lack of enantiopure CDP-ME and difficulty in obtaining pure IspE. Here, enantiopure CDP-ME was chemically synthesized and recombinant IspE from bacterial pathogens were purified and characterized. Although gene disruption was not possible in *Mycobacterium tuberculosis*, IspE is essential in *Mycobacterium smegmatis*. The biochemical and kinetic characteristics of IspE provide the basis for development of a high throughput screen and structural characterization.

INTRODUCTION

Isoprenoids are one of the largest groups of natural products, including 35,000 primary and secondary metabolites (Chang and Keasling, 2006; Hunter, 2007). Despite functional and structural diversity, all isoprenoids are derived from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), produced by the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in most bacterial pathogens, including *Mycobacterium tuberculosis* (tuberculosis), *Burkholderia mallei* (glanders), *Salmonella enterica* serovar *typhimurium* (*S. Typhi*; typhoid fever), and *Vibrio cholerae* (cholera). The early steps in the biosynthesis of bacterial isoprenoids have been demonstrated to be essential in both Gram-positive and Gram-negative bacteria (Brown and Parish, 2008; Buetow et al., 2007; Cornish et al., 2006; Eoh et al., 2007; Freiberg et al., 2001; Sauret-Gueto et al., 2003) and the MEP pathway is not present in mammalian cells. Thus, the MEP pathway is considered to be a good source of potential drug targets (Rohmer, 1998; Eoh et al., 2009; Testa and Brown, 2003).

The fourth enzyme in the MEP pathway (4-diphosphocytidyl-2-C-methyl-D-erythritol [CDP-ME] kinase, IspE, EC2.7.1.148) was first identified in *Escherichia coli* (Luttgen et al., 2000) and tomato (Rohdich et al., 2000). IspE catalyzes the conversion of CDP-ME to 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate (CDP-ME2P) in an ATP-dependent manner (Figure 1) (Eoh et al., 2009; Luttgen et al., 2000). Even though sequence alignments and crystal structures (Miallau et al., 2003) showed that bacterial IspE has a high level of similarity to the ATP-dependent GHMP kinase superfamily (Andreassi and Leyh, 2004; Romanowski et al., 2002; Yang et al., 2002; Zhou et al., 2000), which includes galactose kinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase, significant differences in the catalytic and substrate binding sites were observed, suggesting that bacterial IspE may be a potential drug target (Miallau et al., 2003; Eoh et al., 2009; Sgraja et al., 2008). However, characterization of IspE from bacteria pathogenic to humans has been hampered due to the lack of a source of enantiopure CDP-ME and difficulties encountered in purification of IspE from pathogenic species (Sgraja et al., 2008). Although *in silico* studies indicate that the putative *M. tuberculosis* IspE (Rv1011) has no predicted transmembrane domains and is cytosolic, previous attempts to purify the native form of IspE from *M. tuberculosis* failed (Sgraja et al., 2008). Purification of recombinant *M. tuberculosis* IspE was made possible by generating genetically truncated proteins. Of eight truncated versions of *M. tuberculosis* IspE generated, one showed kinetic properties similar to those obtained using native cytosolic protein isolated from wild-type *M. tuberculosis* H₃₇Rv. The truncation strategy also allowed purification of recombinant IspE enzymes from *B. mallei*, *S. Typhi*, and *V. cholerae*. In the present study, we provide direct evidence of IspE essentiality in *Mycobacterium smegmatis* using allelic disruption (Jackson et al., 2000; Pan et al., 2001). In addition, enantiopure CDP-ME was chemically synthesized, improving the methods we previously reported for chemo-enzymatic synthesis (Narayanasamy et al., 2008), and recombinant IspE from pathogens with potential to be utilized as agents of bioterrorism (Clarke, 2005; Pappas et al., 2006) (*M. tuberculosis*, *B. mallei*, *S. Typhi*, and *V. cholerae*) was purified.

The elucidation of the biochemical and kinetic properties of IspE from human pathogens provides the groundwork for

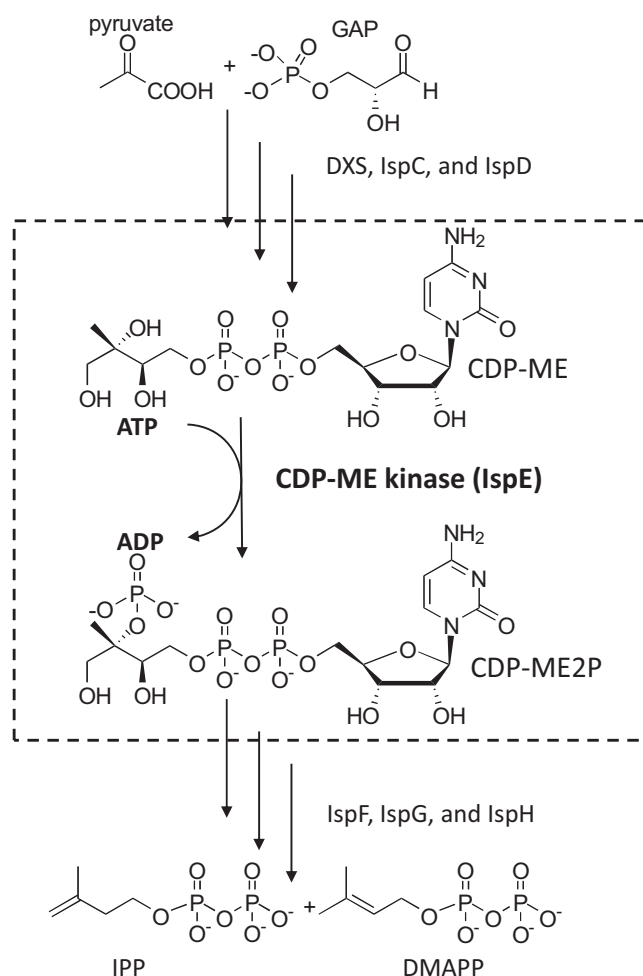


Figure 1. The MEP Pathway and the Reaction Catalyzed by IspE

Starting with pyruvate and glyceraldehyde 3-phosphate (GAP), the sequential activities of DXS, IspC, and IspD produce CDP-ME, the substrate of IspE. IspE phosphorylates the tertiary hydroxyl group of CDP-ME forming CDP-ME2P. The reaction catalyzed by IspE is indicated by the dotted box. Abbreviations; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; IspC, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; IspD, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase; IspE, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; IspF, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; IspG, 1-hydroxyl-2-methyl-2(E)-butenyl 4-diphosphate synthase; IspH, 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate reductase; CDP-ME, 4-diphosphocytidyl-2-C-methyl-D-erythritol; CDP-ME2P, CDP-ME 2 phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate.

development of high-throughput screening (HTS) assays and, potentially, identification of novel antimicrobials.

RESULTS

Synthesis of Enantiopure CDP-ME

Although the synthetic scheme of **3** from **1** is identical to a previous report (Narayanasamy et al., 2008, Figure 2), several steps for synthesizing enantiopure MEP were improved over that report. At first, the tertiary hydroxyl group **3** was protected by benzyl bromide, after treating **3** with KH to yield 90% of **4**.

In this step, KH was found to be faster and better than NaH (Narayanasamy et al., 2008). The aldehyde **5** was reduced with sodium borohydride forming benzyl-protected MEP precursor **6**, which after hydrogenolysis with Pd/C in water/methanol medium and without acid workup led to enantiopure MEP, which was characterized by $^1\text{H-NMR}$, mass spectrometry (MS), and optical rotation. The optical rotations of **3**, **4**, and **6** are newly identified as +33.0, +13.0, and +10.0, respectively (Experimental Procedures and Supplemental Data). Freshly prepared MEP was coupled with cytidine 5'-monophosphate (CMP), which was titrated with triethyl amine leading to the formation of corresponding triethylammonium CMP. The phosphoester moiety was activated by trifluoroacetic anhydride followed by conversion to phosphoramidate by treatment with methylimidazole and then coupled with the tributylammonium salt of MEP. The crude material was purified to obtain CDP-ME. Enantiopure intermediates and CDP-ME were confirmed by $^1\text{H-NMR}$ and high-resolution MS (Experimental Procedures and Supplemental Data and Figure S2). The methyl group at the 2 position of MEP moiety in CDP-ME was observed as a singlet at 1.10 ppm and the cytidine terminus was observed as a doublet at both 7.94 and 6.09 ppm (Rohdich et al., 2000; Cassera et al., 2004; Richard et al., 2001). Optical rotation of the enantiopure CDP-ME was found to be +13.7 in water. Thus enantiopure MEP was synthesized and the coupling reaction of MEP with CMP was satisfactory, yielding enantiopure CDP-ME.

Determination of Mycobacterial IspE Essentiality Using *M. smegmatis*

An allelic disruption strategy was employed to show *M. smegmatis* MSMEG_5436 essentiality as previously described (Jackson et al., 2000). The pPR27::SM *ispE*::Kan^R plasmid has a temperature-sensitive mycobacterial origin of replication that facilitates recombination generating a single homologous-recombination event at the *M. smegmatis* MSMEG_5436 locus. The plasmid also harbors the *sacB* counter selectable marker (Pelicic et al., 1997) and the *xylE* marker (Curcic et al., 1994). Plasmid pPR27::SM *ispE*::Kan^R was electroporated into *M. smegmatis* mc²155, and transformants were selected on LB plates containing 30 $\mu\text{g/ml}$ kanamycin (Kan) at 32°C. One transformant was then propagated in LB broth containing 30 $\mu\text{g/ml}$ Kan at 32°C and plated onto LB plates containing 30 $\mu\text{g/ml}$ Kan at 42°C. The temperature-sensitive origin containing pPR27::SM *ispE*::Kan^R is not able to replicate at 42°C and the Kan-resistant colonies have necessarily integrated part or all of the plasmid into *M. smegmatis* mc²155 chromosome, yielding single-crossover strains. The resulting colonies were analyzed for their XylE phenotype with catechol (Pan et al., 2001) and yellow colonies were further used. Double-crossover events were generated by plating a total of 30 yellow, single-crossover strains onto LB plates containing 30 $\mu\text{g/ml}$ Kan and sucrose at 42°C, leading to the disruption of the *M. smegmatis* MSMEG_5436 gene; no colonies were observed, suggesting that *M. smegmatis* MSMEG_5436 is essential for *M. smegmatis* normal growth. To confirm the essentiality of the gene, a rescue plasmid (pCG76::SM *ispE*) was prepared and electroporated into the single-crossover strains prior to generation of the double crossover. The single-crossover strains containing pCG76::SM *ispE* were grown in LB broth containing 30 $\mu\text{g/ml}$ Kan at 32°C and

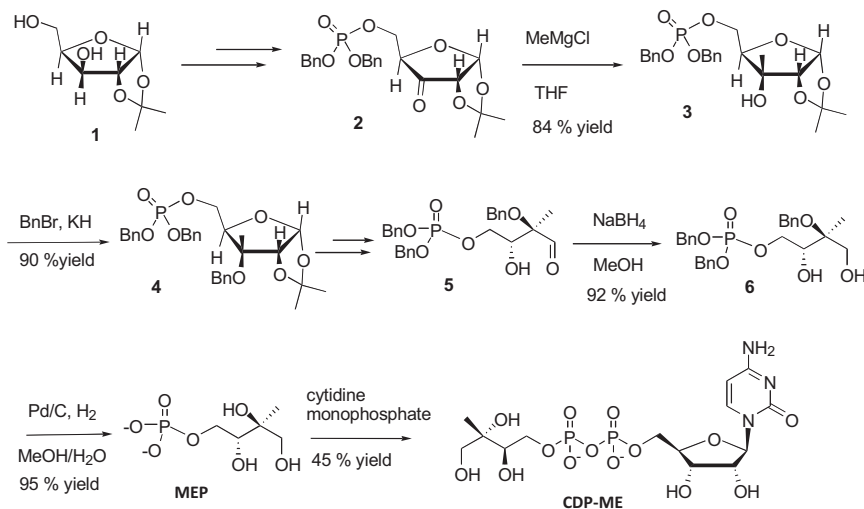


Figure 2. Synthetic Scheme of Enantiopure CDP-ME

Enantiopure 2-C-methyl-D-erythritol 4-phosphate (MEP) was synthesized from commercially available 1,2-O-isopropylidene- α -D-xylofuranose **1**. Subsequently, MEP is coupled with CMP to give a 45% yield of pure CDP-ME.

then plated onto LB plates containing 30 μ g/ml Kan and sucrose at 32°C. The resulting colonies were analyzed for their XylE phenotype with catechol (Pan et al., 2001), and a total of 30 white colonies were obtained as the double-crossover strains. The double-crossover strains were confirmed by polymerase chain reaction (PCR) by using primer sets (MutSmispE-F and R) (Table S3; data not shown). As a final experiment to confirm that *M. smegmatis* MSMEG_5436 is essential for growth, *M. smegmatis* double-crossover strains containing pCG76::SM *ispE* were shown to be unable to grow at 42°C (Figure 3B), due to loss of the plasmid and its insert. On the other hand, *M. smegmatis* double-crossover strains containing pCG76::SM *ispE* were shown to be able to grow at 32°C and *M. smegmatis* wild-type strains grew normally at both 32°C and 42°C. The similar results were observed on the LB plates containing 30 μ g/ml Kan (Figure 3C).

Identification of Putative IspE from *M. tuberculosis*, *B. mallei*, *S. Typhi*, and *V. cholerae*

The putative *M. tuberculosis*, *B. mallei*, *S. Typhi*, and *V. cholerae* IspE proteins have 22%, 44%, 90%, and 59% identity with *E. coli* IspE (Luttgen et al., 2000), respectively. All reported crucial domains, including the glycine-rich domains and substrate binding motifs of the *E. coli* ortholog (Luttgen et al., 2000; Miallau et al., 2003), are well conserved in all IspE orthologs (Figure 4A).

Purification of Active and Soluble Recombinant IspE

Although *M. tuberculosis* IspE is predicted to be cytosolic, purification of soluble, active IspE in native form proved difficult. To facilitate the purification of reasonable quantities of *M. tuberculosis* IspE, eight truncated versions of IspE (Rv1011 I through Rv1011 VIII) were cloned, expressed, and purified (Figure 4B). *M. tuberculosis* IspE contains two predicted functional motifs; a homoserine kinase (ThrB) motif and a GHMP kinase motif (Figure 4B); thus, sequences of the genetic constructs were based on the motif distribution (for example, Rv1011 IV corresponds to the ThrB motif and Rv1011 VIII, the smallest fragment, corresponds to the GHMP kinase motif). The solubility of all of the

expressed proteins was dramatically improved compared with that of intact Rv1011, giving yields of \sim 1 mg purified protein per liter of culture. Of the expressed proteins, only Rv1011 I (Δ 301-305), Rv1011 II (Δ 268-365), and Rv1011 IV showed IspE activity; the other 5 Rv1011 fragments were inactive under the conditions tested. Rv1011 I showed the greatest catalytic activity (Figure 4C).

Rv1011 IV, containing only the ThrB and GHMP kinase domains, showed very weak IspE activity and the activity of Rv1011 II, missing C terminus of ThrB motif was intermediate. Therefore, recombinant Rv1011 I was used for further characterization.

The three IspE orthologs from *B. mallei*, *S. Typhi*, and *V. cholerae* are also predicted to be cytosolic and the native forms also proved difficult to express as soluble, active protein; however, as for the *M. tuberculosis* IspE, enzymes from *B. mallei*, *S. Typhi*, and *V. cholerae* could be expressed and purified after truncation of the C-terminal five amino acids, again resulting in yields of \sim 1 mg purified protein per liter of culture. Three truncated versions of bacterial IspE enzymes of *B. mallei*, *S. Typhi*, and *V. cholerae* were designated mBME, mSTE, and mVCE. The induction and expression of Rv1011 I, mBME, mSTE and mVCE was confirmed by western blot analysis with the expected molecular weights of 30.8, 31.4, 30.2, and 31.3 kDa, respectively (Figure S4).

In Vitro Reaction Requirements of IspE Orthologs

The activities of purified Rv1011 I, mBME, mSTE, and mVCE used in this study were linear with increasing protein up to at least 194.8, 190.9, 198.5, 191.6 pmol, respectively, and all reactions were linear with time up to 30 min. In order to confirm the nature of the product, assay the mixtures were incubated, lyophilized, dissolved in 100 mM aq. ammonium bicarbonate and subjected to size exclusion chromatography on Bio-Gel® P-2 gel fine columns eluted with 100 mM aq. ammonium bicarbonate; fractions containing putative product were then subjected to further chromatography on benzyl DEAE cellulose anion exchange columns, eluted using a gradient of 0- 0.5 M aq. ammonium bicarbonate, and subsequent analysis by MS revealed a peak at 633 m/z corresponding to $[M+2NH_4]^+$.

The optimal pH and divalent cation concentrations for all four IspE orthologs were determined. Rv1011 I was active over a broad pH range (6.0 to 9.0), with optimal activity at pH 7.0 (Figure 5A). The pH optima for mBME, mSTE, and mVCE were similar to that of Rv1011 I, although the pH range at which Rv1011 I was active was somewhat narrower than those of the other three IspE orthologs (Figure S5). Optimal activity for

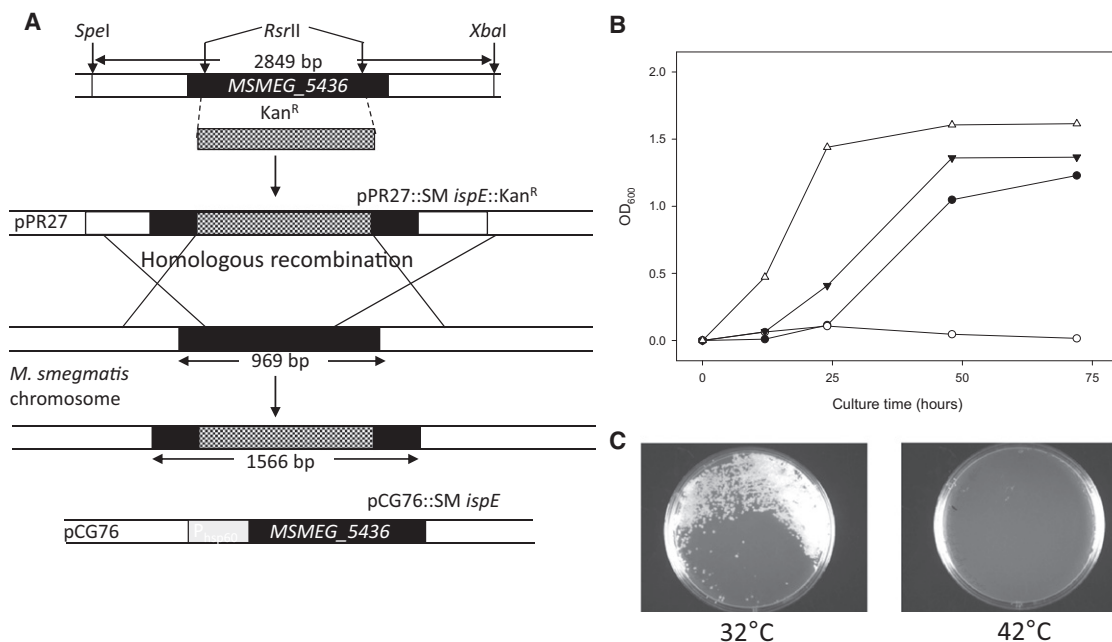


Figure 3. Essentiality of *M. smegmatis* IspE for the Bacterial Growth

(A) Schematic diagram of showing recombination in *M. smegmatis* mc²¹⁵⁵. Black boxes indicate coding sequence of IspE (*MSMEG_5436*). Hatched regions represent the intragenic DNA fragment replaced with a Kanamycin (Kan^R) resistant cassette (Kan^R) from pUC4K. For generation of *MSMEG_5436* disrupted Kan^R construct, Kan^R was cloned into *RsrII* sites of *MSMEG_5436* and ligated with pPR27 yielding pPR27::Sm *ispE*::Kan^R. After homologous recombination, selection for Kan resistance, sucrose resistance and *xylE* phenotype resulted in colonies with a disrupted *MSMEG_5436*, only in the presence of rescue vector pCG76::Sm *ispE*, which contains temperature sensitive origin of replication and heat shock protein 60 promoter (P_{hsp60}) shown in light grey.

(B) Growth curves of *M. smegmatis* mc²¹⁵⁵ strains at 32 and 42°C. *M. smegmatis* mc²¹⁵⁵ containing a rescue plasmid pCG76::Sm *ispE* at 32°C (solid circles) or at 42°C (open circles), *M. smegmatis* mc²¹⁵⁵ wild-type at 32°C (solid triangles) or at 42°C (open triangles). All cultures were grown in LB broth containing appropriate antibiotics (See Experimental Procedures).

(C) Growth patterns of *M. smegmatis* mc²¹⁵⁵ double-crossover strains containing pCG76::Sm *ispE* at 32°C (left) and 42°C (right) on LB plates with appropriate antibiotics.

mBME, mSTE, and mVCE was observed at pH 8.0, 8.0, and 7.5, respectively.

The activity of Rv1011 I is absolutely dependent on the presence of divalent cations (Figure 5B), the addition of 5 mM EDTA to the reaction mixture completely abolished the activity. The activity of Rv1011 I was optimal in the presence of ~6 mM Mg²⁺. Other divalent cations including Mn²⁺, Ca²⁺, or Zn²⁺ supported little or no synthesis of CDP-ME2P at any concentration tested (Figure 5B). Similar results were observed when mBME, mSTE, and mVCE were tested (Figure S3).

Kinetic Properties of the IspE Orthologs

Reaction mixtures containing enantiopure CDP-ME, MgCl₂, [γ -³²P]ATP, and IspE generated a single radioactive product with an R_f value of 0.13 was subjected to thin-layer chromatography (TLC) analysis (Figure 6A); this product corresponds to CDP-ME2P (Kishida et al., 2003; Testa et al., 2006). The apparent K_m^{CDP-ME} and K_m^{ATP} for wild-type *M. tuberculosis* H₃₇Rv (in a crude cytosolic preparation) were calculated to be 206.7 ± 12.5 μM and 20.7 ± 1.2 μM, respectively. The K_m^{CDP-ME} and K_m^{ATP} for purified Rv1011 I (97.2 pmol) were calculated to be 327.8 ± 21.4 μM and 75.2 ± 0.9 μM, respectively (Table 1). The K_m^{CDP-ME} values of mBME, mSTE, and mVCE were 31.3 ± 1.5, 25.4 ± 0.8, and 52.9 ± 11.7 μM, respectively, in the presence of 100 μM ATP. The K_m^{ATP} values of mBME, mSTE, and mVCE

were 76.6 ± 1.1, 9.1 ± 0.2, and 8.8 ± 1.3 μM, respectively, in the presence of 100 μM CDP-ME. The calculated k_{cat} and k_{cat}/K_m values are summarized in Table 1.

Determination of Z'-Factor Values

The kinetic data determined above were used to develop a fluorescence-based in vitro assay that is amenable to HTS. In this assay, the release of ATP is coupled to pyruvate kinase, pyruvate oxidase, and horseradish peroxidase, to produce a fluorescent dye (resorufin) that can be measured spectrophotometrically and is proportional to IspE catalytic activity. The Z'-Factor values for the spectrophotometry based in vitro assay were calculated based on 96 negative controls (without enzyme) and 96 positive controls (with enzyme) in microplates using the equation:

$$Z'\text{-Factor value} = 1 - \frac{(3\sigma_{\text{positive}} - 3\sigma_{\text{negative}})}{|\mu_{\text{positive}} - \mu_{\text{negative}}|}$$

where σ represents the standard deviation and μ is the mean of each set of data points (Zhang et al., 1999). The Z'-Factor value for the 50 μl assays using Rv1011 I was calculated to be 0.82.

Identification of an Unknown Product

Interestingly, a product other than CDP-ME2P was produced, apparently as the sole product, when the reaction mixtures

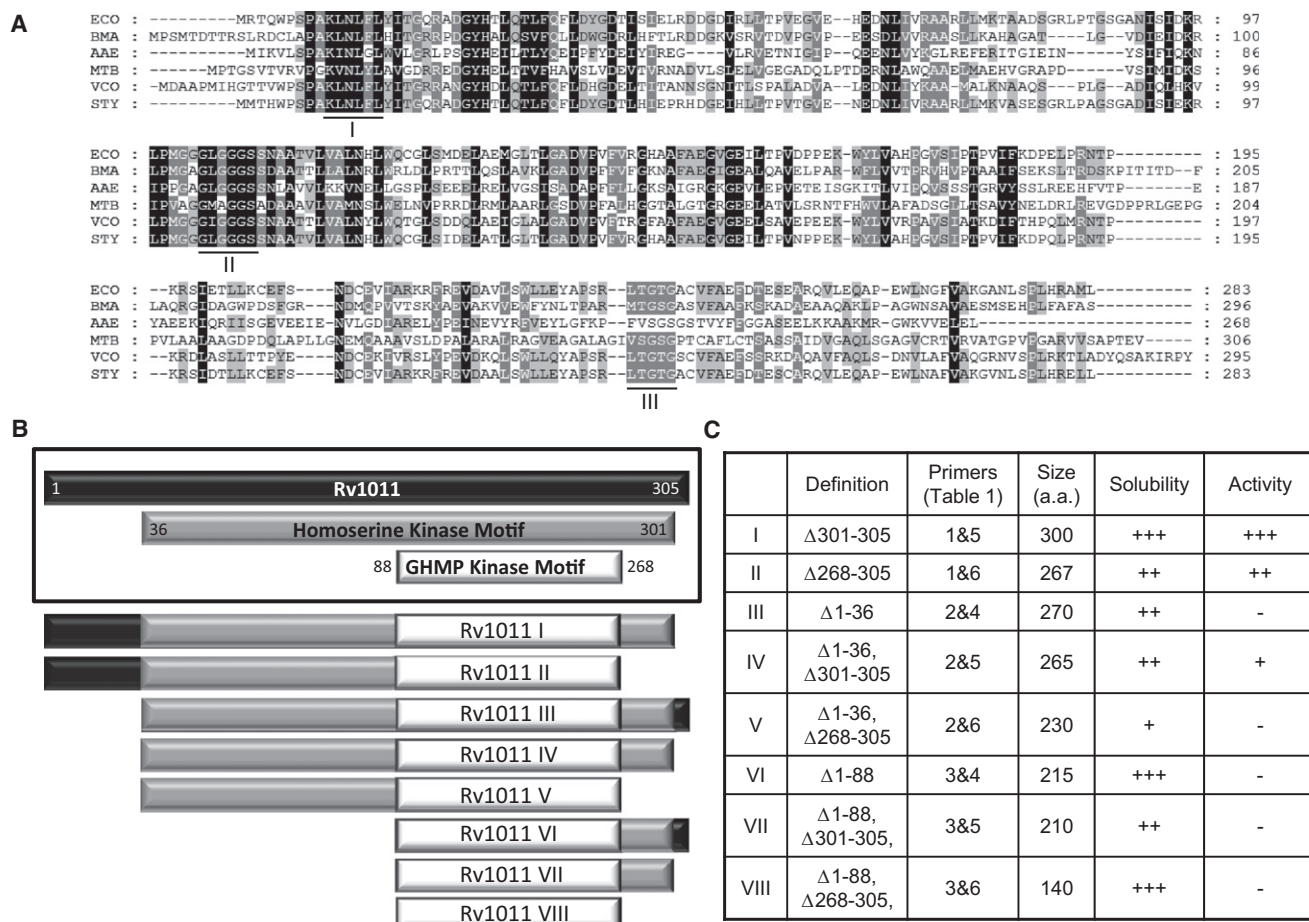


Figure 4. Identification of *M. tuberculosis* IspE and Purification Strategy

(A) Alignment of the amino acid sequence of *E. coli* IspE (ECO) and the putative IspE amino acids of *M. tuberculosis* (Rv1011) (MTB), *B. mallei* (BMA), *S. Typhi* (STY), *V. cholerae* (VCH), and previously characterized *Aquifex aeolicus* (Sgraja et al., 2008). Identities are highlighted in black and similarities in gray. The amino acids reported to be involved in CDP-ME binding (I) and ATP-binding (II + III) reported in *E. coli* IspE (Miallau et al., 2003) are underlined.

(B) Rv1011 contains two predicted kinase motifs, a homoserine (ThrB) kinase (amino acids [aa] 36–301) and a GHMP Kinase (aa 88–268)]. A series of eight fragments was engineered as indicated.

(C) Characteristics of the eight recombinant Rv1011 fragments. The primers used to amplify the fragments are listed in Supplemental Data S3. The solubility was determined by using SDS-PAGE analysis and the activities were determined using the radioisotope based in vitro IspE assay. – no; +, low; ++, intermediate; +++, good.

contained *M. tuberculosis* IspE or mSTE and Mn²⁺ or Zn²⁺ (Figure 6B). TLC analysis showed that the R_f value of the unknown product is 0.34 and the amount of product generated was proportional to the amount of Mn²⁺ or Zn²⁺ added to the reaction mixtures (data not shown). Comigration experiments of the unknown product with 2-C-methyl-D-erythritol 2,4-cyclophosphate (MECPP) showed that the R_f values of two products were similar (Figure 6B), and the unknown product did not comigrate with authentic 2-C-methyl-D-erythritol diphosphate. Thus, the data suggest that the new product could be MECPP. However, reaction mixtures containing cytosolic fractions of wild-type *M. tuberculosis* H₃₇Rv without addition of exogenous divalent cations produced only CDP-ME2P (Figure 6C), indicating *M. tuberculosis* IspE only produces CDP-ME2P under physiological conditions; therefore, the unidentified product was not further characterized.

DISCUSSION

M. tuberculosis IspD and IspF have previously been shown to be essential (Buetow et al., 2007; Eoh et al., 2007), indicating that the *M. tuberculosis* MEP pathway is vital for *M. tuberculosis* growth (Sassetti et al., 2003). Although *M. tuberculosis* IspE has been predicted to be essential (Sassetti et al., 2003), this has yet to be formally demonstrated. Attempts were made to demonstrate *ispE* essentiality in *M. tuberculosis* using previously reported methods (Eoh et al., 2007; Parish and Stoker, 2000). Of more than 140 single-crossover strains tested, none had chromosomal *ispE* deleted, suggesting *M. tuberculosis* *ispE* is essential for the bacillary survival. However, attempts to obtain a chromosomal deletion in the merodiploid background (complemented) were unsuccessful due to unidentified technical difficulties, possibly a lack of site specific recombination in the *ispE*

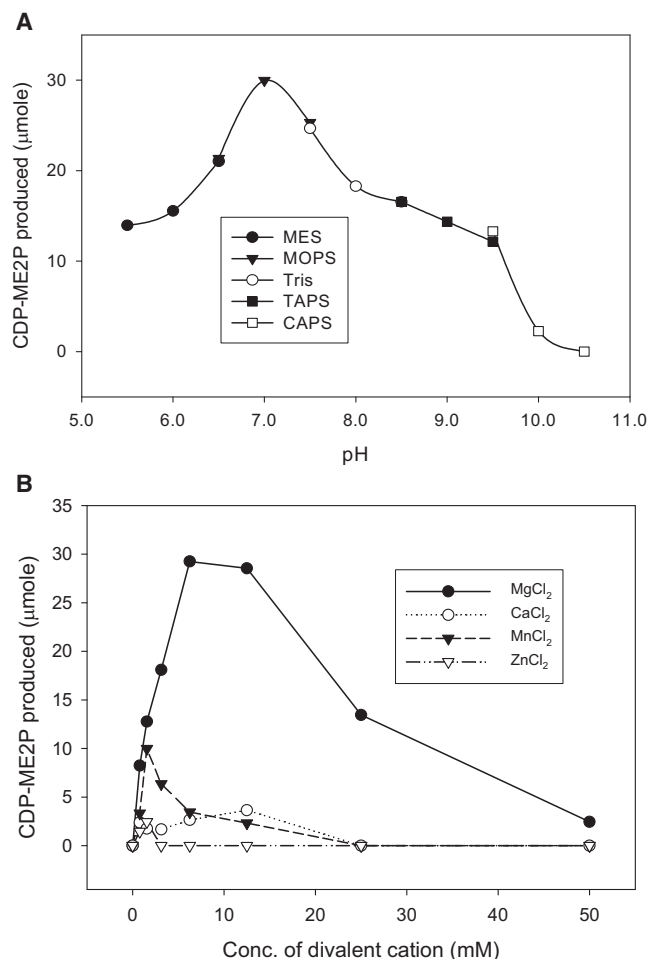


Figure 5. Biochemical Properties of *M. tuberculosis* IspE

(A) The optimal pH for catalytic activity was determined using MES (pH 5.5–7.0), MOPS (pH 7.0–7.5), Tris (pH 7.5–8.5), TAPS (pH 8.5–9.0), and CAPS (pH 9.0–10.5) using appropriate counter ions. (B) Divalent cations (Mg^{2+} , Mn^{2+} , Zn^{2+} , or Ca^{2+}) were added to the reaction mixtures at the indicated concentrations. The reaction mixtures were as described in *Experimental Procedures*.

locus. Therefore, *M. smegmatis* MSMEG_5436 (an *ispE* homolog) was utilized to confirm the essentiality of mycobacterial IspE by an allelic disruption method, in that species. These results strongly suggest that *ispE* is essential in *M. tuberculosis* as was predicted (Sassetti et al., 2003).

Although the protein is predicted to be cytosolic, purification of full-length native *M. tuberculosis* IspE was not possible under any conditions tested, as reported previously (Sgraja et al., 2008). Based on sequence alignments with orthologs from *E. coli* and *Aquifex aeolicus*, which can be expressed as soluble native enzymes, it is not clear why this should be the case; however, this was also true for native IspE from the other pathogens studied here including *B. mali*, *S. Typhi*, and *V. cholerae*. Truncation of the amino acids at N-terminal or C-terminal end of *M. tuberculosis* IspE improved the solubility and retained the activity of the expressed proteins. Notably, the truncated version of *M. tuberculosis* IspE, Rv1011 I (Figure 4B), obtained

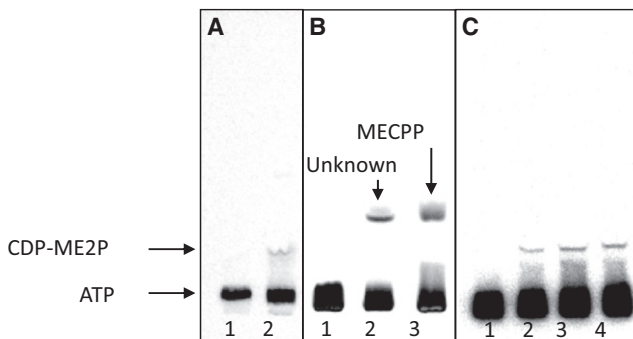


Figure 6. TLC Analysis of the Product Generated by Rv1011 I

(A) The reaction mixtures containing CDP-ME, $MgCl_2$, and $[\gamma\text{-}^{32}P]ATP$ without Rv1011 I (lane 1) and with Rv1011 I (lane 2).

(B) Comigration experiment with a reaction containing $[\gamma\text{-}^{32}P]ATP$, CDP-ME, and Rv1011 I in the presence of $MnCl_2$ (lane 2) and a reaction containing $[\gamma\text{-}^{32}P]ATP$, CDP-ME, Rv1011 I, and *M. tuberculosis* IspF (Eoh et al., 2009) in the presence of $MgCl_2$ (lane 3).

(C) Reaction mixtures containing CDP-ME and different amounts of wild-type *M. tuberculosis* IspE. No (lane 1), 20 μg (lane 2), 40 μg (lane 3), and 60 μg (lane 4) of *M. tuberculosis* IspE were used. The migration positions of ATP, CDP-ME2P, and MECPP are indicated by the arrows.

by removal of five amino acids (A-P-T-E-V) at the C terminus, showed levels of activity comparable to those of wild-type IspE found in *M. tuberculosis* cytosol.

Out of eight truncated versions of *M. tuberculosis* IspE generated in this study, only three (Rv1011 I, II, and IV) showed IspE activity, and only Rv1011 I and II, harboring intact ThrB, GHMP kinase domains and N termini (amino acids 1–36), showed significant IspE activities, suggesting that the three conserved motifs underlined in Figure 4A are critical for the activity. The first region (K-L-N-L-F-L, of *E. coli* IspE) is reported to be involved in CDP-ME binding (Miallau et al., 2003; Sgraja et al., 2008). The second region (G-L-G-G-G-S, of *E. coli* IspE) and third region (L-T-G-T-G, of *E. coli* IspE) constitute an ATP binding motif (Sgraja et al., 2008; Miallau et al., 2003). The N-terminal region (amino acids 1–36) of *M. tuberculosis* IspE is also reported to be involved in forming CDP-ME binding domain (Miallau et al., 2003). The five amino acids removed from *M. tuberculosis* IspE overhang the C terminus of *E. coli* IspE in multiple alignments (Figure 4A) and purified Rv1011 I showed IspE activity similar to wild-type IspE. Therefore, the five amino acids at the C terminus of *M. tuberculosis* IspE are relatively less critical for activity but appear to be a major determinant for solubility of heterologously expressed recombinant proteins. Soluble, active IspE enzymes from *B. mallei*, *S. Typhi*, and *V. cholerae* were also obtained by truncating five amino acids at the C terminus, even though these amino acids do not align with each other, are not conserved between the species and their removal does not result in generation of a conserved C terminus.

The pH and divalent cation usage required for optimal activities of the bacterial IspE orthologs tested here are somewhat different from those of previously reported for the *A. aeolicus* IspE (Sgraja et al., 2008). The activities of four IspE orthologs studied here were retained at acidic pH (5.5) and abolished at basic pH (>10.0). However, the activity of *A. aeolicus* IspE was very low at acidic pH (5.5–6.0) and retained at basic pH (9.0).

Table 1. Calculated Kinetic Parameters for Bacterial IspE Orthologs

Source of IspE	Substrates	K_m (μM)	V_{max} ($\mu\text{mol}/\text{min}$)	k_{cat} (S^{-1})	k_{cat}/K_m ($\text{S}^{-1}\mu\text{M}^{-1}$)
Wild type <i>M. tuberculosis</i> IspE	CDP-ME	206.7 ± 12.5	2.4	N.A.	N.A.
	ATP	20.7 ± 1.2	2.0	N.A.	N.A.
Rv1011 I ($\Delta 301-305$)	CDP-ME	327.8 ± 21.4	2.8	480.1	1.5
	ATP	75.2 ± 0.9	2.5	428.7	5.7
<i>B. mallei</i> IspE	CDP-ME	31.3 ± 1.5	2.7	469.8	15.0
	ATP	76.6 ± 1.1	2.4	417.6	5.5
<i>S. Typhi</i> IspE	CDP-ME	25.4 ± 0.8	2.6	454.0	17.9
	ATP	9.1 ± 0.2	2.1	366.7	40.2
<i>V. cholerae</i> IspE	CDP-ME	52.9 ± 11.7	2.9	487.0	9.2
	ATP	8.8 ± 1.3	2.1	352.7	40.1

The K_m and V_{max} values were calculated from averaged data from three independent experiments, using nonlinear regression analysis (SigmaPlot V.8.02A). In the reaction mixtures, 97.2 pmol Rv1011 I, 95.5 pmol *B. mallei* IspE, 99.2 pmol *S. Typhi* IspE, and 95.8 pmol *V. cholerae* IspE were used. N.A., not applicable.

In terms of divalent cation usage, Mg^{2+} at 6 mM supported optimal IspE activity and concentrations higher than 10 mM Mg^{2+} inhibited the IspE activity for all four species used here. Divalent cations other than Mg^{2+} used in this study supported significantly reduced IspE activity; Mn^{2+} allowed production of CDP-ME2P at low concentrations and Zn^{2+} did not support IspE activity (Figure 5B; Figure S5). Ca^{2+} supported mBME and mVCE activities at concentrations below 25 mM. However, Mn^{2+} supports *A. aeolicus* IspE activity at levels comparable to Mg^{2+} (Sgraja et al., 2008). It should be noted that the characterization of *A. aeolicus* IspE was performed coupled to pyruvate kinase and lactate dehydrogenase; therefore, the reported effects of pH and divalent cations on *A. aeolicus* IspE may be complicated.

The kinetic parameters obtained using Rv1011 I were comparable to those of wild-type IspE in *M. tuberculosis* cytosolic fraction (Table 1). The $K_m^{\text{CDP-ME}}$ value of Rv1011 I is similar to that reported for *E. coli* (150 μM) (Bernal et al., 2005) and *A. aeolicus* (121 μM) (Sgraja et al., 2008). The $K_m^{\text{CDP-ME}}$ values of mBME, mSTE, and mVCE are similar each other, but are lower than those reported for the IspE enzymes of *E. coli*, *A. aeolicus* and Rv1011 I. The K_m^{ATP} values for Rv1011 I and mBME are similar but somewhat higher than K_m^{ATP} values for mSTE and mVCE; however, all four values are lower than those previously reported for *E. coli* (420 μM) (Bernal et al., 2005) and *A. aeolicus* (222 μM) (Sgraja et al., 2008). Although the V_{max} and k_{cat} values of IspE enzymes from all four species studied here are similar, the k_{cat}/K_m values were somewhat different depending upon the origin of enzyme.

Rv1011 I and mSME produced an unknown compound in the presence of Mn^{2+} or Zn^{2+} , which has R_f value of 0.34 as determined by TLC analysis (Figure 6B). Previous reports indicate that MECPP, the product of IspF, has an R_f value of 0.34 on TLC in the same solvent system (Testa et al., 2006; Kishida et al., 2003). Comigration experiments showed that new compound has the same R_f value as MECPP (Figure 6B) although the compound was not rigorously characterized. It is interesting that IspE may be able to generate MECPP from CDP-ME without producing CDP-ME2P under specific in vitro conditions. One report predicted that CDP-ME2P is unstable and naturally hydro-

lyzed (Kishida et al., 2003); however, the amount of the new compound produced was proportional to the amount of added Mn^{2+} or Zn^{2+} in our hands. It should be kept in mind that production of this compound was not observed in reaction mixtures containing wild-type IspE in cytosol and endogenous divalent cations, suggesting that CDP-ME2P is the only product of *M. tuberculosis* IspE under physiological conditions and that the alternate activity is likely an artifact.

To date, characterization and crystallization of IspE from bacterial pathogens have been hampered by a lack of substrate and an inability to isolate a significant quantity of the enzyme. These studies identified a way to obtain the soluble and active IspE enzymes from bacterial pathogens. Moreover, we developed a fluorescence based IspE assay with an excellent Z' -factor value, suggesting that this coupled assay is sufficiently robust to screen for inhibitors. Thus, the results reported here will allow development of HTS and facilitate elucidation of crystal structures to aid in the identification of IspE specific inhibitors.

SIGNIFICANCE

An allelic disruption strategy was employed to confirm the prediction that mycobacterial IspE is essential for bacterial survival; in addition, this study provides first the characterization of IspE from pathogenic bacteria (*M. tuberculosis*, *B. mallei*, *S. Typhi*, and *V. cholera*). A radiochemical based assay was developed and utilized to directly identify the enzymatic properties of IspE orthologs from the bacterial pathogens. The fluorescence-based IspE assay provides a facile and inexpensive HTS assay to screen for IspE specific inhibitors. Because IspE is not targeted by any current antimicrobial drugs, identification of IspE inhibitors could contribute to disease control by providing leads to new drugs for patients with multiple drug-resistant or extensively drug-resistant tuberculosis or those infected with other pathogenic bacteria. This study lays the groundwork for structural-based studies of IspE from pathogenic bacteria and HTS.

EXPERIMENTAL PROCEDURES

Materials

Genomic DNA and a cytosolic fraction isolated from wild-type *M. tuberculosis* H₃₇Rv were provided by TB Vaccine Testing and Research Materials Contract, Colorado State University (NIH NIAID N01-AI-40091). *S. Typhi* (700931D) and *V. cholerae* (39315D) genomic DNA were purchased from the American Type Culture Collection (ATCC, Manassas, VA). *B. mallei* genomic DNA was kindly provided by Dr. Herbert Schweitzer (Colorado State University). All PCR reagents and cloning materials were purchased from QIAGEN (Valencia, CA). His-select nickel affinity resin, ATP, and reagents were obtained from Sigma-Aldrich (St. Louis, MO). [γ -³²P]ATP (6000 Ci/mmol) and PD-10 columns were purchased from Amersham Biosciences (Pittsburgh, PA). The ADP Quest HS Kinase Assay Kit was purchased from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). All other reagents and solvents were of at least analytical grade.

Synthesis of Enantiopure CDP-ME

Approximately 0.54 mmol tertiary alcohol **3** was dissolved in 10 ml freshly distilled dry tetrahydrofuran (THF) and cooled to 0°C. To the cooled solution, 1.08 mmol potassium hydride was added and stirred for 30 min at room temperature. The reaction mixture was cooled to 0°C again and 1.35 mmol benzyl bromide was added slowly, warmed to room temperature, and stirred for an additional 2 hr. The reaction was quenched by addition of saturated ammonium chloride solution and extracted with ethyl acetate. The crude mixture was purified by silica gel column chromatography using ethyl acetate: hexane (7:3, v/v) as the solvent, yielding 0.486 mmol benzylated product (90% yield) **4**.

Data for **3**: ¹H-NMR (CDCl₃, 300 MHz): δ 7.34 (m, 10H), 5.78 (d, 1H, J = 3.6 Hz), 5.02–5.09 (m, 4H), 4.03–4.26 (m, 3H), 3.96 (dd, 1H, J = 2.7 & 7.8 Hz), 1.56 (s, 3H), 1.36 (s, 3H), 1.12 (s, 3H). ¹³C-NMR (CDCl₃, 75 MHz): 135.7, 135.6, 128.6, 128.5, 128.4, 128.0, 127.9, 112.7, 103.4, 83.9, 80.2, 76.1, 69.4, 69.3, 65.9, 26.5, 18.1; IR (neat, cm⁻¹): 3588, 2966, 2362, 2336, 1652, 1614. HRMS (ESI) C₂₃H₃₀O₈P (M+H⁺): calculated 465.1673 and found 465.1680; [α]_D 33.0 (c 0.5, CHCl₃).

Data for **4**: ¹H-NMR (CDCl₃, 300 MHz): δ 7.34 (m, 15H), 5.80 (d, 1H, J = 3.6 Hz), 5.02–5.07 (m, 4H), 4.54–4.67 (m, 2H), 4.08–4.35 (m, 4H), 1.60 (s, 3H), 1.37 (s, 3H), 1.20 (s, 3H). HRMS (ESI) C₃₀H₃₆O₈P (M+H⁺): calculated 555.5758 and found 555.5762; [α]_D 13.0 (c 0.3, CHCl₃).

For MEP and CMP coupling, 0.15 mmol CMP was dissolved in 1 ml acetonitrile followed by addition of 0.6 mmol *N,N*, dimethyl aniline and 0.15 mmol triethylamine at 0°C. Then, 0.76 mmol trifluoroacetic anhydride in acetonitrile was slowly added to the mixture and stirred for 15 min. Excess TFA and anhydride was removed under reduced pressure. To the above reaction mixture, a combination of 0.45 mmol 1-methyl imidazole and 0.76 mmol triethylamine in acetonitrile was added slowly and stirred for 30 min. The activated CMP was added to the mixture of 0.125 mmol MEP and activated 4 Å molecular sieves in acetonitrile at 0°C and stirred for 2 hr. After the reaction was completed, the mixture was extracted with chloroform and the aqueous layer was lyophilized. CDP-ME was purified by sequential chromatography on a Bio-Gel® P-2 gel fine column using 100 mM aq. ammonium bicarbonate followed by further purification on a benzyl DEAE cellulose anion exchange column, using a gradient of 0 to 0.5 M aq. ammonium bicarbonate solution. This resulted in a 45% yield of CDP-ME.

Data for **CDP-ME**: ¹H-NMR (D₂O, 300 MHz): δ 7.94 (d, 1H, J = 7.5 Hz), 6.09 (d, 1H, J = 7.5 Hz), 5.96 (d, 1H, J = 4.2 Hz), 4.27 (m, 6H), 3.95 (m, 1H), 3.80 (d, 1H, J = 10.5 Hz), 3.57 (d, 1H, J = 11.7 Hz), 3.44 (d, 1H, J = 11.7 Hz), 1.10 (s, 3H). HRMS (ESI) C₁₄H₂₅O₁₄N₃P₂ (M-H⁻) calculated 520.0728 and found 520.0724; [α]_D 13.7 (c 0.2, H₂O).

Construction of Vectors for the Determination of Mycobacterial IspE Essentiality

Initially, attempts were made to demonstrate *ispE* essentiality in *M. tuberculosis* using previously reported methods (Eoh et al., 2007; Parish and Stoker, 2000); however, attempts to obtain a chromosomal deletion in the merodiploid background (complemented) were unsuccessful, due to unidentified technical difficulties. Thus, we utilized allelic disruption to demonstrate essentiality in the related species, *M. smegmatis*. The construction of *M. smegmatis* *ispE* ortho-

log (*MSMEG_5436*) replacement plasmid (pPR27::Sm *ispE*::Kan^R) and a rescue plasmid (pCG76-Sm *ispE*) is illustrated in Figure 3A. Briefly, a 2849 bp DNA fragment containing *M. smegmatis* *ispE* ortholog (*MSMEG_5436*), 940 bp upstream, and 940 bp downstream of the gene was amplified by PCR using the MutSmispE-F and R (Table S3). The DNA fragment was cloned to pGEM T plasmid and a 1250 bp Kan resistance cassette (Kan^R) was cut out with HincII from plasmid pUC4K, and inserted into the Klenow fragment (Invitrogen)-filled *RsrII* site of the *MSMEG_5436* gene. A 2849 bp SM *ispE*::Kan^R fragment was cut by *SpeI* and *XbaI* and moved to the same enzyme site of pPR27 (temperature-sensitive mycobacterial origin of replication); *sacB*, *xyIE*, gentamicin resistant (Pellicic et al., 1997), yielding pPR27::SM *ispE*::Kan^R, the vector used to achieve homologous recombination event at the *SMEG_5436* locus of *M. smegmatis*. The pPR27 used in this study has the mycobacterial temperature-sensitive origin of replication. Thus, pPR27::SM *ispE*::Kan^R can replicate at 32°C but is efficiently lost at 42°C and above (Pellicic et al., 1997; Pan et al., 2001). In addition, the plasmid also contains the counterselectable marker *sacB* from *Bacillus subtilis* (Pellicic et al., 1997) for use in selection of the double-crossover event in the presence of sucrose.

The entire *M. smegmatis* *MESG_5436* gene was amplified by PCR with using primer set (SmispE-F and R) (Table S3) and cut with *NdeI* and *BamHI* and ligated into the same enzyme sites downstream of the heat shock promoter P_{Hsp60} in plasmid pCG76 (*E. coli*/Mycobacterium shuttle vector carrying a temperature sensitive origin of replication, and streptomycin resistance), yielding pCG76::SM *ispE* (Figure 3A). Plasmid pCG76 carries the same temperature-sensitive mycobacterial replication origin as pPR27, and thus can replicate at the permissive temperature of 32°C but is abrogated at 42°C and above (Jackson et al., 2000).

PCR Amplification of Putative *ispE* Orthologs of *M. tuberculosis*, *B. mallei*, *S. Typhi*, and *V. cholerae*

Homologs of *E. coli* *IspE* from the genomes of bacterial pathogens were identified by sequence alignment. The *M. tuberculosis* H₃₇Rv genome is available on the Tuberculist website (<http://genolist.pasteur.fr/Tuberculist>) and other three bacterial genome sequences are available on the GenBank of National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/Genbank/>). The DNA sequences were used to design oligonucleotides for amplification of putative *ispE* open reading frames. The PCR primers (Table S3) were designed to contain *NdeI* or *HindIII* restriction enzyme sites in the forward and reverse primers, respectively.

Cloning of Eight *M. tuberculosis* *ispE* Fragments

Purification of the native form of the putative *M. tuberculosis* *IspE* (Rv1011) proved difficult as reported previously (Sgraja et al., 2008); however, soluble *M. tuberculosis* *IspE* was obtained by removal of amino acid residues at either the N terminus, C terminus, or both termini. Genetic modifications were designed using conserved domain searches for *M. tuberculosis* *IspE* as a query performed by tools available in the NCBI (specialized BLAST) (Figure 4B). The PCR products of eight *Rv1011* fragments were amplified using the primer sets shown in Table S3. For amplification of *Rv1011* fragments I through VIII (Figure 4B), primers were designed based on the sequences available in Tuberculist (Table S3) and were synthesized by Macromolecular Resources (Colorado State University). The eight amplified PCR products were digested with *NdeI* and *HindIII*, and ligated into the pET28a(+) vector (EMD Biosciences, Inc., San Diego, CA). The ligation mixtures were used to transform the *E. coli* DH5 α cloning host (Life Technologies, Rockville, MD), generating DH5 α [pET28a(+):*Rv1011* I] through DH5 α [pET28a(+):*Rv1011* VIII], which were subsequently purified using Gene Extraction Kits (QIAGEN, Valencia, CA) and sequenced by Macromolecular Resources.

Cloning of Truncated *ispE* Orthologs of *B. mallei*, *S. Typhi*, and *V. cholerae*

Forward and reverse primers for amplifying *ispE* of *B. mallei*, *S. Typhi*, and *V. cholerae* were designed to remove the five C-terminal amino acids (Table S3), resulting in generation of three truncated versions of bacterial *IspE* enzymes. Expression constructs, pET28a(+):*mBME*, pET28a(+):*mSTE*, and pET28a(+):*mVCE* were generated as described above.

Expression and Purification of the Recombinant Proteins

Recombinant Rv1011 I through VIII, mBME, mSTE, and mVCE were expressed and purified as previously described (Eoh et al., 2007; Mao et al., 2008). Briefly, transformation of expression *E. coli* host, BL21(DE3) (Novagen, Madison, WI), with expression plasmids afforded the recombinant strains, BL21(DE3)[pET28a(+):Rv1011 I], BL21(DE3)[pET28a(+):Rv1011 VIII], BL21(DE3)[pET28a(+):mBME], BL21(DE3)[pET28a(+):mSTE], and BL21(DE3)[pET28a(+):mVCE]. Cells were grown to an OD₆₀₀ of 0.6, treated with 0.5 mM isopropyl-β-D-thiogalactopyranoside at 20°C for 16 hr, harvested by centrifugation, resuspended in binding buffer [50 mM 4-morpholine propane sulfonic acid (MOPS) (pH 7.9), 1 mM MgCl₂, 10% glycerol, and 1 mM β-mercaptoethanol], and disrupted by sonication using a Sanyo Soniprep 150 (Integrated Services, TCP Inc, Palisades Park, NJ) on ice. The recombinant proteins carrying hexa-His tags were purified by immobilized metal affinity chromatography using a Ni-NTA resin (Sigma-Aldrich, St. Louis, MO) and analyzed by SDS-PAGE and western blot analysis with using an anti-His antibody (Sigma-Aldrich). Fractions containing pure recombinant protein were desalted using PD-10 columns and concentrated using a 5000 molecular weight cutoff Centricon (Millipore, Bedford, MA).

In Vitro Radiochemical IspE Assay

For enzymatic characterization, IspE assays were performed in reaction mixtures containing 50 mM Tris-Cl (pH 7.0), 100 μM [γ-³²P]ATP (100 dpm/pmol), 2 mM DTT, 100 μM CDP-ME, and 5 mM MgCl₂ in a 50 μl final reaction volume. Reactions were initiated by addition of recombinant IspE (97.2 pmol Rv1011 I, 95.5 pmol mBME, 99.2 pmol mSTE, and 95.8 pmol mVCE), incubated at 37°C for 30 min, and terminated by the addition of 10 mM of EDTA (pH 8.0). TLC analysis was performed by transferring 10 μl reaction mixture to a TLC plate (Polygam Sil N-HR, Macherey and Nagel) and developing with n-propanol/ethyl acetate/H₂O (6:1:3,v/v/v). Location and quantitation of radiolabeled products on the TLC plates was determined by using a Molecular Dynamics Typhoon 8600 Phosphorimager.

Fluorescence-Based IspE Assay

For HTS purposes, ADP released from the IspE catalyzed reaction was coupled to commercially available the ADP Quest HS Kinase Assay Kit containing pyruvate kinase, pyruvate oxidase, and horseradish peroxidase, to produce a fluorescent dye (resorufin). The reaction mixtures, containing 50 mM Tris-Cl (pH 7.0), 100 μM ATP, 100 μM CDP-ME, 5 mM phosphatase inhibitor (Roche Bioscience, Palo Alto, CA), 5 mM MgCl₂, and 97.2 pmol Rv1011 I in a 50 μl final reaction volume in 96 well black microplates with clear bottom (Costar, NY), were incubated at 37°C for 30 min. Then, 25 μl reagent A and 50 μl reagent B (ADP Quest HS Kinase Assay Kit) were added, sequentially. After incubating for another 15 min at room temperature, fluorescence was measured using a SynergyTM Multi-Detection Microplate Reader (BioTek instruments) at an excitation wavelength of 530 nm and emission wavelength of 590 nm.

Determination of Z'-Factor Value

Z'-factor values for fluorescence-based IspE assays were determined as was previously reported (Zhang et al., 1999). All steps of the assay were performed using an automated fluid transfer system (Precision XS microplate sample processor, BioTek instruments) in 96 well microplates. A total of 96 assays were performed for both positive (with enzyme) and negative controls (without enzyme).

Enzymatic Properties

The effect of ATP concentration on IspE activity was determined using a constant concentration of CDP-ME (100 μM) and varying concentrations of ATP. The effect of CDP-ME concentration was determined using a constant concentration of ATP (100 μM) and varying concentrations of CDP-ME. The *K_m* and *V_{max}* values of substrates for the enzyme were calculated using non-linear regression analysis (SigmaPlot V.8.02A). To determine the pH optima of Rv1011 and IspE orthologs, appropriate buffers (MES, MOPS, Tris, TAPS, or CAPS) were used. Optimal concentrations for divalent cations were determined in assay mixtures containing MgCl₂, CaCl₂, ZnCl₂, or MnCl₂ at the indicated concentrations.

Characterization of an Unknown Product Generated by *M. tuberculosis* IspE

The mobility of the unknown product and *M. tuberculosis* 2-C-methyl-D-erythritol 2,4 cyclodiphosphate (MECPP) on TLC plates was also compared. Authentic MECPP was obtained by enzymatic synthesis in reaction mixtures containing Rv1011 I, CDP-ME, [γ-³²P]ATP, and 112.6 pmol recombinant *M. tuberculosis* IspE (Rv3581c) (Eoh et al., 2009), which had been incubated at 37°C for 30 min.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at [http://www.cell.com/chemistry-biology/supplemental/S1074-5521\(09\)00409-8](http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00409-8).

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