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Cloning, Solubilization, and Characterization of Squalene Synthase from *Thermosynechococcus elongatus* BP-1[▽]

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Squalene synthase (SQS) is a bifunctional enzyme that catalyzes the condensation of two molecules of farnesyl diphosphate (FPP) to give presqualene diphosphate (PSPP) and the subsequent rearrangement of PSPP to squalene. These reactions constitute the first pathway-specific steps in hopane biosynthesis in *Bacteria* and sterol biosynthesis in *Eukarya*. The genes encoding SQS were isolated from the hopane-producing bacteria *Thermosynechococcus elongatus* BP-1, *Bradyrhizobium japonicum*, and *Zymomonas mobilis* and cloned into an *Escherichia coli* expression system. The expressed proteins with a His_6 tag were found exclusively in inclusion bodies when no additives were used in the buffer. After extensive optimization, soluble recombinant *T. elongatus* BP-1 SQS was obtained when cells were disrupted and purified in buffers containing glycerol. The recombinant *B. japonicum* and *Z. mobilis* SQSs could not be solubilized under any of the expression and purification conditions used. Purified *T. elongatus* His_6-SQS gave a single band at 42 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and molecular ion at *m*/*z* 41886 by electrospray mass spectrometry. Incubation with FPP and NADPH gave squalene as the sole product. Incubation of the enzyme with [^{14}C]FPP in the absence of NADPH gave PSPP. The enzyme requires Mg^{2+} for activity, has an optimum pH of 7.6, and is strongly stimulated by detergent. Under optimal conditions, the K_m of FPP is 0.97 ± 0.10 μM and the k_{cat} is 1.74 ± 0.04 s^{-1}. Zaragozic acid A, a potent inhibitor of mammalian, fungal, and *Saccharomyces cerevisiae* SQSs, also inhibited recombinant *T. elongatus* BP-1 SQS, with a 50% inhibitory concentration of 95.5 ± 13.6 nM.

Squalene synthase ([SQS] farnesyl diphosphate:farnesyl diphosphate transferase; EC 2.5.1.21) catalyzes the condensation of two molecules of farnesyl diphosphate (FPP) to form a c1′-2-3-linked triterpene intermediate, presqualene diphosphate (PSPP), and the subsequent NADPH-dependent rearrangement and reduction of PSPP to produce squalene (SQ), as outlined in Fig. 1 (36, 37). These transformations are the first pathway-specific reactions in the hopanoid biosynthetic pathway in *Bacteria* (7, 33, 42, 48) and the sterol pathway in *Eukarya* (37). Studies of yeast SQS show that substrate addition is ordered with two molecules of FPP added first, followed by NADPH (24, 39). When NADPH is present in the buffer, PSPP is converted directly to SQ without dissociating from the active site. Formation of PSPP or a prior conformational change in SQS is the rate-limiting step in the overall conversion of FPP to SQ. PSPP is formed and released when NADPH is absent. Under these conditions, SQS catalyzes a slow "solvolysis" of PSPP to give triterpene alcohols and hydrocarbons with irregular isoprenoid skeletons (14).

SQS has been cloned from a variety of eukaryotes, including fungal (15, 21, 57), protozoan (45), rat (22), mouse (12), human (41, 50), and plant sources (27). The eukaryotic enzyme is associated with membranes, where it appears to be anchored by a hydrophobic membrane-spanning α-helix at the C terminus of the protein (47, 49, 57). The poor solubility of SQS has hampered attempts to purify native soluble protein. Small quantities of soluble SQS from yeast microsomes were purified with deoxycholate and detergents (1, 18). A purified sample of membrane-free, C-terminally truncated, rat hepatic microsomal SQS was obtained by trypsin digestion (47). A modestly soluble recombinant version of SQS was obtained from *Saccharomyces cerevisiae* (21, 57), *Trypanosoma cruzi* (45), rats (22), and humans (52) by truncation of the coding region for the C-terminal α-helix.

Hopanoids are pentacyclic triterpene lipids found in many bacteria (42), including a number of nitrogen-fixing organisms, and several species of cyanobacteria (7, 33, 48). Hopanoids are localized in bacterial membranes, where they exert many of the same stabilizing effects as membrane sterols in eukaryotes (16, 31). In contrast to eukaryotic SQS, little is known about the bacterial enzyme. Predicted secondary structures from amino acid sequences for the bacterial enzyme do not show the C-terminal α-helix seen in the eukaryotic proteins. There are no reports of solubilization or purification of a native or recombinant bacterial SQS. We now describe construction of an *Escherichia coli* clone with the full-length gene for SQS (tll1096) from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 and the solubilization, purification, and characterization of the recombinant enzyme.

MATERIALS AND METHODS

Materials. Genomic DNA for *T. elongatus* BP-1 was provided by Satoshi Tabata (Kazusa DNA Research Institute, Japan) (25). *Bradyrhizobium japonicum* USDA 110 (NRRL B-4361) and *Zymomonas mobilis* ZM4 (ATCC 31821) strains were obtained from Agricultural Research Service Culture Collection at the U.S. Department of Agriculture and American Type Culture Collection, respectively (17, 46). Magnesium chloride, Tween 80, dithiothreitol (DTT), bovine serum albumin (BSA), SQ, NADPH, and zaragozic acid A were purchased from Sigma. EDTA-free protease inhibitor cocktail tablets were purchased from Roche Diagnostics. Ni-nitrilotriacetic acid (NTA) agarose resin was purchased from Qiagen. All restriction endonucleases and T4 DNA ligase were purchased

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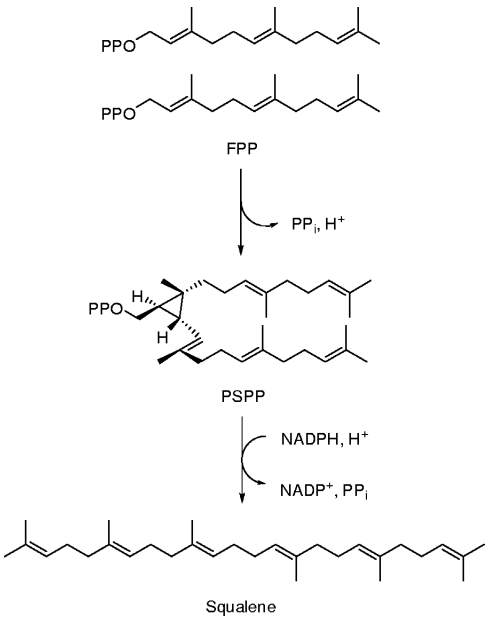

FIG. 1. Conversion of FPP to SQ.

from New England Biolabs. pGEM-T Easy vector and *Taq* DNA polymerase were purchased from Promega. Deoxynucleoside triphosphates, a BenchMark protein ladder, and an Easy-DNA Genomic DNA Isolation Kit were purchased from Invitrogen. Easy-A PCR cloning enzyme, *Pfu* Ultra DNA polymerase, and XL1-Blue competent cells were purchased from Stratagene. pET28b(+) vector, BL21(DE3), and Rosetta (DE3) competent cells were purchased from Novagen. Microcon and Centriprep centrifugal filter devices were purchased from Millipore. Attempts to refold SQS after solubilization of inclusion bodies were conducted with a Spin-column Membrane Protein Folding Screen Kit from ProFoldin Protein Folding Service. Recombinant *E. coli* FPP synthase was provided by Mo Chen (unpublished data). Geranyl diphosphate (GPP) and FPP were synthesized by the procedure of Davisson et al. (8). [1-^{14}C]isopentenyl diphosphate (IPP) and [1-^{3}H]FPP were purchased from GE Healthcare.

General methods. Mini-preparations of plasmid DNA for restriction analysis were obtained by using a Qiagen plasmid mini-prep kit. Genomic DNA was prepared from *B. japonicum* and *Z. mobilis* cells using an Easy-DNA Genomic DNA Isolation Kit. DNA fragments were purified by agarose gel electrophoresis using a GFX PCR DNA and gel purification kit from GE Healthcare. Restriction digestion, ligation, and transformation of competent cells were conducted as described by Sambrook et al. (44). PCR was performed using a PTC-200 Petier Thermal Cycler from MJ Research. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the discontinuous buffer system of Laemmli (19). Gels were stained with Gelcode blue stain reagent from Pierce. Protein concentrations were determined with a Coomassie plus protein assay reagent (Pierce) using a BSA standard. Oligonucleotide primers were synthesized by Integrated DNA Technologies. DNA was sequenced at the Health Sciences Center Sequencing Facility, Eccles Institute of Human Genetics, University of Utah. The molecular masses of proteins were determined by positive-ion electrospray mass spectrometry in the University of Utah Chemistry Department mass spectrometry laboratory. Homology searches were performed at the National Center for Biotechnology Information website. Pairwise sequence alignments were performed with the Vector NTI (Informax) and EMBL-EBI (European Bioinformatics Institute) servers (www.ebi.ac.uk/Tools/clustalw/index.html) using the ClustalW algorithm.

Cloning of bacterial SQS. Nested PCR primers were used to amplify DNA fragments containing the SQS-coding gene tll1096 (EMBL nucleotide sequence accession no. BAC08649) with Easy-A PCR cloning enzyme from *T. elongatus* BP-1 genomic DNA as follows: forward primer, 5′-TTGTGGAAGGCATCCCCTGCTGGGTTGCGATC-3′; reverse primer, 5′-TAGCGATGACTGGCGGCAAACTCAGCACGG-3′. PCR conditions (25 cycles) were as follows: initial denaturation at 95°C for 120 s, denaturation at 95°C for 45 s, annealing at 66.4°C for 45 s, extension at 72°C for 90 s, and a final extension at 72°C for 7 min. Additional PCR primers were designed incorporating a 5′ NdeI site (underlined) and a 3′ HindIII site (underlined) complementary to tll1096 from nested PCR products as follows: NdeI 5′-CAT**ATG**CGTGTAGGAGTGAACC-3′ and HindIII 5′-AAGCTTA**TTA**AAGTCCGCCAAGGATGAAAGG-3′. The stop codon was mutated from TAG to TAA. The start and stop codons are shown in bold. PCR conditions were identical to those described for nested PCR, except that the annealing steps were modified (52.7°C for 45 s). The gel-purified 1.1-kb PCR product was tailed with an A residue and ligated into the subcloning vector pGEM-T Easy using T4 DNA ligase to give pgBP1Q. pgBP1Q was transformed to XL1-Blue by electroporation for blue-white screening. Individual colonies from Luria-Bertani (LB)–ampicillin (100 μg/ml)–tetracycline (12 μg/ml) agar plates were picked, and the plasmid DNA was isolated. pgBP1Q was sequenced to verify that the 1.1-kb insert was identical to the deposited sequence for tll1096 from *T. elongatus* BP-1 (25).

pgBP1Q was digested with NdeI and HindIII and ligated using T4 DNA ligase into the doubly digested expression vector pET28b(+) with the same restriction enzymes to give the expression plasmid ptBP1QX. After tll1096 was subcloned to pET28b(+), the resulting plasmid (ptBP1QX) was transformed to XL1-Blue by electroporation. Individual colonies from LB-kanamycin (LB-Kan; 30 μg/ml) agar plates were picked, and the plasmid DNA was isolated and sequenced. This construct encoded a version of *T. elongatus* BP-1 SQS with an N-terminal His_6 tag and a thrombin proteolytic site.

B. japonicum strain USDA 110 was cultivated in a yeast-mannitol medium to prepare genomic DNA as previously described (23). Nested PCR was used to amplify DNA fragments containing the long-version (EMBL nucleotide sequence accession no. BAC48266) and short-version (34) SQS genes from *B. japonicum* genomic DNA. Additional PCR primers were used to incorporate a 5′ NdeI site and a 3′ HindIII site complementary to the *B. japonicum* SQS gene from nested PCR products as follows for the long version of SQS (restriction sites are underlined, and start and stop codons are in boldface): for NdeI, 5′-[CAT**ATG**GGTGCCGCGGCGGCATCGCCG-3′; for HindIII, 5′-AAGCTTA**TTA**AGCGTCATGTGCAGTCCCCGGTCTGG-3′. GGG next to the start codon in the forward primer was changed to GGT to introduce a silent mutation. For the short version of SQS, the primers were the following: for NdeI, 5′-CAT**ATG**ACCTCTGCGAGCGAATTGCGATCCGGC-3′; for HindIII, 5′-AAGCTTA**TTA**AGCGTCATGTGCAGTCCCCGGTCTGG-3′, were used. The stop codon was mutated from TAG to TAA. PCR conditions were identical to those used for nested PCR except for modification of the annealing steps (62.3°C for 45 s). *Z. mobilis* strain ZM4 was cultivated in RM medium (glucose [20 g/liter], yeast extract [10 g/liter], KH_2PO_4 [2 g/liter] [pH 6.0]) to prepare genomic DNA as previously described (4). The PCR primers used to incorporate a 5′ NdeI site and a 3′ HindIII site complementary to the *Z. mobilis* SQS gene (EMBL nucleotide sequence accession no. AAV89493) from *Z. mobilis* genomic DNA were as follows: for NdeI, 5′-CAT**ATG**GAAGGGGCGTGCGCAAGCACGTATAG-3′; for HindIII, 5′-AAGCTTA**TTA**AGAAAATAATCTCCCTGCGGCGGCGAC-3′. The start and stop codons were mutated from GTG to ATG and TAG to TAA, respectively. PCR conditions were identical to those described for *T. elongatus* BP-1 SQS except for modifications to the annealing steps (58.3°C for 45 s). Procedures for cloning *B. japonicum* and *Z. mobilis* SQS after PCR were performed as described for *T. elongatus* BP-1 SQS cloning using pGEM-T Easy and pET28b(+) vectors. The final constructs encoded versions of *B. japonicum* and *Z. mobilis* SQSs with N-terminal His_6 tags and thrombin proteolytic sites. The generated plasmids and *E. coli* strains are listed in Table 1.

Expression and purification of recombinant *T. elongatus* BP-1 SQS. ptBP1QX was transformed into Rosetta (DE3) to create strain ptBP1QXRO. LB medium was used for all growth conditions (44). The bacterial strains used in this study are listed in Table 1 and were grown at 30°C in LB medium supplemented with chloramphenicol (Cam; 34 μg/ml) and Kan (30 μg/ml) as necessary. Cultures were grown by a three-stage fermentation protocol. LB-Cam-Kan cultures (5 ml) inoculated with a single colony of ptBP1QXRO were incubated by shaking at 250 rpm at 37°C for 10 h. These starter cultures were used to inoculate 100 ml of LB-Cam-Kan and grown overnight at 37°C. Inocula (10 ml) from the stage II culture were used to inoculate 1 liter of LB-Cam-Kan cultures. The cultures were grown to an optical density at 600 nm of approximately 0.6, induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and incubated for an additional 6 h at 30°C. Transformation and expression of ptBJQlgX, ptBJQstX, and ptZMQX were identical with the previous procedures for ptBP1QX.

For purification of recombinant *T. elongatus* BP-1 SQS, the cells from strain ptBP1QXRO were harvested by centrifugation for 20 min at 4°C at 5,000 × *g*. All steps in the purification were performed at 4°C. Cell paste (11 g) was suspended in 20 ml of lysis buffer consisting of 50 mM sodium phosphate (pH 8), 300 mM NaCl, 10 mM imidazole, 50% (vol/vol) glycerol, and a protease inhibitor cocktail tablet. The cells were disrupted by sonication (5 sets of 30-s pulses), and the resulting homogenate was centrifuged at 12,000 × *g* to remove cellular debris.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli		
XL1-blue	Host for cloning vector; Tet^r	Stratagene
XL-pgBP1Q	XL1-Blue containing pgBP1Q	This work
XL-ptBP1QX	XL1-Blue containing ptBP1QX	This work
XL-pgBJQlg	XL1-Blue containing pgBJQlg	This work
XL-ptBJQlgX	XL1-Blue containing ptBJQlgX	This work
XL-pgBJQst	XL1-Blue containing pgBJQst	This work
XL-ptBJQstX	XL1-Blue containing ptBJQstX	This work
XL-pgZMQ	XL1-Blue containing pgZMQ	This work
XL-ptZMQX	XL1-Blue containing ptZMQX	This work
Rosetta(DE3)	Host for protein expression; provides rare codon tRNAs; Cam^r	Novagen
ptBP1QXRO	Rosetta (DE3) containing ptBP1QX; Kan^r Cam^r	This work
Thermosynechococcus elongatus BP-1	Genomic DNA having SQS gene (tll1096)	S. Tabata; 25
Bradyrhizobium japonicum USDA 110	Proteobacteria strain having SQS gene	USDA
Zymomonas mobilis ZM4	Proteobacteria strain having SQS gene (zmo0869)	ATCC
Plasmids		
pGEM-T Easy	Cloning vector for PCR products; Amp^r	Promega
pgBP1Q	pGEM-T Easy with PCR product containing ORF tll1096	This work
pgBJQlg	pGEM-T Easy with PCR product containing ORF blr3001	This work
pgBJQst	pGEM-T Easy with PCR product containing short version of SQS; bp 3312158–3313043 in *B. japonicum* genome[a]	This work
pgZMQ	pGEM-T Easy with PCR product containing ORF zmo0869	This work
pET28b(+)	Expression vector with an N-terminal His_6 tag; Kan^r	Novagen
ptBP1QX	pET28b(+) with a 1.1-kb NdeI-HindIII insert corresponding to ORF tll1096	This work
ptBJQlgX	pET28b(+) with a 1.0-kb NdeI-HindIII insert corresponding to ORF blr3001	This work
ptBJQstX	pET28b(+) with a 0.9-kb NdeI-HindIII insert corresponding to short version of SQS	This work
ptZMQX	pET28b(+) with a 0.9-kb NdeI-HindIII insert corresponding to ORF zmo0869	This work

[a] See reference 34.

Glycerol-free lysis buffer was added to the resulting supernatant to lower the glycerol concentration to 20%, Ni-NTA (7 ml in a 50% slurry) was added to the resulting mixture, and the suspension was swirled at 4°C at 100 rpm for 1 h on a rotary shaker. The slurry was poured into a 100-ml fritted glass column, and the flowthrough was collected. The Ni-NTA resin was washed with 30 ml of a 20% (vol/vol) glycerol, 50 mM imidazole, and 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl; samples were further washed with 30 ml of 20% (vol/vol) glycerol, 70 mM imidazole, and 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl. The recombinant protein was eluted with 20 ml of 20% (vol/vol) glycerol, 250 mM imidazole, and 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and analyzed by SDS-PAGE. The protein was concentrated via ultrafiltration (YM-10; Centriprep) and dialyzed against 20% (vol/vol) glycerol–50 mM HEPES, pH 7.5, containing 0.1 mM DTT to yield 10.3 mg of enzyme that gave a single band (>97% purity) by SDS-PAGE. The protein was flash-frozen and stored at −80°C until needed.

SQS assays. SQS was assayed by modification of the procedure of Zhang and Poulter (58). In a typical assay, 10 μM [1-^{3}H]FPP (50 μCi ^{3}H/μmol) was incubated with 30 ng of SQS in 200 μl of 50 mM morpholinepropanesulfonic acid (MOPS) buffer, pH 7.2, containing 10 mM $MgCl_2$, 1 mM DTT, 1 mg/ml BSA, 1 mM NADPH, and 1% (vol/vol) Tween 80 (buffer A). The reaction was initiated by adding 10 μl of a solution of SQS in the same buffer to 190 μl of the assay cocktail. The reaction mixture was incubated at 37°C for 5 min and quenched with 300 μl of methanol–40% (wt/vol) aqueous KOH (1:1, vol/vol). After NaCl (~0.4 g) was added to saturate the mixture, ligroin (2 ml) containing 0.5% (vol/vol) SQ was added, and the resulting mixture was vortexed vigorously for 30 s. A 1-ml portion of the ligroin layer was loaded onto a 0.5- by 6-cm alumina column (80 to 200 mesh; Fisher), packed in a Pasteur pipette, and preequilibrated with 2 ml of ligroin containing 0.5% (vol/vol) SQ. The column was eluted with five 1-ml portions of toluene containing 0.5% (vol/vol) SQ. The radioactivity of the eluent was measured in 10 ml of Ultima Gold liquid scintillation cocktail (PerkinElmer) by liquid scintillation spectrometry (Packard).

Electrospray mass spectrometry of recombinant *T. elongatus* BP-1 SQS. Concentrated protein (10 μl) was diluted into 500 μl of water-acetonitrile-formic acid (80:20:0.2) and concentrated via ultrafiltration (YM-10; Microcon). The protein was resuspended in the same solution, concentrated, and resuspended in 200 μl of water-acetonitrile-formic acid (50:50:0.2) to give a final protein concentration of 15 μM. Positive-ion electrospray mass spectrometry was performed on a Waters Micromass Quattro II triple-quadrupole mass spectrometer.

Product studies of recombinant *T. elongatus* BP-1 SQS with GC and GC-mass spectrometry. FPP (50 μg; 0.12 μmol) and SQS (360 μg; 9.1 nmol) in 1 ml of 50 mM MOPS buffer, pH 7.2, containing 10 mM $MgCl_2$ and 1 mM NADPH were incubated at 37°C for 2 h. NaCl (~1 g) was added, and the mixture was extracted with 5 ml of methyl *tert*-butyl ether. The extracts were concentrated to ~100 μl with a gentle stream of N_2, and a 1-μl portion of the extract was analyzed by gas chromatography (GC) on an Agilent 6890N gas chromatograph with flame ionization detection using a 30-m by 0.318-mm (bore size) by 0.25-μm (film thickness) HP-5 capillary column (J & W Scientific). Compounds were eluted with a temperature gradient from 60 to 260°C at 10°C increase/min; temperature was then maintained at 260°C for 20 min with He, with a flow rate of 1.3 ml/min. Similar conditions were used to analyze samples by GC-mass spectrometry on an HP 5971A mass spectrometer. The mass spectra of eluted peaks were compared with authentic SQ.

Formation of [^{14}C]PSPP and [^{14}C]SQ by recombinant *T. elongatus* BP-1 SQS. Formation of PSPP in the absence of NADPH by recombinant *T. elongatus* BP-1 SQS was measured using [1-^{14}C]FPP synthesized by incubation of [1-^{14}C]IPP and GPP with FPP synthase. The incubation mixture consisted of 18 μg of recombinant *E. coli* FPP synthase in 38 μl of 50 mM MOPS buffer, pH 7.2, 50 μM [1-^{14}C]IPP (50 μCi ^{14}C/μmol), 135 μM GPP, 10 mM $MgCl_2$, 1 mM DTT, and 1 mg/ml BSA. After 10 min at 37°C, 0.6 μg of recombinant *T. elongatus* BP-1 SQS in 2 μl of MOPS buffer was added. The incubation was continued at 37°C for 2 h with occasional mixing before the reaction was quenched by the addition of 10 μl of 250 mM EDTA. A 5-μl portion of the resulting reaction mixture was spotted on a silica gel 60 F_{254} thin-layer chromatography (TLC) plate (Merck), which was developed with 30:70:13:8 chloroform-formic acid-pyridine-water. Radioactivity on the TLC plate was measured with a Typhoon 8600 imaging analyzer (GE Healthcare). In a parallel experiment, SQ formation was measured under the same conditions by adding 1 mM NADPH to the reaction mixture. The TLC plate was developed with 4:1 hexane-toluene.

Dependence on metal ions, NADPH, pH, temperature, and detergents. Assays were typically run in 50 mM MOPS buffer, pH 7.2, containing 10 μM [1-^{3}H]FPP (50 μCi ^{3}H/μmol), 1 mM DTT, and 1 mg/ml BSA (buffer B). The reactions were worked up as described for the standard assay. For studies of Mg^{2+} dependence, the assays contained 0.2 to 30 mM $MgCl_2$, 1 mM NADPH, and 1% (vol/vol) Tween 80 in buffer B. For studies of the NADPH dependence of SQS, assays contained 0 to 3.0 mM NADPH, 10 mM $MgCl_2$, and 1% (vol/vol) Tween 80 in buffer B. Activity at pHs ranging from 5.3 to 9.0 at 37°C was measured in buffer A using a 50 mM concentration of a different buffer system, depending on the pH (pH 5.3, acetate; pH 5.7 to 6.4, morpholinethanesulfonic acid; pH 6.7 to 7.6, MOPS; pH 7.9 to 8.2, HEPES; pH 8.6 to 9.5, CHES [2-(cyclohexylamino) ethanesulphonic acid]). Activity at different temperatures (25 to 70°C) was measured using buffer A. The dependence of activity on Tween 80 concentration (0 to 3%, vol/vol) was measured in buffer B containing 1 mM NADPH and 10 mM $MgCl_2$.

Steady-state kinetic measurements. Assays were performed in duplicate in 50 mM MOPS buffer, pH 7.2, containing 10 mM $MgCl_2$, 1 mM DTT, 1 mg/ml BSA, and 1% (vol/vol) Tween 80 at 37°C. For kinetic measurements to determine the K_m and k_{cat} of FPP (K_m^{FPP} and k_{cat}^{FPP}, respectively), the assay buffer contained 0.25 to 20 μM [1-^{3}H]FPP (37.5 to 750 μCi ^{3}H/μmol) and 1 mM NADPH. For the K_m^{NADPH} and k_{cat}^{NADPH}, the assay buffer contained 0.05 to 3 mM NADPH and 10 μM [1-^{3}H]FPP (50 μCi ^{3}H/μmol). Conversion of FPP to SQ was limited to 10% or less. Values for K_m and k_{cat} were calculated by fitting initial velocities at different substrate concentrations to the appropriate form of the Michaelis-Menten equation (39).

Inhibition of recombinant *T. elongatus* BP-1 SQS by zaragozic acid A. The 50% inhibitory concentration of zaragozic acid A for *T. elongatus* BP-1 SQS was determined by measuring the conversion of FPP to SQ in the presence of different concentrations of zaragozic acid A. [1-^{3}H]FPP (1 μM; 50 μCi ^{3}H/μmol) and 0 to 160 nM zaragozic acid A were incubated with 5 ng of SQS in 200 μl of buffer A. The assays were then performed as described above in "SQS assays".

RESULTS AND DISCUSSION

Sequence analysis of genes encoding bacterial SQS. Genes encoding SQS have been cloned from a variety of eukaryotic sources, including yeast (15, 21, 57), protozoa (45), rat (22), mouse (12), human (41, 50), and plant (27). In bacteria, hopanoid biosynthesis gene clusters containing the gene for SQS, *hpnC*, were cloned from *Z. mobilis* and *B. japonicum*, and synthesis of SQ was detected in cell extracts of the transformants (34). We conducted a database search for proteins homologous to biochemically characterized SQSs from fungi, animals, and plants and found hits in cyanobacteria, proteobacteria, and archaea, including *T. elongatus* BP-1, *Gloeobacter violaceus* PCC 7421 (EMBL nucleotide sequence accession no. BAC91999) (26), *Methylococcus capsulatus* (EMBL nucleotide sequence accession no. CAA71097) (53), *Halobacterium salinarum* NRC-1 (EMBL nucleotide sequence accession no. AAG19173) (29), and *Haloarcula marismortui* ATCC 43049 (EMBL nucleotide sequence accession no. AAV46402) (3). Additional hits for SQS were found, as expected, in the hopane-producing bacteria *B. japonicum*, *Z. mobilis*, and *M. capsulatus* (33, 40, 53). *T. elongates* BP-1 also has a putative SQ-hopane cyclase (EMBL nucleotide sequence accession no. BAC09861).

Figure 2 shows amino acid alignments of representative SQSs from bacteria and eukaryotes. *T. elongatus* BP-1 SQS, which is typical of the bacterial SQSs, has ~30% overall similarity with the mouse, rat, human, *S. cerevisiae*, and *Schizosaccharomyces pombe* proteins (Fig. 2) (15, 22, 27, 41, 50, 51, 57). Eukaryotic SQSs have four conserved regions (Fig. 2, I to IV), which kinetic studies with site-directed mutants and a crystal structure indicate are important for catalysis (10, 32). Regions I, II, and III are involved in the first half-reaction, the condensation of two molecules of FPP to give PSPP. Mutation of the highly conserved Tyr171 in region II of rat SQS appears to be particularly important (10). Regions I and III are likely involved in binding of the diphosphate units in FPP via bridging Mg^{2+} (10, 32). Region IV is thought to be required for the rearrangement of PSPP to SQ. Although SQS does not have a "typical" NADPH binding motif, likely candidates are the FC AIPQVMAIATL sequence found in region IV and the VKIRK sequence located downstream from region IV in the case of rat and human SQSs (10, 32). VKIRK is the most flexible part of human SQS in its crystal structure and is thought to be stabilized by NADPH binding (32). A related enzyme, phytoene synthase (PS), converts geranylgeranyl diphosphate to phytoene (13, 28). SQS and PS both catalyze the cyclopropanation and rearrangement reactions shown in Fig. 1. However, the rearrangement step in phytoene synthesis is terminated by loss of a proton, and the enzyme does not utilize NADPH (13, 50). Eukaryotic and bacterial PSs do not have sequences that correspond to the putative NADPH binding motifs found in SQSs.

The degree of similarity between eukaryotic and bacterial SQSs is highest in regions I and II and drops considerably in regions III and IV. The FCAIPQVMAIATL putative NADPH binding motif in region IV of eukaryotic SQSs is not highly conserved in the bacterial enzymes, while the VKIRK motif is well conserved in both groups. Finally, bacterial SQS does not have a consensus sequence for the membrane-spanning C-terminal α-helix that anchors eukaryotic SQS to membrane (47, 49, 57).

Cloning, solubilization, and purification of SQS. Eukaryotic SQSs are associated with microsomes and can be solubilized with detergents. However, when expressed in *E. coli*, SQS is found in inclusion bodies and cannot be reconstituted to give soluble active enzyme. Zhang et al. discovered that soluble recombinant yeast SQS could be obtained by deletion of a putative C-terminal membrane-spanning α-helix (57). This approach has been used to obtain soluble recombinant enzyme from other eukaryotes (1, 18, 45, 47, 52). However, bacterial SQSs do not have a C-terminal sequence predicted to give a membrane-spanning helix, and the basis for membrane affiliation by the bacterial enzymes is not apparent.

Three bacteria, *T. elongatus* BP-1, *B. japonicum*, and *Z. mobilis*, were selected as sources for the SQS gene. Previously, SQ synthesis was detected in *E. coli* transformants harboring the hopane gene cluster from *B. japonicum* and *Z. mobilis* (34). In addition, a SQ-hopane cyclase from *B. japonicum* and *Z. mobilis* has been expressed in *E. coli* (33, 40). The moderate thermophile, *T. elongatus*, has been the source of genes for (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase, an enzyme in the 2-*C*-methyl-D-erythritol phosphate pathway for isoprenoid biosynthesis (30), the circadian clock proteins (KaiA, KaiB, and KaiC), and photoreceptor proteins (9, 55, 56).

The DNA sequence for *hpnC*, the putative SQS gene in the *B. japonicum* hopanoid biosynthesis gene cluster (34), did not have the 84 bp at the N terminus reported in the genomic DNA sequence for the same bacterium (blr3001; EMBL nucleotide sequence accession no. BAC48266) (17). Both versions of *B. japonicum* SQS were cloned into an *E. coli* expression system. The DNA sequence for the *Z. mobilis* SQS gene from the

FIG. 2. Amino acid sequence alignments for SQSs from *Rattus norvegicus* (rSQS), *Homo sapiens* (hSQS), *S. cerevisiae* (ySQS), *Arabidopsis thaliana* (pSQS), *Z. mobilis* (zSQS), *B. japonicum* (bSQS), and *T. elongatus* BP-1 (tSQS). The ClustalW algorithm was used to generate the alignment (www.ebi.ac.uk/Tools/clustalw/index.html). Dashes indicate gaps that were introduced to maximize the alignment. The conserved regions I, II, III, and IV are indicated above the sequences.

hopanoid biosynthesis gene cluster (34) was also slightly different from the one (zmo0869; EMBL nucleotide sequence accession no. AAV89493) from *Z. mobilis* genomic DNA (37). *hpnC* from *Z. mobilis* SQS uses ATG as a start codon instead of GTG for zmo0869 (34, 46). Overall, 10 bp (12 amino acids in the protein) in *hpnC* from *Z. mobilis* SQS are different from those of zmo0869. These differences mostly code for amino acids found at the N terminus of the protein. The sequence of our PCR clone of *Z. mobilis* SQS matched the sequence for *Z. mobilis* genomic DNA (zmo0869) (Fig. 2). A comparison of flanking regions showed that our clone was located in the hopanoid biosynthesis gene cluster, as described previously (34, 46).

tll1096 from *T. elongatus* BP-1 has 1,080 bp and encodes a 359-amino-acid protein (~39.9 kDa). The open reading frame (ORF) was cloned by PCR using *T. elongatus* BP-1 genomic DNA as a template. Initial attempts with primers containing NdeI and HindIII restriction sites failed. A 1.3-kb DNA fragment containing tll1096 was amplified using primers without the restriction sites. The gel-purified 1.3-kb DNA fragment was used as the template for a second round of PCR using primers to introduce the flanking NdeI and HindIII restriction sites. The PCR product was cloned into pGEM-T Easy to give pgBP1Q. The sequence of tll1096 in pgBP1Q was identical to that reported for *T. elongatus* BP-1 genomic DNA (25). tll1096 from pgBP1Q was subcloned into the expression vector pET28b(+) to give ptBP1QX, which encoded SQS with an N-terminal His_6 tag. A parallel set of experiments was carried out for the SQS genes from *B. japonicum* and *Z. mobilis*.

IPTG-induced expression in ptBP1QX gave high levels of SQS as judged by SDS-PAGE of cell extracts. Initially, *T. elongatus* BP-1 SQS was found exclusively in inclusion bodies as an inactive enzyme. Attempts to solubilize and refold the protein under a variety of conditions were unsuccessful. Different media (LB or M9 minimal media), temperatures (18°C, 30°C, and 37°C), and *E. coli* hosts [BL21(DE3) and Rosetta (DE3)] gave similar results. Eventually, we were able to obtain soluble active *T. elongatus* BP-1 SQS (~3 to 6% of total cytosolic protein) by adding glycerol (20 to 50% vol/vol) to the lysis and purification buffers and by lowering the incubation temperature to 30°C. Different buffer additives, including glycerol, Tween 20, Tween 80, NaCl, and polyethylene glycol, were

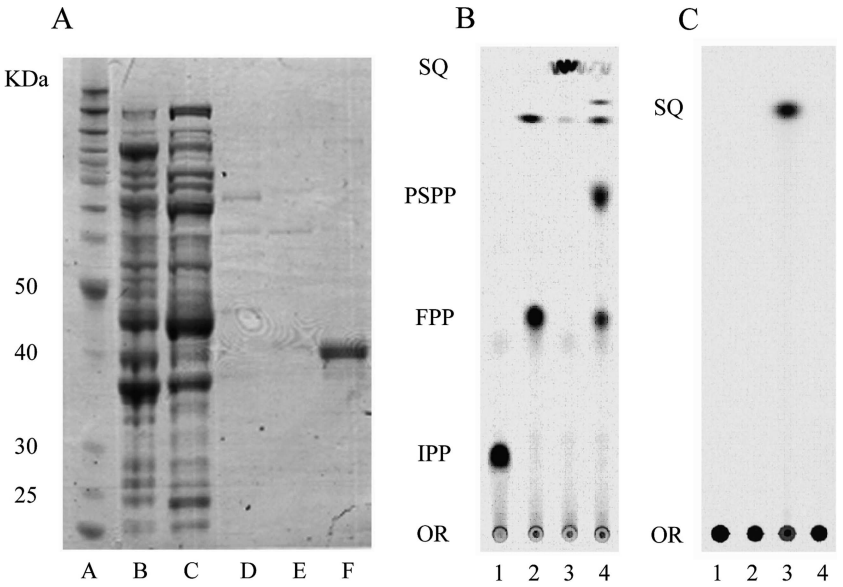

FIG. 3. Characterization of recombinant of *T. elongatus* BP-1 SQS. (A) SDS-PAGE of samples from the purification of recombinant *T. elongatus* BP-1 SQS. Lane A, molecular weight markers; lane B, cell extract from ptBP1QXRO; lane C, flowthrough; lane D, washed fraction with 50 mM imidazole-buffer; lane E, washed fraction with 70 mM imidazole-buffer; lane F, eluted SQS with 250 mM imidazole-buffer. (B and C) Autoradiograms of TLC plates of products from recombinant *T. elongatus* BP-1 SQS. In panel B, the TLC plate was developed with 30:70:13:8 chloroform-formic acid-pyridine-water. In panel C, development was with 4:1 hexane-toluene. Lane 1, [^{14}C]IPP. Other lanes are as follows: [^{14}C]IPP and GPP with recombinant *E. coli* FPP synthase (lane 2), with *T. elongatus* BP-1 SQS in the presence of NADPH (lane 3), and with *T. elongatus* BP-1 SQS in the absence of NADPH (lane 4). The positions of [^{14}C]IPP, [^{14}C]FPP, [^{14}C]PSPP, [^{14}C]SQ, and the chromatographic origin (OR) are indicated.

examined, but none except glycerol gave active soluble *T. elongatus* BP-1 SQS. Glycerol seems to be more important for solubility rather than stability for the *T. elongatus* BP-1 SQS. Once BP-1 SQS is solubilized, the protein remains active and soluble even after glycerol is reduced or removed from the buffer. Unfortunately, similar attempts to obtain soluble recombinant SQS from *B. japonicum* and *Z. mobilis* failed.

In a typical purification, cells were disrupted in buffer containing 50% (vol/vol) glycerol, and the glycerol concentration was lowered to 20% (vol/vol) to minimize problems associated with viscosity during subsequent steps. The cell extract was chromatographed on Ni-NTA. After a washing step with buffer containing 50 mM and 70 mM imidazole, SQS eluted as a sharp peak with buffer containing 250 mM imidazole. SDS gel electrophoresis of purified *T. elongatus* BP-1 SQS (2 to 3 mg/ml) gave a single band (>97% purity) at approximately 42 kDa, consistent with the predicted molecular mass for the His_6-tagged enzyme (Fig. 3A). Purified protein was flash-frozen in liquid N_2 and stored at −80°C until needed. The enzyme was stable for at least 6 months under these conditions.

Product analysis. Incubation of *T. elongatus* BP-1 SQS with FPP in the presence of NADPH gave a single product with a GC retention time of 24.5 min that comigrated with an authentic sample of SQ. A GC-mass spectrum of the product had a prominent peak at *m/z* 410 and a fragmentation pattern identical to that produced by an authentic sample of SQ.

Synthesis of PSPP by *T. elongatus* BP-1 SQS, when incubated with FPP in the absence of NADPH, was measured using [^{14}C]FPP. [^{14}C]FPP was synthesized from [^{14}C]IPP and GPP using recombinant *E. coli* FPP synthase. Recombinant *T. elongatus* BP-1 SQS was then added to the same reaction mixture in the absence of NADPH to investigate PSPP formation. Product studies with TLC analysis required greater concentrations of substrate and *T. elongatus* BP-1 SQS than the kinetic assays because of the difference in the sensitivity of the two methods (2, 27, 38). Formation of PSPP was detected by TLC as a radioactive product (Fig. 3B) at an R_f of 0.73, which was identical to that of PSPP prepared with recombinant yeast SQS as described by Jarstfer et al. (14). SQ ran at the solvent front when the TLC plate was developed with 30:70:13:8 chloroform-formic acid-pyridine-water. Possible solvolysis products of FPP and PSPP were also detected at an R_f of ~0.9 (Fig. 3B, lanes 2 and 4). Farnesol, neoridol, and hydrocarbons are known solvolysis products of FPP by FPP synthase (43). PSPP also undergoes SQS-catalyzed solvolysis to give a mixture of (*Z*)-dehydrosqualene, (*R*)-12-hydroxysqualene, and (10*S*,13*S*)-10-hydroxybotryococcene (14). In 4:1 hexane-toluene, SQ was detected at an R_f of 0.88, while IPP, FPP, and PSPP remained at the origin (Fig. 3C) (38). When NADPH was added to the incubation mixture, recombinant *T. elongatus* BP-1 SQS converted FPP directly to SQ without formation of detectable amounts of PSPP (Fig. 3C).

Characterization of recombinant *T. elongatus* BP-1 SQS. Positive-ion electrospray mass spectrometry of His_6-SQS gave a peak corresponding to a mass of *m/z* 41886. This value is within experimental error of a predicted mass of 41,888 Da for the protein without the N-terminal Met, presumably removed by methionyl aminopeptidase in the *E. coli* expression system (11). The activity of *T. elongatus* BP-1 SQS increased in a hyperbolic manner with increasing concentrations of NADPH and Tween 80, with maximal activity at 2.0 mM NADPH and 2% (vol/vol) Tween 80 (Table 2 and Fig. 4D). Similar behavior

TABLE 2. Kinetic constants for SQS

SQS source	SQS type	k_{cat} (s^{-1})	K_m^{FPP} (μM)	K_m^{NADPH} (μM)	k_{cat}/K_m^{FPP} ($\mu M^{-1} s^{-1}$)	Reference
Human	Microsomal		2.3			52
	N- and C-terminally truncated	1.44	2.8		0.51	52
Rat	Microsomal		1.8			52
Yeast	C-terminally truncated	0.53	2.5	530	0.21	21
T. cruzi	Microsomal		2.3	33		54
	N- and C-terminally truncated	1.05	5.3	23	0.20	45
L. mexicana	Microsomal		2.8	57		54
T. elongatus BP-1		1.74 ± 0.04	0.97 ± 0.10	241 ± 13	1.80 ± 0.02	This work

is seen for the eukaryotic enzyme. Presumably the detergent facilitates turnover by providing a hydrophobic reservoir for the water-insoluble product of the reaction (57). Enzyme activity gradually increased from 25°C to a maximum at 60°C (Fig. 4C). A plot of activity versus pH gives a well-defined bell curve (Fig. 4B) with an optimum at pH 7.6, which is somewhat higher than the value of 7.2 reported for the yeast enzyme (57). *T. elongatus* BP-1 SQS has a strict requirement for Mg^{2+}, with maximal activity at ~20 mM (Fig. 4A). No activity was observed when the enzyme was assayed in Mg^{2+}-free buffer. This behavior is typical for enzymes in the isoprenoid biosynthetic pathway that processes diphosphate substrates.

The rate of SQ synthesis from FPP was linear at the limits of substrate consumption (1 to 15%) and reaction times (1 to 10 min) used for the assays. Michaelis constants for FPP and NADPH were determined at saturating concentrations of the other substrate, and the values are listed in Table 2. The catalytic efficiencies of the bacterial and eukaryotic enzymes are similar. K_m^{FPP} for *T. elongatus* BP-1 SQS is slightly lower than the values of the eukaryotic enzymes while k_{cat}^{FPP} is higher.

Zaragozic acid A, a fungal metabolite, is a potent inhibitor of mammalian and fungal SQSs, which are thought to mimic FPP and PSPP (5, 6, 35). Initially, zaragozic acid is a competitive inhibitor against FPP in rat SQS, which is followed by irreversible inactivation of the enzyme (20). When *T. elongatus* BP-1 SQS activity was measured in the presence of ~K_m concentration of FPP, zaragozic acid A showed dose-dependent inhibition. Zaragozic acid A is also a potent inhibitor of recombinant *T. elongatus* BP-1 SQS, with a 50% inhibitory concentration of 95.5 ± 13.6 nM.

Conclusions. In summary, the tll1096 gene in *T. elongatus* BP-1 encodes SQS. The protein shows modest (~30%) overall similarity to eukaryotic SQSs. The highest degree of similarity was seen in regions I and II, thought to be responsible for the conversion of FPP to PSPP. The similarity in regions III and IV is considerably lower. Bacterial SQSs, including the *T. elongatus* BP-1 enzyme, do not have the C-terminal membrane-spanning motif found in the eukaryotic proteins. When expressed in *E. coli*, the bacterial SQSs were located in inclusion bodies and could not be reconstituted to give soluble active enzymes. After considerable experimentation, we were able to solubilize

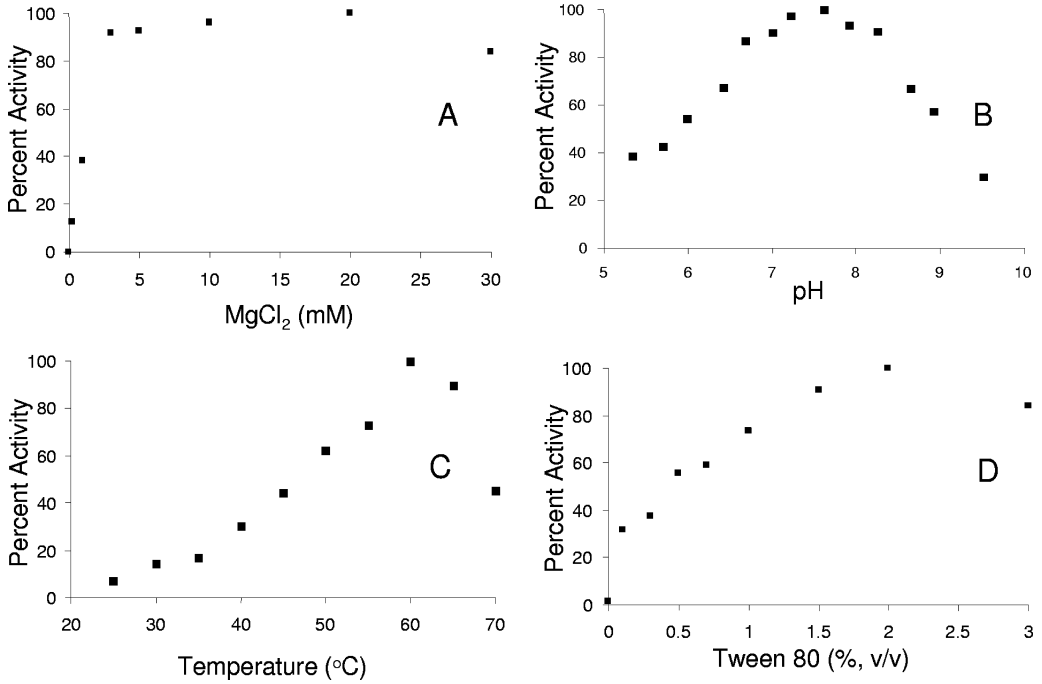

FIG. 4. Dependence of SQS activity from *T. elongatus* BP-1 on $MgCl_2$ (A), pH (B), temperature (C), and Tween 80 (D).

and purify the recombinant His_6-tagged enzyme from *T. elongatus* BP-1 (>97% homogeneity) by including high concentrations of glycerol in the disruption and purification buffers. We were not, however, able to find conditions that gave active soluble enzyme for the proteins from *Z. mobilis* and *B. japonicum*. The metal ion dependence, pH dependence, and kinetic properties of the *T. elongatus* enzyme were similar to those of its eukaryotic counterparts. The *T. elongatus* BP-1 SQS gene contains conserved regions similar to those associated with catalysis by the enzyme in *Eukarya*. Zaragozic acid A, a potent inhibitor of mammalian, fungal, and yeast SQS, is also a potent inhibitor of recombinant *T. elongatus* BP-1 SQS.

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