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Biosynthesis of Squalene from Farnesyl Diphosphate in Bacteria: Three Steps Catalyzed by Three Enzymes

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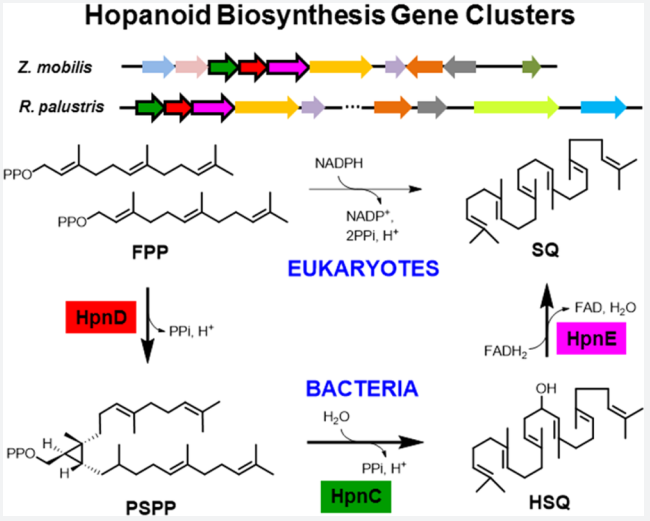
Supporting Information

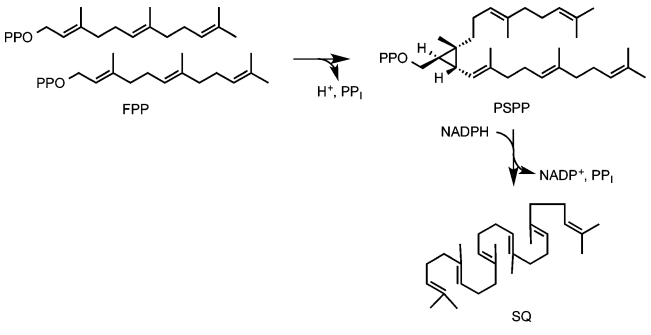
ABSTRACT: Squalene (SQ) is an intermediate in the biosynthesis of sterols in eukaryotes and a few bacteria and of hopanoids in bacteria where they promote membrane stability and the formation of lipid rafts in their hosts. The genes for hopanoid biosynthesis are typically located on clusters that consist of four highly conserved genes—*hpnC*, *hpnD*, *hpnE*, and *hpnF*—for conversion of farnesyl diphosphate (FPP) to hopene or related pentacyclic metabolites. While *hpnF* is known to encode a squalene cyclase, the functions for *hpnC*, *hpnD*, and *hpnE* are not rigorously established. The *hpnC*, *hpnD*, and *hpnE* genes from *Zymomonas mobilis* and *Rhodopseudomonas palustris* were cloned into *Escherichia coli*, a bacterium that does not contain genes homologous to *hpnC*, *hpnD*, and *hpnE*, and their functions were established *in vitro* and *in vivo*. HpnD catalyzes formation of presqualene diphosphate (PSPP) from two molecules of FPP; HpnC converts PSPP to hydroxysqualene (HSQ); and HpnE, a member of the amine oxidoreductase family, reduces HSQ to SQ. Collectively the reactions catalyzed by these three enzymes constitute a new pathway for biosynthesis of SQ in bacteria.



Squalene (SQ) is an intermediate in the biosynthesis of sterols in eukaryotes and sterols, hopanoids, and related pentacyclic triterpenes in bacteria. These metabolites perform numerous essential functions in their hosts, including the stabilization of lipid membranes and formation of membrane rafts.[1−3] Squalene synthase (SQase) couples the hydrocarbon moieties from two molecules of farnesyl diphosphate (FPP). SQase was first identified in rat liver homogenates[4] and has subsequently been studied in other animals,[5,6] plants,[7−9] fungi,[10,11] and a few bacteria.[12,13] These enzymes catalyze two reactions—the coupling of two molecules of FPP to give presqualene diphosphate (PSPP) and the subsequent NADPH-dependent reductive rearrangement of PSPP to SQ[14] (Scheme 1)—without releasing PSPP from the active site.[15]

The bacterial genes for biosynthesis of hopanoids and related pentacyclic triterpenes are typically located in clusters. A representative example of this organization is illustrated by the genomic location of 14 putative hopanoid biosynthesis genes, *hpnA*−*hpnM*, in the ethanol producing bacterium *Zymomonas mobilis* (Figure 1).[16] A large continuous cluster contains *hpnA*−*hpnH*, followed by *ispH* and *hpnM*. While not directly involved in hopanoid biosynthesis, *ispH* is an essential enzyme in the methylerythritol phosphate pathway for biosynthesis of

Scheme 1. Conversion of FPP to SQ in Two Steps by Eukaryotic SQases



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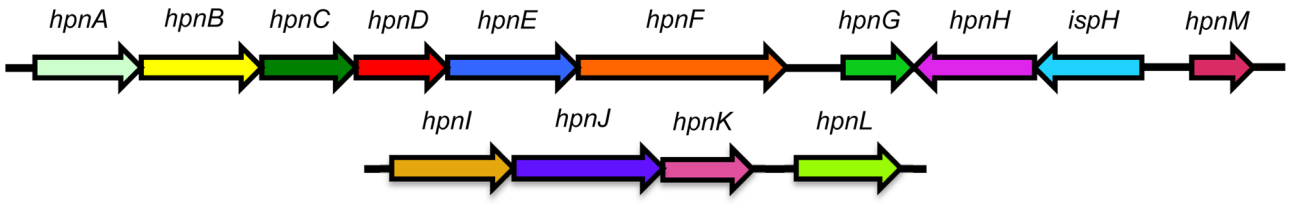

hpnA – sugar epimerase
hpnB – glycosyl transferase
hpnC – "squalene synthase like" protein
hpnD – "squalene synthase like" protein
hpnE – FAD-dependent amino oxidase; phytoene desaturase; unknown function
hpnF – squalene-hopene cyclase
hpnG – phosphatase
hpnH – radical SAM
ispH – hydroxydimethylallyl diphosphate reductase
hpnM – membrane associated ABC transporter
hpnI – glycosylase
hpnJ – radical SAM
hpnK – polysaccharide hydrolyase
hpnL – membrane spanning protein

Figure 1. Hopanoid biosynthesis clusters in *Z. mobilis*.

isopentenyl and dimethylallyl diphosphates, the C_5 building blocks for FPP. *HpnI*–*hpnL* are located downstream from the larger cluster in the same region of the genome. Three hopanoid genes, *hpnC*, *hpnD*, and *hpnF*, are commonly associated with the conversion of FPP to hopene. *HpnC* and *hpnD* belong to the SQ-phytoene synthase family (PF00494) and are commonly annotated as "squalene synthase like proteins".[17] *HpnF* encodes the proton-initiated SQ-hopene cyclase.[18,19] The function of a fourth gene, *hpnE*, located between *hpnD* and *hpnF* is cryptic. HpnE encodes a putative flavoprotein in the amine oxidoreductase family (PF01593) and is assigned a number of different functions, none of which are obviously related to SQ biosynthesis.[17] However, *hpnE* is commonly found in a four-gene *hpnC*–*hpnF* cassette in the genomes of a wide variety of other bacteria, including species in the Acidobacteria (Acidobacteriaceae), Actinobacteria (Actinomycetales), Firmicutes (bacilli), Nitrospirae (*Nitrospira*), Planctomycetes (Planctomycetacia), Proteobacteria (Alphaproteobacteria and Betaproteobacteria), and Verrucomicrobia (*Methylacidiphilum*) phyla.

Only a few bacterial SQases have been characterized biochemically. The reactions catalyzed by SQases from *Thermosynechococcus elongatus*[12] and *Methylococcus capsulatus*[13] are identical to those catalyzed by eukaryotic SQases. These organisms produce hopanoids[12] and sterols,[20,21] respectively, and each has a single SQase gene instead of a *hpnC-hpnD* pair. The co-occurrence of *hpnC*, *hpnD*, and *hpnE* in hopanoid biosynthesis clusters raises questions about why two SQase-like proteins are apparently required to convert FPP to SQ and what the function is of the vaguely annotated flavoprotein. We now report the rigorous functional annotation of *hpnC*, *hpnD*, and *hpnE* from *Zymomonas mobilis* and *Rhodopseudomonas palustris*, which collectively encode enzymes in a novel three-step pathway for biosynthesis of SQ from FPP in bacteria.

Functional annotations for *hpnC*, *hpnD*, and *hpnE* in *Z. mobilis* ZM4 and *R. palustris* strains BisB5 and CGA009 were determined with His_6-tagged recombinant enzymes obtained from *E. coli* clones. HpnC and HpnD were obtained as pure proteins upon chromatography on a Ni^{2+} column; however, HpnC lost a substantial amount of activity during chromatography. As a result, it was studied in cell-free homogenates. HpnE eluted as a pure protein in a bright yellow fraction, which gave a yellow filtrate following deflavination and membrane filtration. UPLC–MS analysis of the yellow filtrate gave a single peak with the mass (m/z 784 $[M - H]^-$) expected for FAD. The functions of the three enzymes were established by radio-TLC on silica gel and C18 reversed-phase plates and by GC/MS with comparisons to authentic samples. The *E. coli* host does not have genes homologous to *hpnC*, *hpnD*, or *hpnE*, and control experiments established that the host lacks the ability to catalyze reactions analogous to those for the HpnC, HpnD, and HpnE proteins.

Cell free *E. coli* homogenates containing heterologously expressed HpnC from *Z. mobilis* ZM4 and *R. plaustris* CGA009 were incubated with [^{14}C]FPP. The incubation mixtures were extracted with methyl *tert*-butyl ether (MTBE). The aqueous layer was then treated with acid phosphatase and extracted again with MTBE. Products were compared with those from identical incubations of yeast SQase, which in the absence of NADPH slowly gives (1*R*,2*R*,3*R*)-PSPP, followed by the much slower conversion of PSPP to (*R*)-12-hydroxysqualene (HSQ), isohydroxysqualene (isoHSQ), *cis*-dehydrosqualene (DSQ), (*S*)-hydroxybotryococcene (HBO), and isohydroxybotryococcene (isoHBO) (Figure 2; Figure 3, lanes ySQase-NADPH) from PSPP (Figure 1).[22,23]

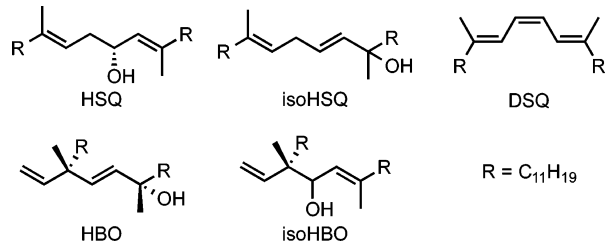

Figure 2. Promiscuous products from incubation of yeast SQase with FPP in the absence of NADPH.

Incubation of cell-free homogenates containing overexpressed HpnC and FPP, without and with added NADH or NADPH, gave detectable radioactivity in MTBE extracts that comigrated with farnesol (FOH) presumably generated by hydrolysis by *E. coli* phosphatases in the homogenate. Higher levels of FOH were found in MTBE extracts after the aqueous layer was treated with acid phosphatase (Figure S1, parts A and B; lanes zHpnC + NADH, zHpnC + NADPH, rHpnC + NADH, rHpnC + NADPH, data not shown for HpnC without NADPH or NADH). Collectively, these experiments establish that FPP is not a substrate for the enzyme. Incubations with purified *Z. mobilis* HpnD and FPP gave trace amounts of extractable radioactivity before treatment with phosphatase, which comigrated with HSQ (Figure 3, part A; lane zHpnD),

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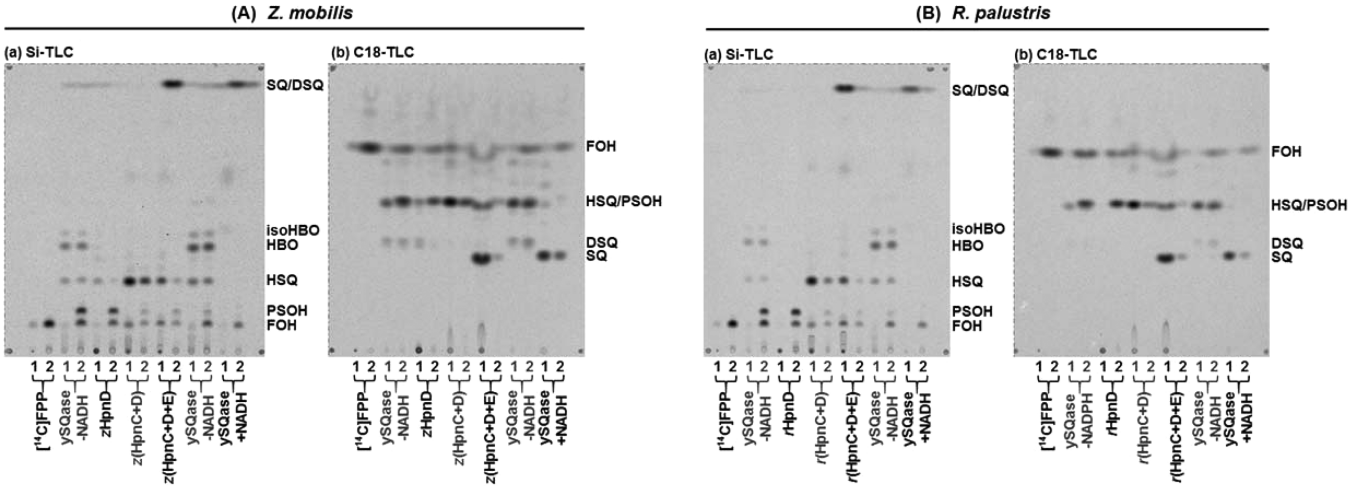

Figure 3. Radio-TLC activity assays for HpnC-E from (A) *Z. mobilis* and (B) *R. palustris*. After incubation at 37 °C for 2 h in buffer containing $MgCl_2$, each reaction was first (1) extracted with MTBE. The aqueous layer was then subjected to phosphate hydrolysis by acid phosphatase at 30 °C for 15−16 h. The hydrolyzed products were extracted again (2) with MTBE. The MTBE solvent was removed, and residues were spotted on normal and C18 reversed-phase TLC plates.

while no radioactivity for HSQ was seen in a similar experiment with *R. plaustris* (Figure 3, part B; lane rHpnD). These results were unchanged for incubations in buffer containing $MgCl_2$ with added NADH or NADPH (Figure S1, part A, lanes zHpnD + NADH, zHpnD + NADPH, and part B, lanes rHpnD + NADH, rHpnD + NADPH). Larger amounts of HSQ were detected for incubations in buffer containing $MnCl_2$ (Figure S2, part A, lanes zHpnD + NADH, zHpnD + NADPH, and part B, lanes rHpnD + NADH, rHpnD + NADPH). UPLC−MS analysis of incubation mixtures from the *Z. mobilis* and *R. plaustris* enzymes gave a product with peaks at m/z 585 $[M - H]^-$ and m/z 505 $[M - PO_3H_2]^-$, typical for a triterpene diphosphate. After phosphatase treatment, an intense band was seen with an R_f identical to that of presqualene alcohol (PSOH) (Figure 3, part A, lane zHpnD, part B, lane rHpnD), whose identity was confirmed by GC−MS (Figure S3). Incubations with HpnE gave results similar to those for HpnC (Figures S1 and S2; part A, lanes zHpnE + NADH, zHpnE + NADPH, part B, lanes rHpnE + NADH, rHpnE + NADPH). These results establish that HpnD is a PSPP synthase (PSPPase), HpnC and HpnE do not catalyze reactions with FPP, and *Z. mobilis* HpnD has a low level of promiscuous activity for synthesis of HSQ.

Incubation of a mixture of a cell-free extract containing HpnC and purified HpnD with FPP gave an MTBE-extractable product whose R_f on silica gel and C18 reversed-phase TLC plates was similar to that of HSQ[22,23] (Figure 3; part A, lane z(HpnC + D), and part B, lane r(HpnC + D)). A GC−MS spectrum of the extract, after treatment with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide, matched the spectrum of the TMS derivative of yeast HSQ (Figure S4). These results were unchanged upon incubation with NADH or NADPH (Figures S1 and S2; part A, lanes z(HpnC+D) + NADH, z(HpnC+D) + NADPH, and part B, lanes r(HpnC+D) + NADH, r(HpnC+D) + NADPH) and indicate that HpnC is a HSQ synthase (HSQase) that coverts PSPP to HSQ. This assignment was confirmed as follows. FPP was incubated with rHpnD, and the incubation mixture was extracted with n-butanol to give PSPP. Incubation of this material with NADH and zHpnC or rHpnC gave HSQ (Figures S5 and S6, lanes zHpnC + ^{14}C-PSPP, rHpnC + ^{14}C-PSPP), while incubation with yeast SQase and NADH gave SQ (Figures S5 and S6, lane ySQase + ^{14}C-PSPP). Conversely, incubation of HpnC with PSPP synthesized by yeast SQase gave HSQ (Figure S7, lanes zHpnC + ySQase, rHpnC + ySQase, rHa-HpnC + ySQase) Thus, (1*R*,2*R*,3*R*)-PSPP is product of HpnD and the substrate for HpnC.

Finally, incubation of FPP with a mixture of HpnC, HpnD, HpnE, FAD, and NADH gave an MTBE-extractable product with an R_f on a C18 TLC plate that matched that of SQ (Figure 3, part A, lane z(HpnC+D+E), and part B, lane r(HpnC+D+E)). GC-MS analysis of the extracts gave a peak with the same retention time and mass spectrum as SQ (Figure S8). The cryptic HpnE protein is a novel FAD-dependent SQase, and the reactions catalyzed by HpnC-HpnE constitute a new three-step route for biosynthesis of SQ from FPP.

The pathway was reconstructed in an *E. coli* host, a bacterium that lacks the ability to synthesize SQ and whose genome does not have homologues for *hpnC*, *hpnD*, or *hpnE*. *E. coli* cells were transformed with plasmids containing *Z. mobilis* and *R. plaustris* *hpnD*, *hpnC-hpnD*, and *hpnC-hpnD-hpnE*. To augment the low level of FPP production in *E. coli*, the recombinant strains were cotransformed with plasmid pBbA5c-MevT(CO)-T1-MBIS-(CO, *ispA*).[24] The transformants were incubated for 4 h following induction with IPTG and then analyzed for the presence of PSPP by LC-MS and of HSQ and SQ by GC-MS. The results are summarized in Table 1 and Figure S9. Cell-free homogenates from *E. coli* controls transformed with pBbA5c-MevT(CO)-T1-MBIS(CO, *ispA*), but not the *hpn* genes, did not contain PSPP, HSQ, or SQ. The *hpnD* transformants

Table 1. Relative Levels of PSPP, HSQ, and SQ in *E. coli* and *E. coli hpnD*, *hpn(C+D)*, and *hpn(C+D+E)* Transformants[a]

	PSPP	HSQ	SQ
E. coli control	nd	nd	nd
zhpnD	++++	tr	nd
zhpn(C+D)	+	+++	nd
zhpn(C+D+E)	+	++++	+++++
rhpnD	+++++	tr	nd
rhpn(C+D)	+	++++	nd
rhpn(C+D+E)	+	+	+++++

[a]Not detected, nd; trace, tr.

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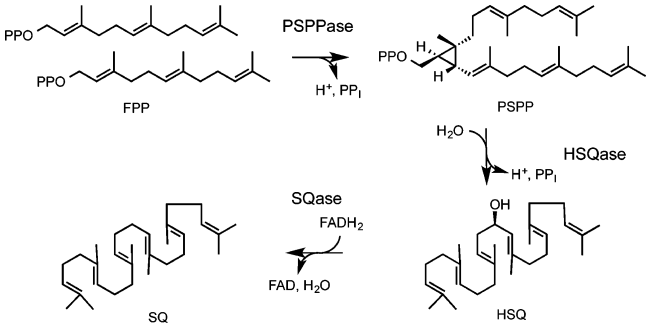
synthesized PSPP, as expected from our results with the purified enzymes. In addition, the homogenates from the *Z. mobilis* and *R. palustris hpnD* transformants contained small amounts of HSQ, consistent our TLC results with purified HpnD. These results suggest that HpnD from both bacteria has a low level of promiscuous activity for conversion of PSPP to HSQ. Cell free homogenates from the *hpnC-hpnD* transformants contained HSQ and smaller quantities of PSPP, but no detectable levels of SQ. Cell free homogenates from the *hpnC-hpnD-hpnE* transformants gave SQ along with smaller quantities of HSQ and PSPP. Thus, all three genes are required for heterologous biosynthesis of SQ in an *E. coli* host. Collectively, our experiments establish the functions of *hpnC*, *hpnD*, and *hpnE* in hopanoid biosynthesis gene clusters and reveal a new, unanticipated three-step/three-enzyme pathway for biosynthesis of SQ from FPP in bacteria (Scheme 2).

Scheme 2. Conversion of FPP to SQ in Bacteria: Three Steps/Three Enzymes



Interestingly, HSQ is a poor substrate for SQ-hopene cyclase. HSQ accumulated when *Streptomyces coelicolor* A3(2) HpnF was incubated with FPP and *Z. mobilis* or *R. palustris* Hpn(C+D), with no evidence for formation of a pentacyclic C_{30} alcohol (Figure S10; lanes z(HpnC+D), z(HpnC+D) + Sco-SHC, r(HpnC+D), r(HpnC+D) + Sco-SHC). In contrast, a similar incubation with yeast SQase and *S. coelicolor* SQ-hopene cyclase gave hopene (Figure S10, lane ySQase + Sco-SHC). Cyclization of HSQ would place a polar hydroxyl group in the C ring of the resulting pentacyclic structure that would compromise its ability to insert into membranes and would need to be removed in the ultimate pentacyclic metabolites.

Our results have interesting implications for evolution of enzymes for SQ biosynthesis. The pathway in bacteria begins with cyclopropanation of FPP to give PSPP. It is likely that PSPPase activity arose from ubiquitous chain elongation enzymes (PF00348) in the isoprenoid synthase superfamily.[25,26] There is precedent for the independent emergence of cyclopropane-forming activity in chrysanthemyl diphosphate synthase from a FPPase in plants as recently as ∼50 million years ago (MYA).[23] Eukaryotic SQase, which synthesizes PSPP from FPP in the absence of NADPH, also converts PSPP to HSQ.[22,23] This observation, along with documented promiscuous activities for yeast SQase, supports a scenario for evolution of PSPPase from an ancestral chain elongation enzyme and of HSQase from PSPPase. In this scenario, the HSQ reductase activity in HpnE, which is a necessary step in the biosynthesis of hopanoid metabolites that insert properly into membrane, evolved independently from a protein in the flavin-dependent amino oxidase family.

There is a recent report of PSPPase activity in the colonial eukaryotic photosynthetic alga *Botryococcus braunii* race B. *B. braunii* has a gene for SQase (BSS)[9] and three "SQase-like (SSL)" genes.[27] The BSS protein converts FPP to SQ like other eukaryotic SQases. SSL1 converts FPP to PSPP, SSL2 catalyzes an NADPH-dependent reductive rearrangement/reduction of PSPP to SQ, and SSL3 catalyzes the NADPH-dependent reductive rearrangement of PSPP to botryococcene (BO), a triterpene isomer of SQ. SQ, BO, and their methylated derivatives are pumped into a translucent membrane surrounding individual cells in the *B. braunii* colony, where they accumulate to 30−86% of the dry weight of the algal mass and provide flotation for maximal exposure of the organism to sunlight.[28] BSS and the three SSL genes are estimated to have separated from a common ancestor ∼500 MYA.[27] A brief phylogenetic analysis of the four *B. brauni* genes places them firmly within a group of other eukaryotic SQase genes in fungi, plants, and animals (Figure S11). Most likely, the *B. braunii* PSPPase evolved from a eukaryotic SQase to facilitate regulation of the flux of FPP into sterol biosynthesis independently from the flux into biosynthesis of the massive amounts of methylated SQ and BO required for flotation. In contrast, *hpnD* in both Gram-positive and Gram-negative bacteria is distantly related to eukaryotic SQases, consistent with an ancient common ancestor for the bacterial and eukaryotic enzymes.

Contraction of the three-enzyme bacterial pathway to a one-enzyme eukaryotic pathway could logically begin by acquisition of NADPH binding capacity by HSQase in order to catalyze the synchronous rearrangement/reduction of PSPP to SQ, followed by reacquisition of cyclopropanation activity by the enzyme to generate the modern eukaryotic SQase. We have preliminary TLC data for HpnD and HpnC homologues from *Burkholderia pseudomallei* that the "HpnD-like" protein is a PSPPase and the "HpnC-like" protein is an NADPH-dependent SQase that requires PSPP as its isoprenoid substrate, similar to the *B. braunii* enzyme.

The alcohol reductase activity of the flavin-dependent SQase is unusual. While the mechanism of this reaction is not known, a likely scenario utilizes reduced flavin as an acid−base catalyst where protonation activates the alcohol moiety in HSQ as a leaving group and the resulting allylic carbocation is reduced by the dihydroflavin anion. This mechanism is similar to those proposed for the dihydroflavin-dependent isomerization of isopentenyl diphosphate to dimethylallyl diphosphate,[29] cyclization of lycopene,[30] and *cis*−*trans* isomerization of phytoene.[31]

During their studies of *hpnC*, *hpnD*, and *hpnE*, Perzl and co-workers reported formation of hydrocarbon products from *E. coli* strains containing *hpnC* (*B. japonicum*), *hpn(C+D)* (*Z. mobilis*) and *hpn(C+D+E)* (*Z. mobilis*) that were not synthesized by the parental *E. coli* strain.[18] The *hpn(C+D+E)* strain gave SQ consistent with our results. However, they reported that *hpn(C+D)* strain gave a mixture of SQ and DSQ, although the experimental evidence for this claim was not presented. We occasionally saw trace amounts of DSQ in extracts from an *E. coli hpn(C+D)* clone, which we attributed to decomposition of HSQ. HSQ is a sensitive allylic alcohol that is prone to elimination during analysis by gas chromatography. However, we did not detect formation of SQ by *hpn(C+D)* clones in our TLC and GC-MS analysis of extracts from *in vitro* or *in vivo* experiments. In related work, Ghimire and co-workers reported functions for the SQase-like *Streptomyces peucetius*[32]

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enzymes equivalent to HpnC and HpnD based on *in vivo* studies of the heterologous expressed proteins and concluded that the HpnC and HpnD proteins both produced SQ from FPP.[33] Their results are inconsistent with our *in vitro* and *in vivo* studies. Since we have shown that *E. coli* expression clones for HpnC, HpnD, or Hpn(C+D) are incapable of synthesizing SQ from FPP, their observation would require that both enzymes convert FPP to SQ, which seem unlikely claims given the widespread distribution of the *hpnC*, *hpnD*, *hpnE* trio of genes in bacteria.

In summary, we discovered a new pathway for biosynthesis of SQ from FPP in bacteria through the concerted actions of the enzymes encoded by *hpnC*, *hpnD*, and *hpnE*. These enzymes present interesting targets for disrupting bacterial membranes by blocking hopanoid biosynthesis.

■ ASSOCIATED CONTENT

Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.5b00115.

Experimental materials and methods and Figures S1–S11 (PDF)

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Notes

The authors declare no competing financial interest.

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