Supporting Information

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

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Experimental materials and methods

Reagents. Common chemicals were purchased from Sigma-Aldrich. HPLC-grade MTBE and hexanes were purchased from Macron Fine Chemicals™ and Fisher Scientific, respectively. Wheat germ acid phosphatase [specific activity (SA) = 0.5 unit/mg] was purchased from MP Biomedicals. [4-^{14}C]IPP (SA = 50 – 60 μCi/μmol) was purchased from PerkinElmer. [^{14}C]FPP was synthesized by the FPP synthases characterized previously[1] using [4-^{14}C]IPP and DMAPP. After enzymatic reactions, [^{14}C]FPP was extracted with 1-butanol (saturated with water) and the solvent was removed on a SpeedVac (Thermo Scientific). The extracted [^{14}C]FPP was verified by radio-TLC and quantified on a Tri-Carb 2910TR liquid scintillation analyzer (PerkinElmer). Non-radioactive DMAPP and FPP were synthesized according to the published procedures.[2]

Protein cloning/expression/purification.

Table S1

Plasmid	Vector	Marker
pZMK-HpnC	pSGC	Km
pZMK-HpnD	pSGX3	Km
pZMK-HpnE	pSGC-His	Km
pZMA-HpnC	pET-Duet-1	Amp
pZMA-HpnC-HpnD	pET-Duet-1	Amp
pRPK-HpnC	pSGC	Km
pRPK-HpnD	pHS-SUMO30	Km
pRPK-HpnE	pSGC-His	Km
pRPA-HpnC	pET-Duet-1	Amp
pRPA-HpnC-HpnE	pET-Duet-1	Amp

Plasmids used in this work are listed in Table S1. *HpnC* and *HpnE* from *Zymomonas mobilis* ssp. mobilis ZM4 (ATCC 31821), *HpnC* and *HpnE* from *Rhodopseudomonas palustris* CGA009 and *HpnD* from *Rhodopseudomonas palustris* BisB5 were amplified from genomic DNA by PCR and cloned by ligation independent cloning into His-tag expression vectors pSGC, pSGC-His or pSGX3. *HpnD* from *Zymomonas mobilis* ssp. mobilis ZM4 (ATCC 31821) was synthetized and optimized for expression in *Escherichia coli* and cloned into pSGX3 expression vector. Primers GS1F (5'-AGATATACCCATGGACCATCATCATCA-3') and GS1R (5'-TCCTTTCGGGCTTTGTTAGCAG-3') were used to amplify *Zymomonas mobilis HpnC* from plasmid pZMK-*HpnC* and *Rhodopseudomonas palustris HpnC* from plasmid pRPK-HpnC. The PCR products were digested with *NcoI-HindIII* and cloned into pETduet-1 (Novagen®) to generate plasmids pZMA-*HpnC* and pRPA-*HpnC*, respectively. Plasmid pRPA-*HpnC-HpnE* was constructed by subcloning the NdeI-XhoI fragment containing *Rhodopseudomobas palustris HpnE* from plasmid pRPK-*HpnE* into pRPA-*HpnC*. Plasmid pZMA-HpnC-HpnD resulted from subcloning the NdeI-XhoI fragment containing *Zymomonas mobilis HpnD* from plasmid pZMK-*HpnD* into pZMA-HpnC.

Expression. The plasmids were transformed into Rosetta™ 2(DE3) competent cells (Novagen®), which were used to inoculate 2 mL LB overnight cultures containing 50 μg/mL kanamycin and

34 μg/mL chloramphenicol. The overnight cultures were then used to inoculate 400 mL LB-based ZYM-5052 auto-induction medium[3] containing the same antibiotics. The cultures were grown at 37 °C for 4 h with rigorous shaking (350 rpm), followed by another 24 h continued shaking at 22 °C. The cells were harvested by centrifugation at 6000 rpm for 15 min, and then kept at -80 °C until purification.

Purification. Cells were resuspended in the lysis buffer containing 50 mM sodium phosphate, pH 7.6, 300 mM NaCl and 10 mM imidazole, and lysed by sonication. The lysates were cleared by centrifugation at 10,000 rpm for 30 min, and the supernatants were loaded on the Ni-NTA column (Qiagen) preequilibrated with lysis buffer. The column was then washed with wash buffer containing 20 mM imidazole. Finally, the protein was eluted with elution buffer containing 250 mM imidazole. The collected eluent was concentrated, dialyzed [against 20 mM HEPES, pH 7.6, 150 mM NaCl, 10 mM β-mercaptoethanol (BME) and 15% glycerol] and analyzed by SDS-PAGE protein gels. The purified protein was flash-frozen by liquid nitrogen and stored at -80 °C. For HpnCs, large-scale expression and purification did not yield measurable protein (on SDS-PAGE) even with the help of glycerol in the purification buffers. Thus, small-scale 5 mL cultures were expressed in the LB-based ZYM-5052 autoinduction medium at 20 °C for 72 h, and the lysates were used directly after sonication in a buffer containing 50 mM HEPES, pH 7.6, 500 mM NaCl, 10 mM BME and 25% glycerol.

Radio-TLC assays. The radio-TLC assays for the *in vitro* enzyme activity and product characterization followed the protocol as that for the chain-elongation polyprenyl diphosphate synthases[1] with some modifications. Typical enzymatic incubations (40 μL) were carried out at 37 °C for 2 h in a reaction buffer containing 35 mM HEPES (pH 7.6), 10 mM $MgCl_2$, 5 mM BME and 0.25 % Triton X-100 along with 100 μM [^{14}C]FPP (SA = 5.5 μCi/μmol) and 5 – 20 μg of each individual enzyme, unless otherwise specified. After incubation, the reaction mixture was extracted with 3x100 μL methyl *t*-butyl ether (MTBE), and the aqueous layer was subjected to the diphosphate hydrolysis using wheat germ acid phosphatase (2 units) at 30 °C, overnight (15 – 16 h). The hydrolyzed products were extracted with 3x300 μL MTBE. The MTBE from both extractions was removed on the SpeedVac, and the residues were spotted on the normal (silica) or C18 reversed-phase TLC plates. Normal-phase TLC plates were generally developed with hexanes:MTBE (9:1 v/v), and C18 reversed-phase TLC plates with acetone:water (94:6, v/v) unless otherwise specified. The TLC plates were exposed to storage phosphor screens, and then imaged on a Typhoon 8600 phosphorimager (GE Healthcare).

Ultra performance liquid chromatography (UPLC)-mass spectrometry (MS) analysis of flavin-compounds in HpnEs. The purified HpnEs (500 μL) were concentrated to a minimal volume on 0.5 mL Amicon® centrifugal filter units (MWCO 30,000), followed by addition of 150 μL deflavination buffer (2 M KBr, 2 M guanidine chloride, 1X PBS, pH 7.4), and let sit for 10 min at room temperature. The yellow filtrates were collected by centrifugation at 13,200 rpm for 10 min, and then analyzed by UPLC-MS. The UPLC-MS analysis was carried out on a Waters ACQUITY UPLC H-Class system equipped with a TQ (tandem quadrupole) detector with MassLynx v4.1 software. The UPLC conditions were as follows: C18 column (Waters ACQUITY BEH C18 column, 1.7 μm, 2.1 mm x 100 mm) at a flow rate of 0.4 mL/min using a

mobile phase with linear gradient of A: 25 mM NH_4HCO_3 and B: methanol, 0-1 min: 85A15B, 1-4 min: 85A15B → 50A50B, 4-6 min: 50A50B, 6-8 min: 50A50B → 85A15B. The UPLC runs were simultaneously monitored by a photodiode array UV detector and the equipped mass spectrometer. The mass spectrometer was operated under negative electrospray ionization (ESI) mode with parameters set as follows: acquisition m/z range 100-1000, capillary voltage 2.5 kV, cone voltage 40 V, source temperature 110 °C, desolvation temperature 220 °C, cone gas flow 10 L/h and desolvation gas flow 550 L/h.

Gas chromatography (GC)-MS analysis of presqualene alcohol (PSOH). Presqualene diphosphate (PSPP) was produced from either HpnDs or ySQase in the scale-up 5 mL reactions containing 300 μM FPP and ~1500 μg enzymes and in the absence of NADH/NADPH. After incubations at 37 °C for 2 h for HpnDs and 10 min for ySQase, the reaction mixtures were lyophilized and the residues were hydrolyzed by 100 mg acid phosphatase in a 2 mL reaction mixture at 30 °C overnight. The hydrolyzed PSOH was extracted with 3x4 mL MTBE. After concentrating to ~100 μL, the samples were directly used for the GC-MS analysis. The GC-MS analysis was conducted on an Agilent 7890A GC system equipped with an Agilent 7000 Series Triple Quadrupole mass spectrometer. The GC was run on a HP-5ms capillary column (Agilent 19091S-433, 30 m x 250 μm x 0.25 μm, 325 °C max) with a temperature program: 220 °C, hold for 1 min; ramp up to 280 °C at 1 °C/min; hold for 5 min. The injection volume was 1 μL with a splitless setting, and the flow rate of the helium carrier gas was maintained at 1 mL/min. For the MS parameters, the standard 70 eV electron impact (EI) ionization mode was employed with an acquisition m/z range 40 – 600. The inlet temperature was set at 230 °C, source temperature 240 °C, and interface temperature 285 °C.

GC-MS analysis of HSQ. HSQ was produced from co-incubations of HpnCs and HpnDs in the scale-up 5 mL reactions containing 300 μM FPP and 1 mL of HpnCs' lysates and ~1500 μg HpnDs and in the absence of NADH/NADPH. After incubations at 37 °C for 2 h, the HSQ product was extracted with 3x10 mL MTBE. After concentrating to ~100 μL, 10 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and 10 μL of anhydrous pyridine were added to the samples for trimethylsilyl (TMS) derivatization. The sealed reaction in a GC vial was let sit at room temperature for overnight, then directly injected into the GC-MS system described above. The temperature program for GC runs was as follows: 100 °C, hold for 1 min; ramp up to 200 °C at 5 °C/min; ramp up to 280 °C at 10 °C/min; hold for 15 min. The MS parameters were the same as those in the PSOH analysis, except for the higher inlet temperature at 260 °C. The standard HSQ from ySQase-catalyzed solvolytic reactions was purified by HPLC according to our previously published procedures.[4] The standard HSQ was also TMS-derivatized using MSTFA before GC-MS analysis.

GC-MS analysis of SQ. SQ was produced from co-incubations of HpnCs, HpnDs and HpnEs in the scale-up 5 mL reactions containing 300 μM FPP and 1 mL of HpnCs' lysates and ~1500 μg of HpnDs and HpnEs and in the presence of NADH. Triton X-100 was omitted in the HpnC-E co-incubations since it was shown to inhibit the HpnE's activity in the radio-TLC assays. After incubations at 37 °C for 2 h, the SQ product was extracted with 3x10 mL MTBE. After concentrating to ~500 μL, the extracted MTBE was loaded onto a silica glass pipette column pre-

equilibrated with a solvent system of hexanes:MTBE = 9:1. The column was eluted with the same solvent system and SQ was collected in the first column volume. The GC-MS analysis was conducted on an Agilent 5975C inert GC/MSD system equipped with an Agilent 6850 GC system. The GC-MS conditions were the same as those in the HSQ analysis, except for the lower source temperature at 230 °C in the MS settings.

Metabolomic analysis. To analyze the squalene biosynthesis and its intermediates in *Escherichia coli* BL21(DE3) strain, cells were transformed with plasmids harboring *Zymomonas mobilis* or *Rhodopseudomonas palustri*s *HpnC, HpnD and HpnE* in different combinations (*HpnD, HpnD-HpnC and HpnD-HpnC-HpnE*). To increase the amount of FPP, the recombinant strains were also co-transformed with plasmid pBbA5c-MevT(CO)-T1-MBIS(CO, ispA)[5] (a gift from Jay Keasling & Taek_Soon Lee (Addgene plasmid # 35152)). Cells were grown in LB-medium at 37 ^{o}C to OD_{600} = 0.5, induced with 0.1 mM IPTG, an shaken for 4 h. The cells were then collected by centrifugation and the pellets stored at -80 ^{o}C until analysis.

Samples were analyzed at the Metabolomics Center, Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign.

Sample pellets were treated by ultrasound for 10 minutes using Microson XL2000 Ultrasonic Homogenizer (QSonica, CT, USA). Samples were subsequently extracted at room temperature with 1 mL of MTBE and 0.3 mL isopropanol for SQ/HSQ and 60% MeOH for PSPP and FPP Every extraction was accompanied by centrifugation (5 min at 15,000 g), and supernatants for FPP/PSPP and SQ/HSQ were collected into different tubes. SQ/HSQ extracts later were dried under N_2 gas and stored at -20 ^{o}C prior to derivatization. FPP/PSPP extracts were analyzed by LC/MS and SQ/HSQ by GC/MS.

The LC/MS system consisted of a 1200 series HPLC system (Agilent Technologies, Santa Clara, CA), a degasser, an autosampler, a binary pump and a 5500 QTRAP LC/MS/MS system (AB Sciex, Foster City, CA). The LC separation was performed on a Phenomenex 3μ C18 Gemini column (2 x 100 mm, 3 μm. Torrance, CA) with mobile phase A (25 mM NH_4HCO_3 in water) and mobile phase B (methanol). The flow rate was 0.2 mL/min. The linear gradient was as follows: 0-1 min, 80%A; 6-14 min, 0%A; 14.5-19 min, 80%A. The autosampler was set at 5°C. The injection volume was 10 μL. Mass spectra were acquired under negative electrospray ionization (ESI) with the ion spray voltage of -4500 V. The source temperature was 450 °C. The curtain gas, ion source gas 1, and ion source gas 2 were 33, 65, and 50, respectively. Multiple reaction monitoring (MRM) was used to measure PSPP (m/z 585.2 → m/z 79.1).

For GC/MS analysis dried extracts were derivatized with 70 μL MSTFA + 30 μL of pyridine (2 hours at 50 °C) and analyzed using a GC-MS system (Agilent Inc, CA, USA) consisting of an Agilent 6890 gas chromatograph, an Agilent 5973 mass selective detector and a HP 7683B autosampler. Gas chromatography was performed on a ZB-5MS (60 m × 0.32 mm I.D. and 0.25 μm film thickness) capillary column (Phenomenex, CA, USA). The inlet and MS interface temperatures were 300 °C, and the ion source temperature was adjusted to 230 °C. An aliquot of 2 μL was injected with the split ratio of 7:1. The helium carrier gas was kept at a constant flow rate of 2 mL/min. The temperature program was as follows: 2 min at 220 °C, an increase of 10 °C/min to 315 °C, and 4.5 min at 315 °C. The mass spectrometer was operated in positive electron impact mode (EI) at 69.9 eV ionization energy at m/z 50-600 scan range.

Standards used in the identification of the compounds were purchased from Sigma-Aldrich (FPP and SQ) or enzyme-synthesized (PSPP and HSQ).

Supporting Information References

1. Wallrapp, F. H., Pan, J. J., Ramamoorthy, G., Almonacid, D. E., Hillerich, B. S., Seidel, R., Patskovsky, Y., Babbitt, P. C., Almo, S. C., Jacobson, M. P., and Poulter, C. D. Prediction of function for the polyprenyl transferase subgroup in the isoprenoid synthase superfamily, *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, E1196-1202.
2. Davisson, V. J., Woodside, A. B., Neal, T. R., Stremler, K. E., Muehlbacher, M., and Poulter, C. D. Phosphorylation of isoprenoid alcohols, *J. Org. Chem.* **1986**, *51*, 4768-4779.
3. Studier, F. W. Protein production by auto-induction in high density shaking cultures, *Protein Expr. Purif.* **2005**, *41*, 207-234.
4. Pan, J.-J., Bugni, T. S., and Poulter, C. D. Recombinant Squalene Synthase. Synthesis of Cyclopentyl Non-Head-to-Tail Triterpenes, *J. Org. Chem.* **2009**, *74*, 7562-7565.
5. Peralta-Yahya, P. P., Ouellet, M., Chan, R., Mukhopadhyay, A., Keasling, J. D., and Lee, T. S. Identification and microbial production of a terpene-based advanced biofuel, *Nat. Commun.* **2011**, *2*, article no. 483.

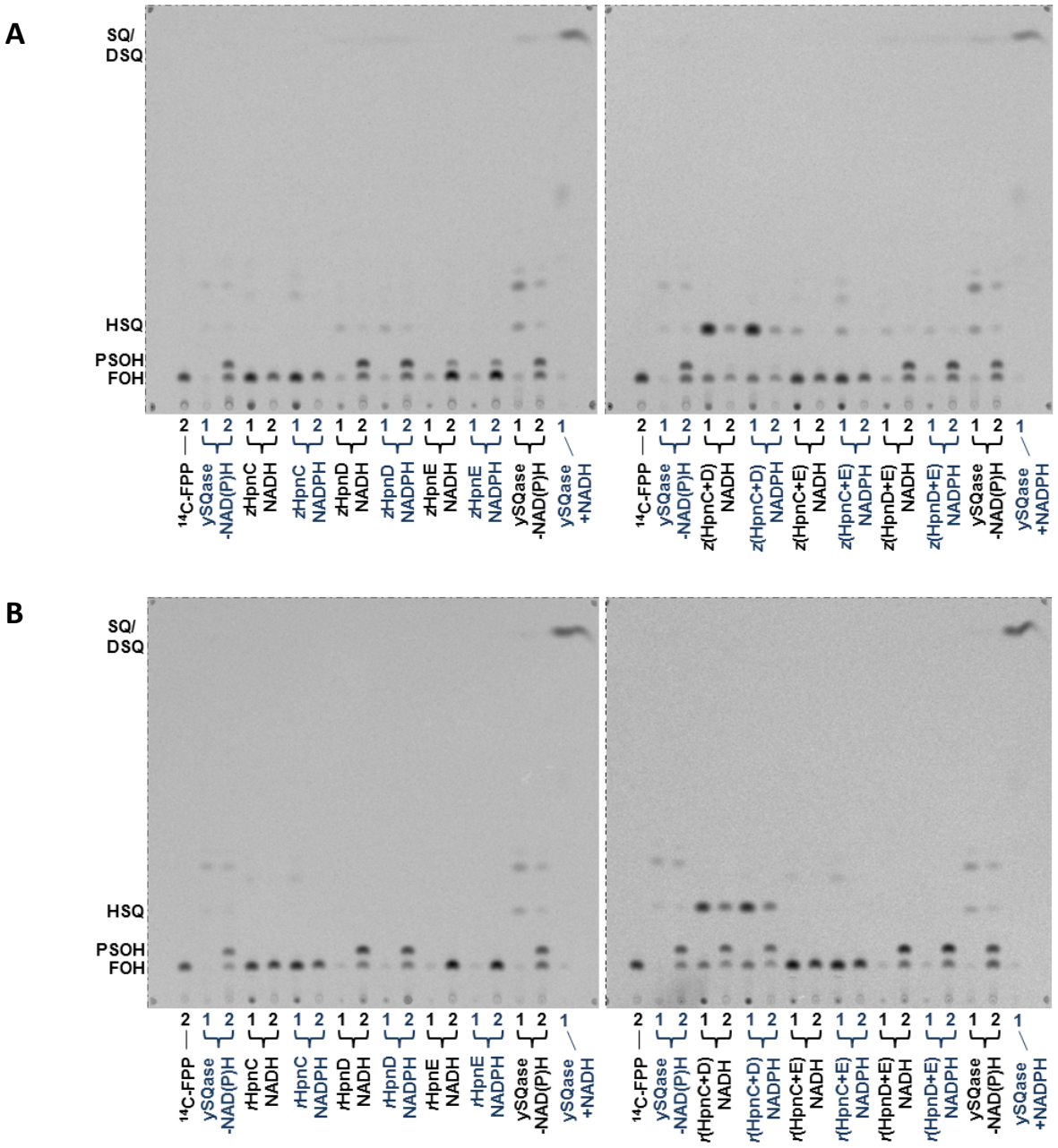


Figure S1. Radio- TLC (silica) assays for HpnCs, HpnDs and HpnEs from both *Z. mobilis* and *R. palustris* using [^{14}C]FPP in the presence of $MgCl_2$ combined with the presence or absence of NADH and NADPH. Top: HpnC-E from *Z. mobilis*. Bottom: HpnC-E from *R. palustris*. 1: 1st MTBE extraction directly after each reaction is finished; 2: 2nd MTBE extraction after the aqueous layer was treated with acid phosphatase to hydrolyze the diphosphate compounds.

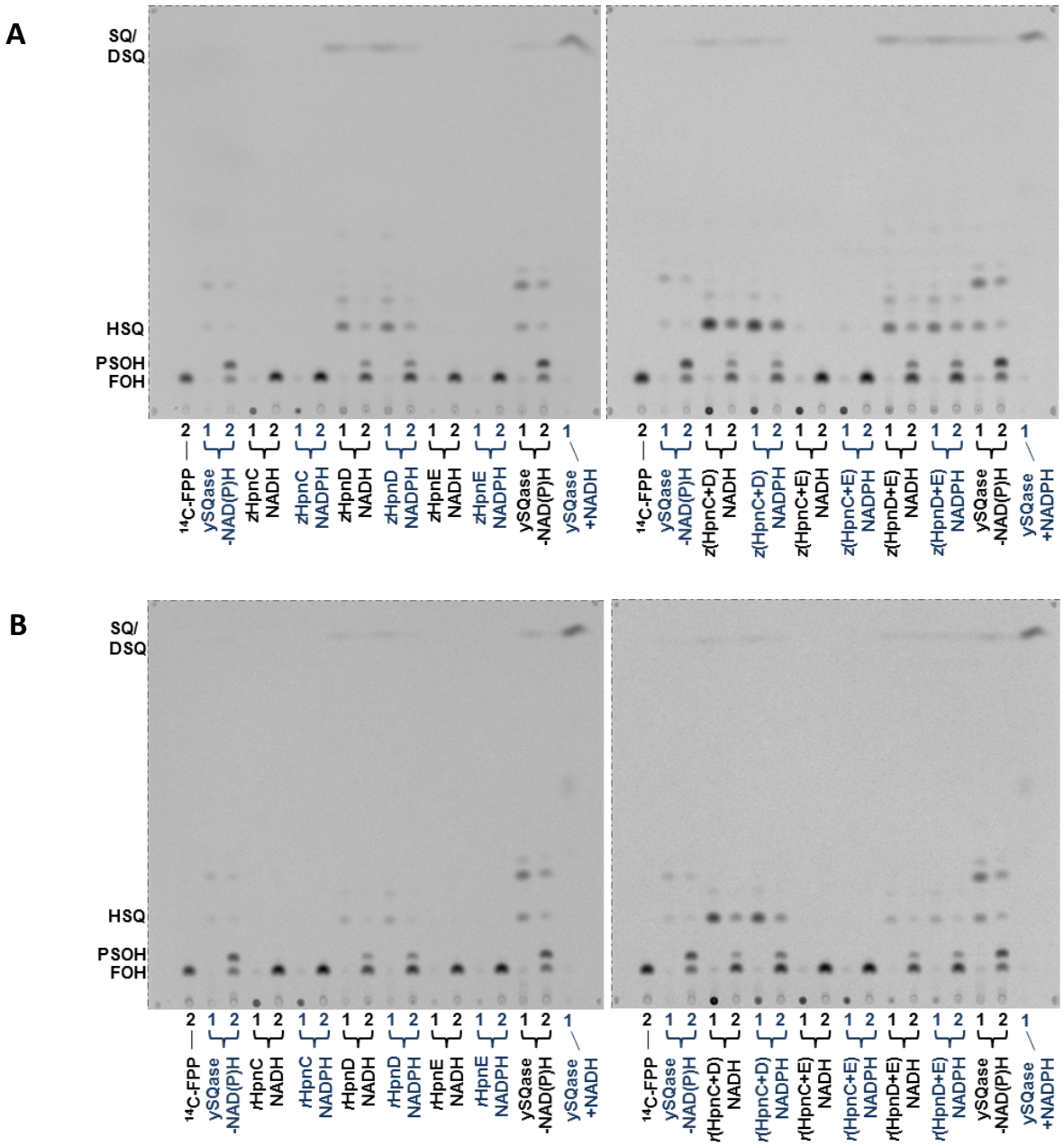


Figure S2. Radio-TLC (silica) assays for HpnCs, HpnDs and HpnEs from both *Z. mobilis* and *R. palustris* using [^{14}C]FPP in the presence of $MnCl_2$ combined with the presence or absence of NADH and NADPH. Top: HpnC-E from *Z. mobilis*. Bottom: HpnC-E from *R. palustris*. 1: 1st MTBE extraction directly after each reaction is finished; 2: 2nd MTBE extraction after the aqueous layer was treated with acid phosphatase to hydrolyze the diphosphate compounds.

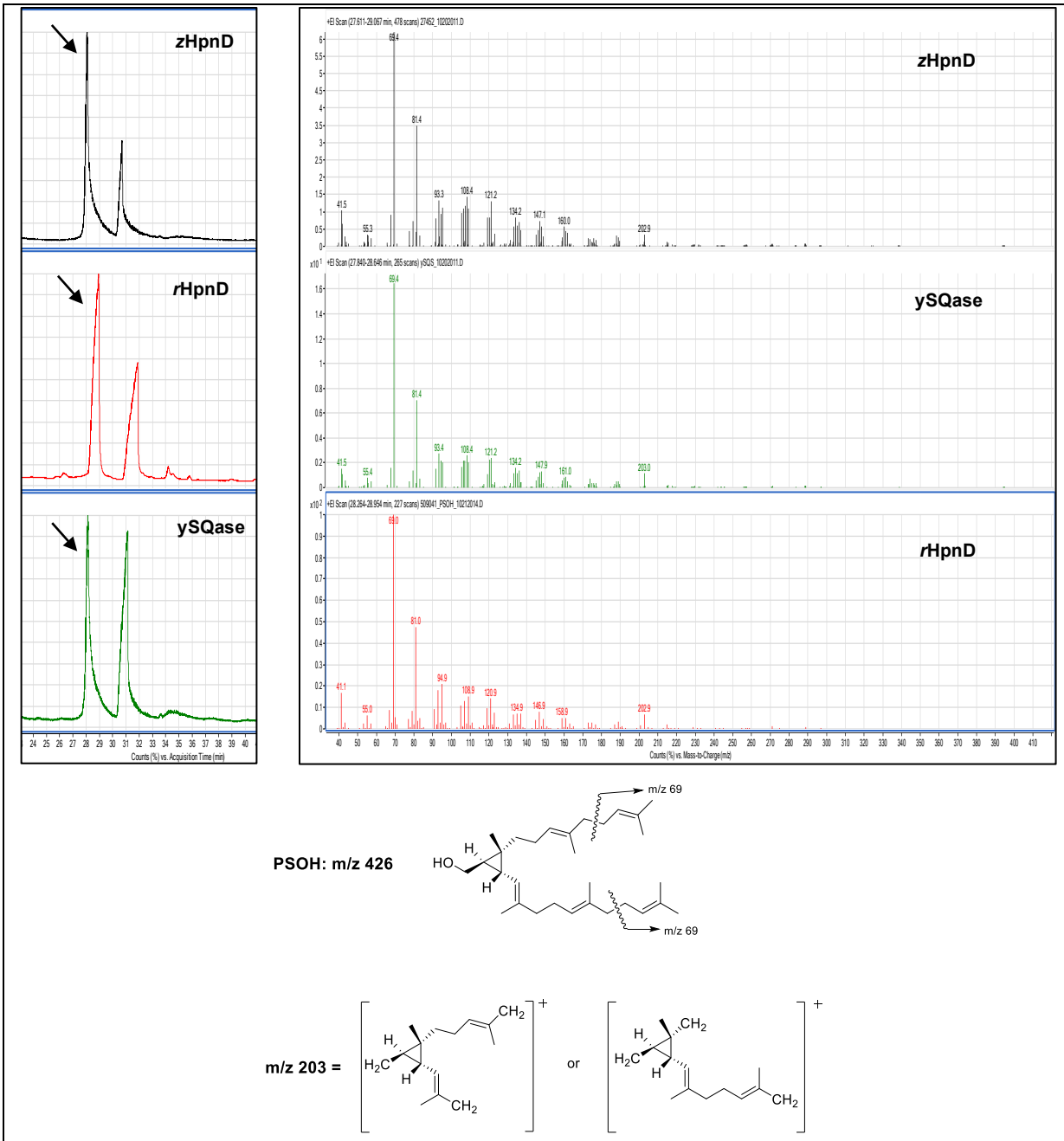
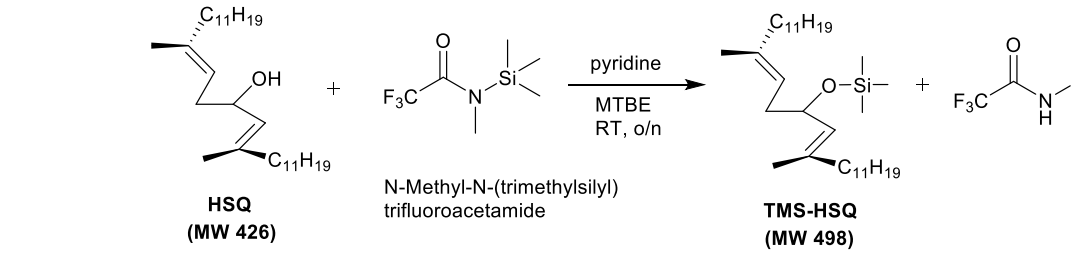


Figure S3. GC-MS analysis of PSPP products from HpnDs from both *Z. mobilis* and *R. palustris*. The PSPP products were hydrolyzed by acid phosphatase to produce PSOH for GC-MS analysis. Top left: GC chromatograms of PSOH (arrow indicated) from *z*HpnD, *r*HpnD and ySQase. Top right; MS spectra of the corresponding PSOH peaks from the GC chromatograms. Bottom: the prominent and distinct fragmentation m/z's of PSOH.

HSQ TMS-derivatization:



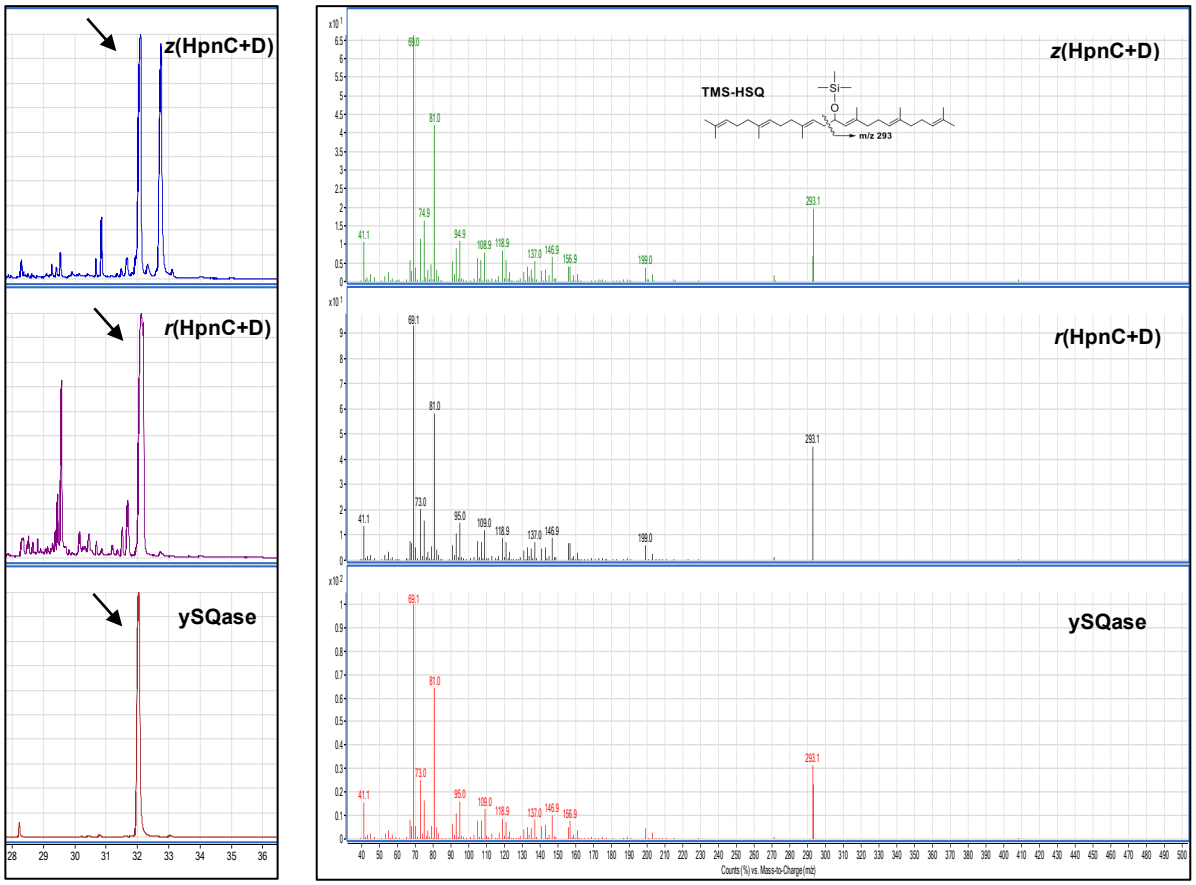


Figure S4. GC-MS analysis of trimethylsilyl-derivatized HSQ (TMS-HSQ) products from combinations of HpnC and HpnD from both *Z. mobilis* and *R. palustris*. Top: HSQ TMS-derivatization using *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide. Bottom left: GC chromatograms of TMS-HSQ (arrow indicated) from *z*(HpnC+HpnD), *r*(HpnC+HpnD) and ySQase. Bottom right: MS spectra of the corresponding TMS-HSQ peaks from the GC chromatograms.

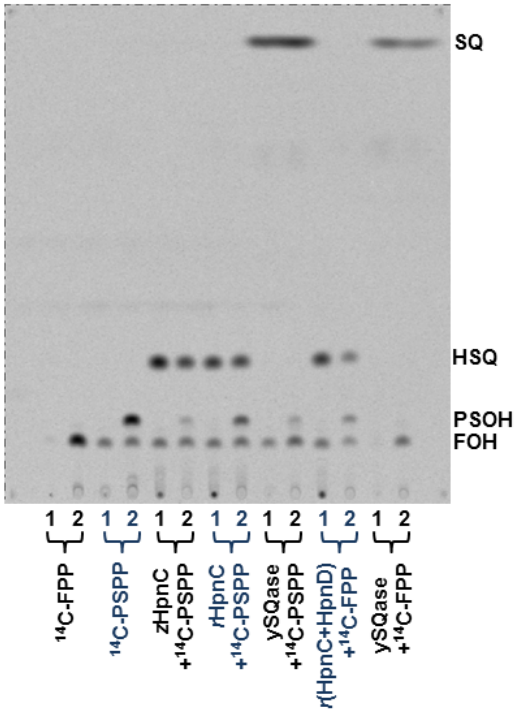


Figure S5. Radio-TLC (silica) assays for HpnCs from both *Z. mobilis* and *R. palustris* using [^{14}C]PSPP. [^{14}C]PSPP from an incubation of *r*HpnD and [^{14}C]FPP was extracted with water-saturated butanol. The butanol was removed on a SpeedVac. The residue was dissolved in 25 mM NH_4HCO_3 and used as a substrate for incubations with HpnC and yeast SQase. All reactions were carried out under standard incubation conditions and in the presence of NADH.

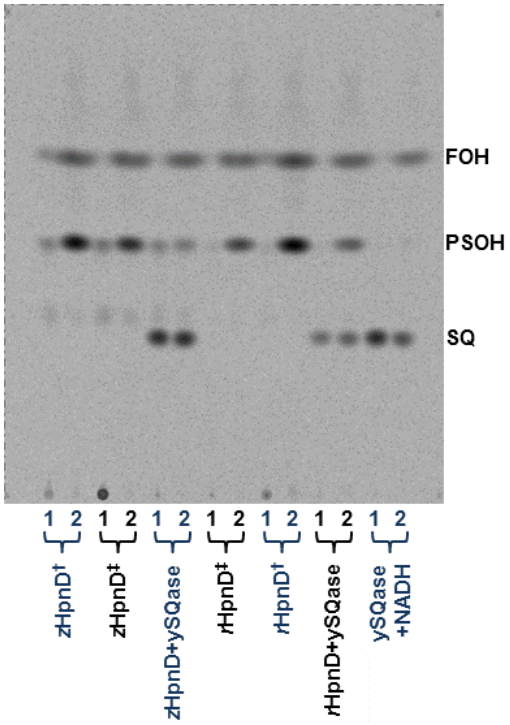


Figure S6. Radio-TLC (C18) assays for ySQase toward PSPP produced by HpnDs from both *Z. mobilis* and *R. palustris*. †HpnD incubations at 37 °C, 1 hr; ‡HpnD incubations at 37 °C, 2 hr. The one-hour incubations were then added ySQase and NADH, then continued incubation for another 1 hr. 1: 1st MTBE extraction directly after each reaction is finished; 2: 2nd MTBE extraction after the aqueous layer was treated with acid phosphatase to hydrolyze the diphosphate compounds.

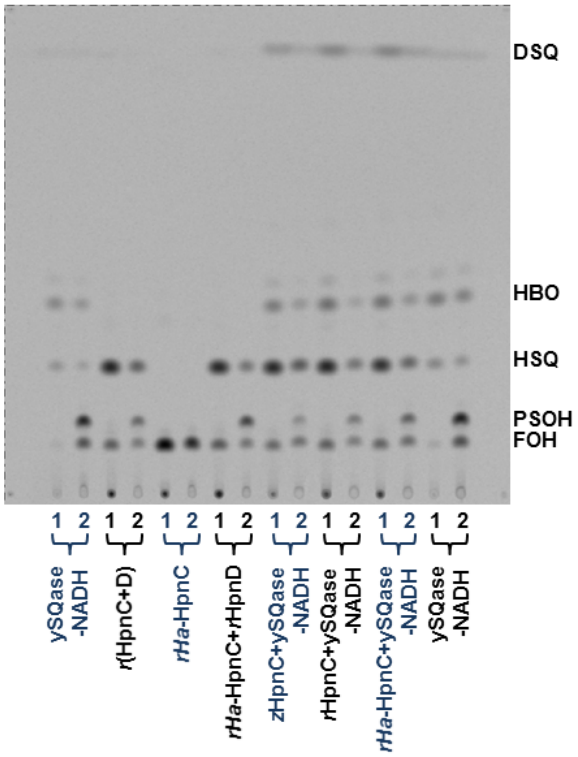
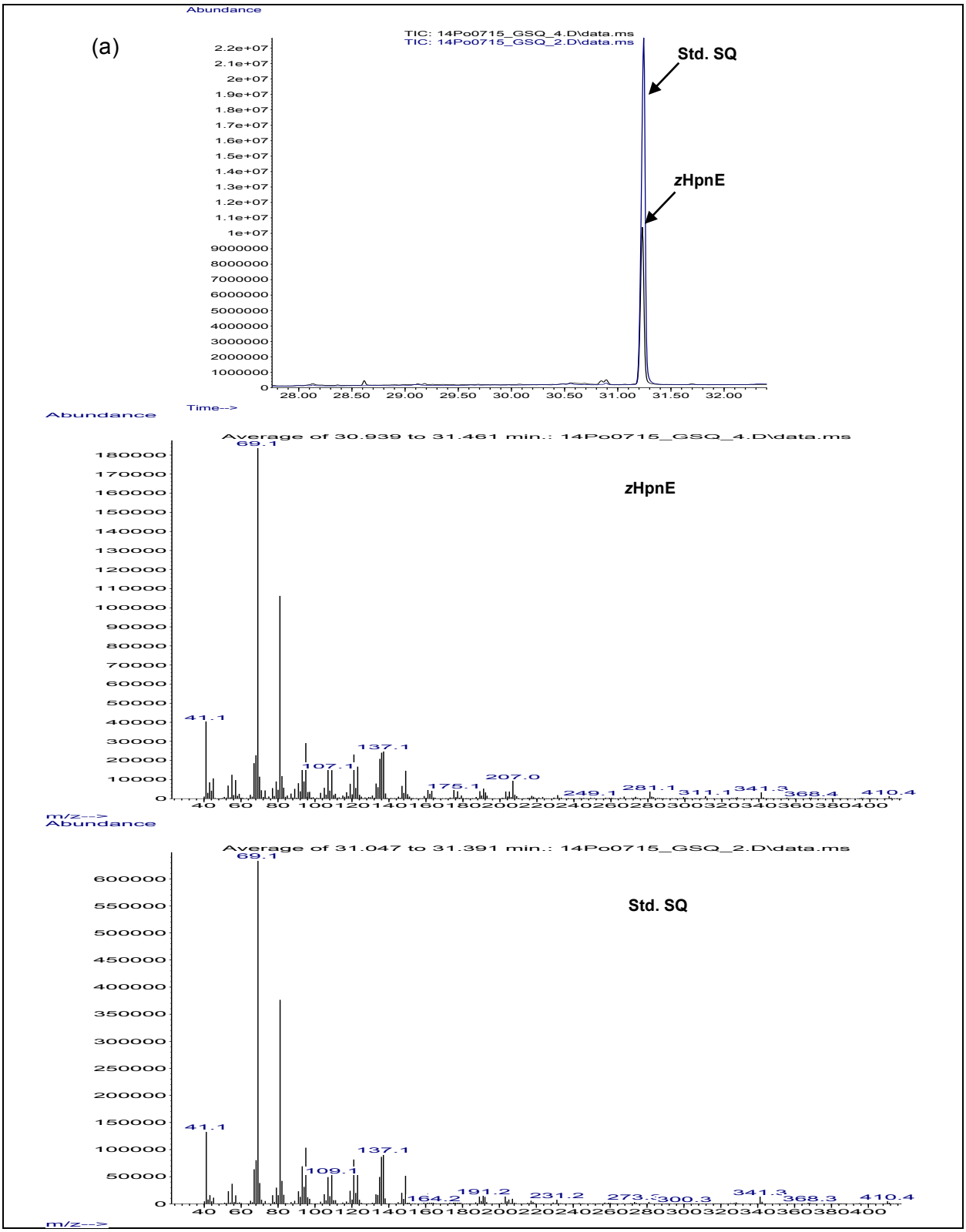


Figure S7. Radio-TLC (silica) assays for incubations of HpnCs from *Z. mobilis*, *R. palustris,* and the HaA2 strain of *R. palustris* with PSPP synthesized by ySQase (- NAD(P)H). *z*HpnC, *r*HpnC or *rHa*HpnC. ySQase was incubated with [^{14}C]FPP at 37 °C in buffer containing $MgCl_2$ for 5 min, followed by addition of *z*HpnC, *r*HpnC or *rHa*HpnC, and incubation was continued for 2 h. 1: Lane 1, samples were extracted with MTBE following incubation. Lane 2, aqueous layer from the extraction was treated with acid phosphatase and then extracted with MTBE. Note enhanced radioactivity for HSQ relative to HBO for incubations with ySQase and zHpnC, rHpnC, or rHaHpnC relative to incubations with ySQase.


(a)
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TIC: 14Po0715_GSQ_2.D\data.ms
Std. SQ
zHpnE
Time-->
Abundance
Average of 30.939 to 31.461 min.: 14Po0715_GSQ_4.D\data.ms
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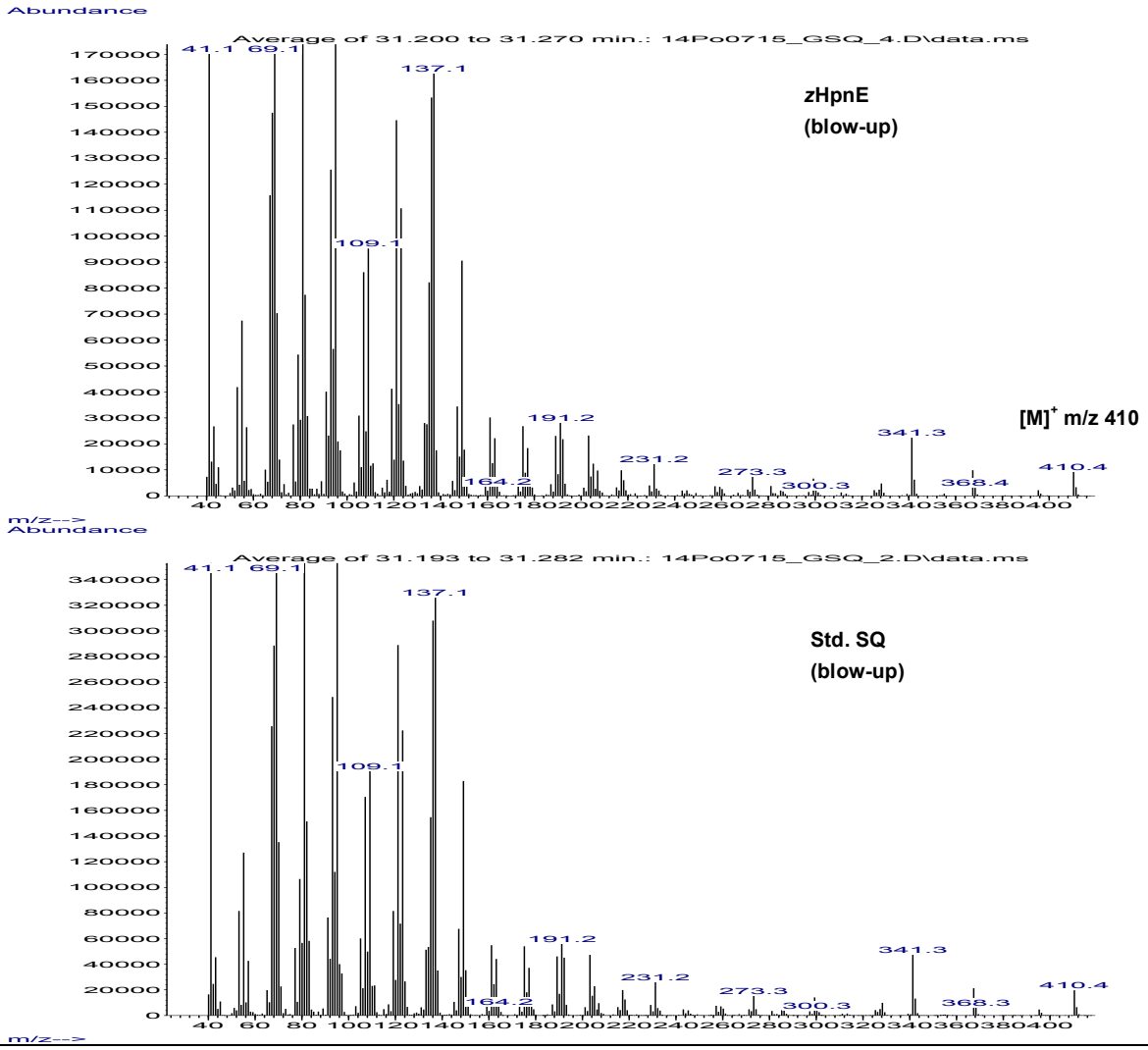
m/z-->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137.1
109.1
191.2
231.2
273.3
300.3
341.3
368.4
410.4
164.2
zHpnE
(blow-up)
[M]+ m/z 410
m/z-->
Abundance
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137.1
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191.2
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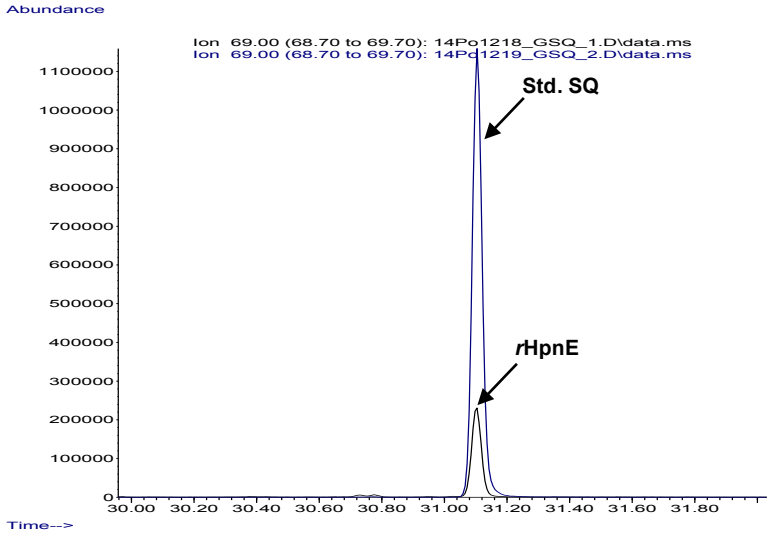
m/z-->

(b)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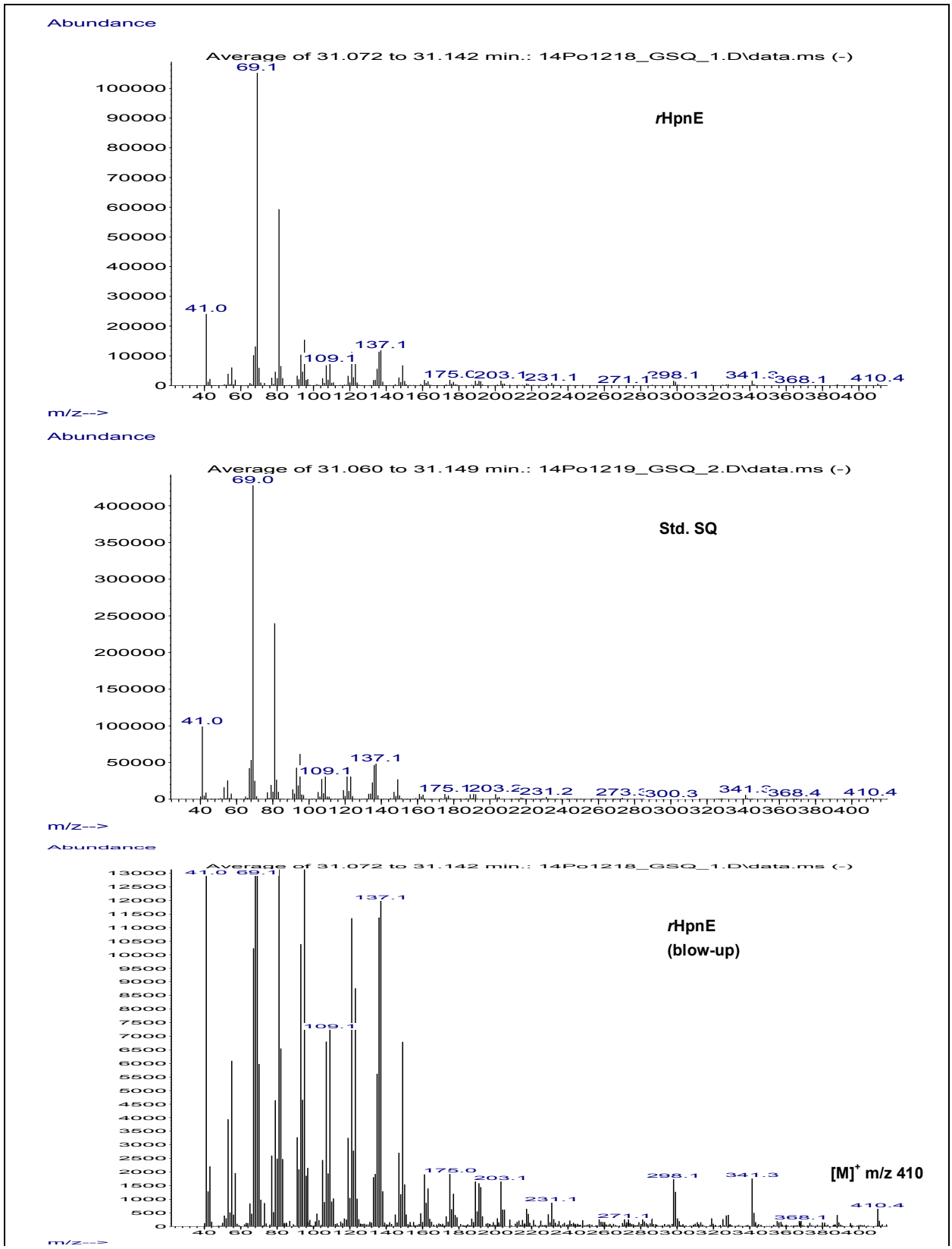
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Std. SQ
41.0
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m/z-->

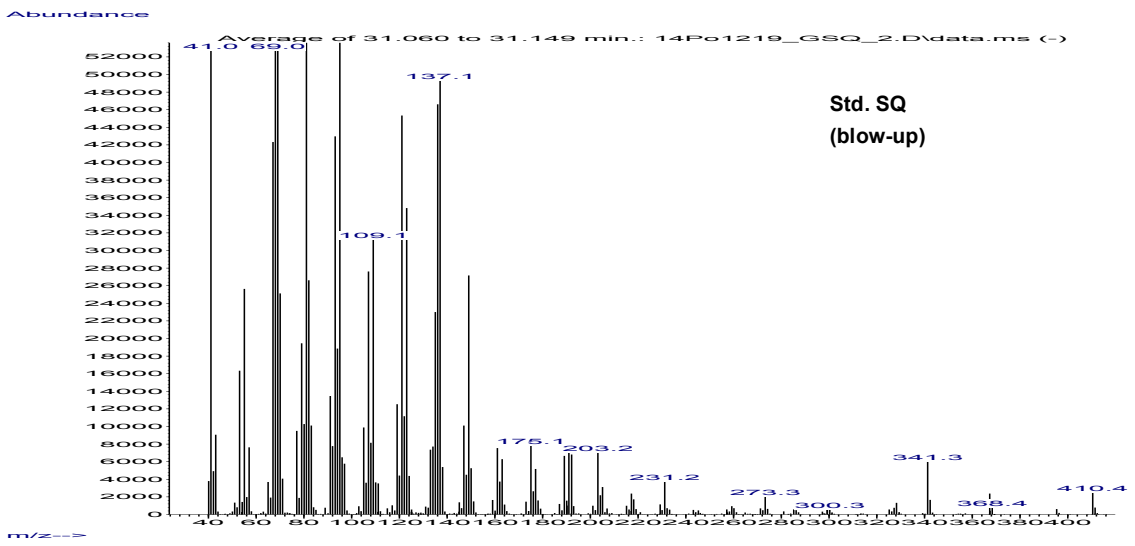

Figure S8. GC-MS analysis of the SQ product from HpnC-E reactions from (a) *Z. mobilis*, and (b) *R. palustris*.

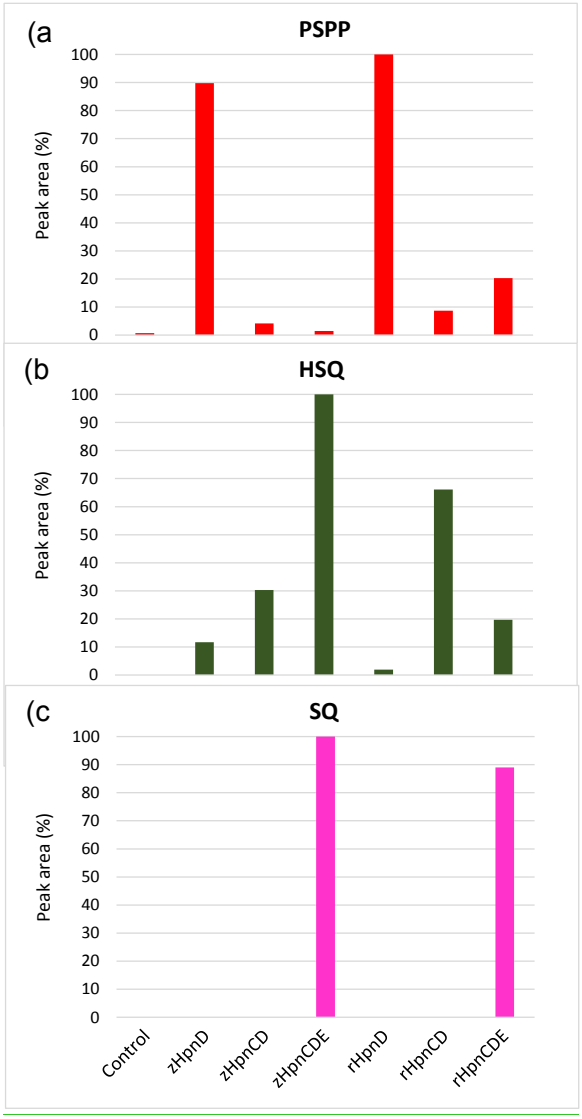

Figure S9. Distribution of PSPP, HSQ, and SQ in *E. coli hpnC*, *hpnC-hpnD*, and *hpnC-hpnD-hpnE* transformants of *Z. mobilis* (z) and *R. palustris* (r). Part a, *hpnC* transformants, part b, *hpnC-hpnD* transformants, and part c, *hpnC-hpnD-hpnE* transformats. The y-axis in each part shows the relative intensities of peaks at the appropriate m/z values for PSPP, HSQ, and SQ, normalized to the most intense signal for that metabolite.

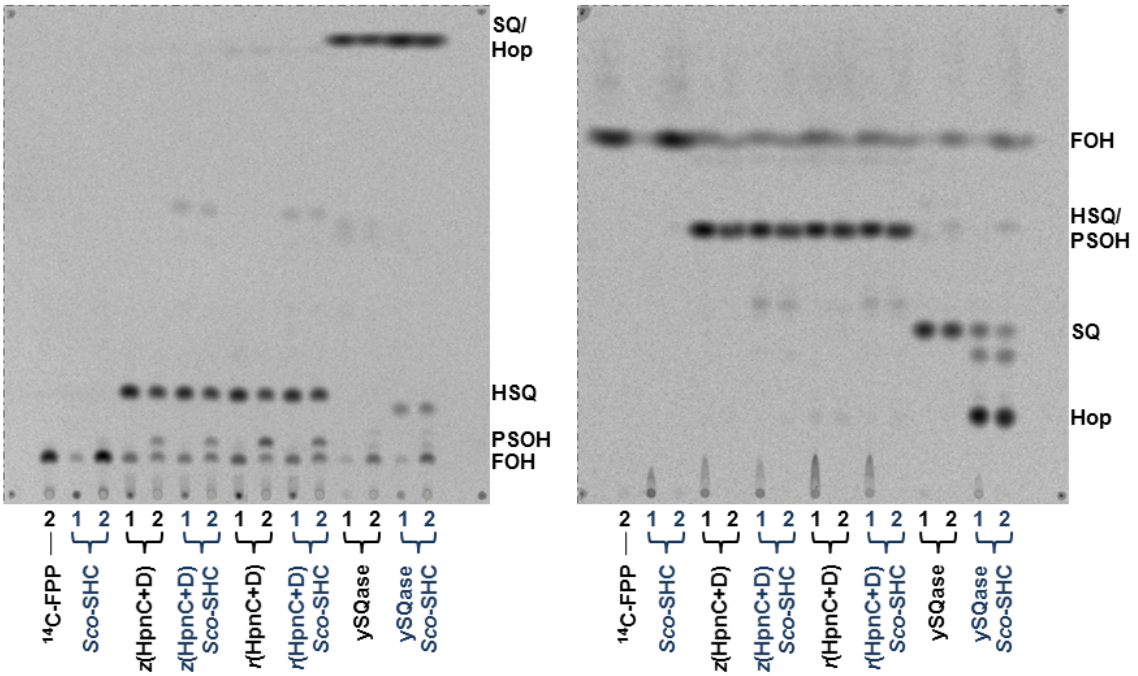

Figure S10. Radio-TLC activity assays for the putative squalene-hopene cyclase (SHC) from *S. coelicolor* A3(2) toward HSQ and SQ. Each reaction was analyzed on both Si- and C18-TLCs. *Sco*-SHC was co-incubated with *z*(HpnC+D) or *r*(HpnC+D) to check its activity toward HSQ; or co-incubated with ySQase to check its activity toward SQ. 1: 1st MTBE extraction directly after each reaction is finished; 2: 2nd MTBE extraction after the aqueous layer was treated with acid phosphatase to hydrolyze the diphosphate compounds.

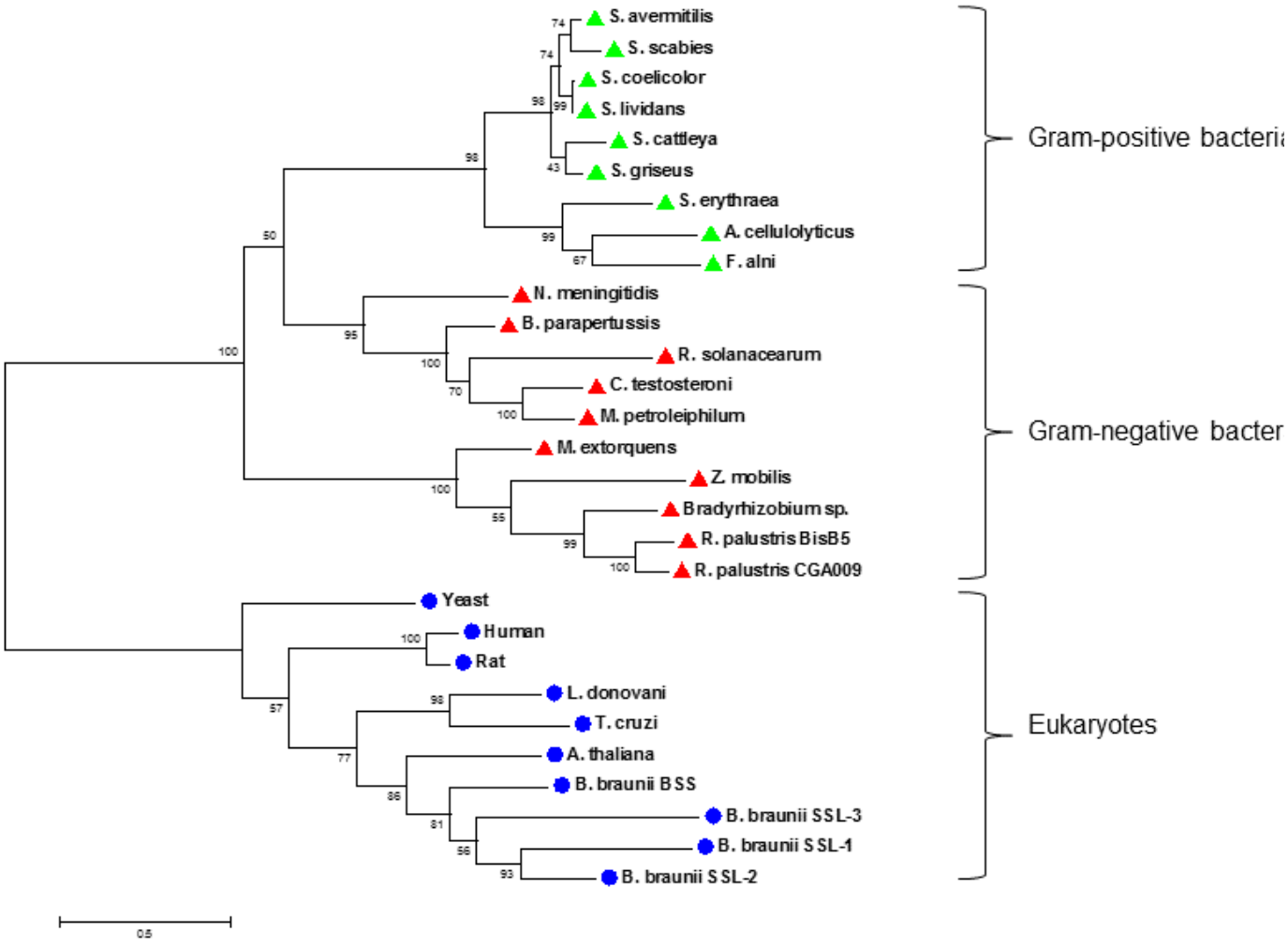

Figure S11. A representative maximum-likelihood phylogenetic tree for eukaryotic SQases (including *B. braunii* BSS, SSL-1, SSL-2 and SSL-3) and bacterial PSPPases (HpnDs). The protein sequences were aligned using the MUSCLE program (http://www.drive5.com/muscle/), and the phylogenetic tree was constructed using the MEGA software (http://www.megasoftware.net/) based on the Jones-Taylor-Thornton (JTT) model of amino acid evolution with default parameters. 500 bootstrap replicates were calculated, and the bootstrapped percentages were shown at each node. The scale bar represents 0.5 substitutions per site.