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

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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 homogenates4 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 SQ14 (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–hpnF, 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
isopentenyl and dimethylallyl diposphates, the C₅ 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 hpnE, 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.²⁰ 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 (Nitrospirota), Planctomycetes (Planctomycetacia), Proteobacteria (Alphaproteobacteria and Betaproteobacteria), and Verrucomicrobia (Methylicacidiphilum) phyla.

Only a few bacterial SQases have been characterized biochemically. The reactions catalyzed by SQases from Thermosynechococcus elongatus²¹ and Methyllococcus capsulatus²² are identical to those catalyzed by eukaryotic SQases. These organisms produce hopanoids and sterols,²³,²⁴ respectively, and each has a single SQase gene instead of a SQase cassette in the genomes of a wide variety of other bacteria, including species in the Acidobacteria (Acidobacteriaceae), Actinobacteria (Actinomycetales), Firmicutes (bacilli), Nitrospirae (Nitrospirota), Planctomycetes (Planctomycetacia), Proteobacteria (Alphaproteobacteria and Betaproteobacteria), and Verrucomicrobia (Methylicacidiphilum) phyla.

Functional annotations for hpnC, hpnD, and hpnE in Z. mobilis ZM4 and R. palustris CGA009 were determined with His₆-tagged recombinant enzymes obtained from E. coli clones. HpnC and HpnD were obtained as pure proteins upon chromatography on a Ni²⁺ 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 radioactive 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. palustris CGA009 were incubated with [¹⁴C]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 (1R,2R,3R)-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 1. Hopianoid biosynthesis clusters in Z. mobilis.](Image)

![Figure 2. Promiscuous products from incubation of yeast SQase with FPP in the absence of NADPH.](Image)
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 MgCl₂ 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 MnCl₂ (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 – PO₃H₂]⁻, typical for a triterpene diphosphate. After phosphatase treatment, an intense band was seen with an Rᵣ 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, and 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 HpnD and purified HpnD with FPP gave an MTBE-extractable product whose Rᵣ on silica gel and C18 reversed-phase TLC plates was similar to that of HSQ²²,²³ (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 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 converts 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 + ¹⁴C-PSPP, rHpnC + ¹⁴C-PSPP), while incubation with yeast SQase and NADH gave SQ (Figures S5 and S6, lane ySQase + ¹⁴C-PSPP).

Conversely, incubation of HpnC with PSPP synthesized by yeast SQase gave HSQ (Figure S7, lanes zHpnC + ySQase, rHpnC + ySQase, rHa-HpnC + ySQase) Thus, (1R,2R,3R)-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 Rᵣ 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 pBbAsc-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 pBbAsc-MevT(CO)-T1-MBIS(CO, ispA), but not the *hpn* genes, did not contain PSPP, HSQ, or SQ. The *hpn* transformants did not have homologues for *hpnD*, 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.

![Figure 3](image-url) 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 MgCl₂, 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.

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*

|          | 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) | + | +++ | +++ |

*Not detected, nd; trace, tr.*
There is a recent report of PSPPase activity in the colonial eukaryotic photosynthetic alga *Botryococcus braunii*. *B. braunii* has a gene for SQase (BSS) and three “SQase-like (SSL)” genes. 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. 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. BSS and the three SSL genes are estimated to have separated from a common ancestor ~500 MYA. A brief phylogenetic analysis of the four *B. braunii* 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 distinctly 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 isopenyl 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*.
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. 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, our observation would require that both enzymes convert FPP to SQ, which seem unlikely to change 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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