Title

Utilization of an intermediate of the methylerythritol phosphate pathway, (*E*)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate, as the prenyl donor substrate for various prenyltransferases

Running title

Utilization of HMBPP by prenyltransferases

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Abstract

(*E*)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate (HMBPP) is an intermediate of the methylerythritol phosphate pathway. Utilization of HMBPP by lycopene elongase from *Corynebacterium glutamicum*, which is a UbiA-family prenyltransferase responsible for C_{50} carotenoid biosynthesis, was investigated using an *Escherichia coli* strain that contained the exogenous mevalonate pathway as well as the carotenoid biosynthetic pathway. Inhibition of the endogenous methylerythritol phosphate pathway resulted in loss of the production of C_{50} carotenoid flavuxanthin, while C_{40} lycopene formation was retained. Overexpression of *E. coli ispH* gene, which encodes HMBPP reductase, also decreased the production of flavuxanthin in *E. coli* cells. These results indicate the preference of lycopene elongase for HMBPP instead of the previously proposed substrate, dimethylallyl diphosphate. Furthermore, several (all-*E*)-prenyl diphosphate synthases, which are classified in a distinct family of prenyltransferase, were demonstrated to accept HMBPP, which implies that the compound is more widely used as a prenyl donor substrate than was previously expected.

Keywords: isoprenoid; prenyltransferase; lycopene elongase; flavuxanthin; carotenoid.

Introduction

C_{50} carotenoids have been discovered in various organisms such as part of the bacteria of class Actinobacteria and most of the archaea of class Halobacteria.[1)] Their structures are varied and could be either cyclic or acyclic, but they generally have hydroxyl groups at both ends of a molecule, as with hydroxylated C_{40} carotenoids such as zeaxanthin and lutein. The biosynthetic routes of the C_{50} carotenoids are similar (Figure 1): 1) Phytoene, an acyclic, colorless C_{40} precursor of carotenoid, is formed from two molecules of geranylgeranyl diphosphate, and then is desaturated to form a reddish C_{40} carotenoid, lycopene. 2) Two C_5 isoprene units are condensed with lycopene at its terminal double bonds to form a C_{50} skeleton. 3) Cyclization occurs, if needed, at the termini of the lycopene moiety, which does not include newly added C_5 units. The ways hydroxyl groups are acquired, however, differ among organisms. Bacterioruberin, an acyclic C_{50} carotenoid from halophilic archaea such as *Halobacterium salinarum* and *Haloarcula japanica*, possesses four hydroxyl groups at its 1, 1', 3'', and 3''' positions. The two at the 1 and 1' positions are acquired when C_5 dimethylallyl groups are transferred from dimethylallyl diphosphate (DMAPP) to the C2 and C2' of lycopene by the action of the UbiA-family lycopene elongase Lye, which suggests that hydroxylation is the result of carbocation quenching by water.[2, 3)] The hydroxyl groups at the 3'' and 3''' positions are added by the action of a homolog of CruF-type carotenoid 1,2-hydratase to the transferred dimethylallyl groups. In contrast, bacterial C_{50} carotenoids generally have a hydroxyl group at the ω-methyl group of each of the transferred C_5 isoprene units, rather than at the termini of C_{40} skeletons, which are often cyclized. In other words, C_{50} carotenoids such as decaprenoxanthin, sarcinaxanthin, and C.p. 450 produced by *Corynebacterium glutamicum*, *Micrococcus luteus*, and *Dietzia* sp.

CQ4, respectively, possess 4-hydroxy-3-methylbut-2-enyl groups.[4-6] The acquisition of hydroxyl groups is thought to be catalyzed by lycopene elongase after the transfer of dimethylallyl groups from DMAPP, because the recombinant expression of *C. glutamicum* lycopene elongase CrtEb in a lycopene-producing *E. coli* strain results in the formation of monohydroxylated C_{45} acyclic carotenoid nonaflavuxantion and dihydroxylated C_{50} acyclic carotenoid flavuxanthin, both of which are the intermediates of decaprenoxanthin biosynthesis.[6, 7] This UbiA-family prenyltransferase, however, obviously has no homology to known hydroxylases that would specifically act toward the ω-methyl group of acyclic isoprenoids, such as geraniol 8-hydroxylase,[8, 9] farnesol ω-hydroxylase,[10] and methyl-branched lipid ω-hydroxylase.[11] Given these findings and the fact that some bacterial C_{50} carotenoid producers such as *C. glutamicum* and *M. luteus* possess the methylerythritol phosphate (MEP) pathway, we devised the simple idea that the prenyl donor substrate used by bacterial lycopene elongases is (*E*)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate (HMBPP), an intermediate of the MEP pathway, rather than DMAPP.

In this study, we intended to elucidate the prenyl donor substrate of lycopene elongase from *C. glutamicum*, by constructing a flavuxanthin-producing *E. coli* strain that also possesses the exogenous mevalonate (MVA) pathway, which enabled the inhibition of the endogenous MEP pathway. In addition, the effect of the overexpression of HMBPP reductase on carotenogenesis was investigated. We also evaluated the ability of HMBPP to act as a substrate for (all-*E*)-prenyl diphosphate synthases, which utilize DMAPP as a proper substrate and are known to catalyze the carbocation-involving prenyltransfer reaction.

Materials and Methods

Cultivation of microorganisms and preparation of genome DNA– *C. glutamicum* was provided by Dr. Takaaki Kojima, Nagoya University, and was cultivated in a 5 mL of medium containing 5 g/L polypeptone, 2 g/L NaCl, 10 g/L glucose, 1 g/L K_2HPO_4, 200 mg/L $Mg_2SO_4 \cdot 7H_2O$, and 3 g/L beef extract, at 30 °C overnight prior to being harvested. *Lactobacillus brevis* was provided by the RIKEN BRC through the Natural Bio-Resource Project of the MEXT, Japan, and was cultivated in a 5 mL of MRS medium containing 5 g/L Bacto Peptone (Difco), 1 g/L beef extract, 5 g/L yeast extract, 20 g/L glucose, 1 g/L Tween 80, 2 g/L K_2HPO_4, 5 g/L CH_3COONa, 2 g/L ammonium citrate, 200 mg/L $Mg_2SO_4 \cdot 7H_2O$, and 50 mg/L $MnSO_4 \cdot 5H_2O$, at 30 °C overnight prior to being harvested. *Flavobacterium johnsoniae* was cultivated as described elsewhere.[12] The *E. coli* Top10 strain was cultivated in a LB medium. Genome DNA was extracted from the microorganisms using a Cica Geneus® DNA extraction reagent (Kanto Chemical, Japan). Genome DNA of *Saccharomyces cerevisiae* was extracted as previously reported.[13]

Construction of plasmids– The gene of *C. glutamicum* lycopene elongase, *crtEb*, was amplified from *C. glutamicum* genome using KOD plus DNA polymerase (Toyobo, Japan) and origonucleotide primers 5'-attcgagctcggtacaaggagatatattatgatggaaaaaataagactaatt-3' and 5'-aggatccccgggtaccttatatctgatgaattgctattagc-3'. The amplified DNA fragment was inserted into a *Kpn*I-treated pBAD18 plasmid using an InFusion HD Cloning Kit (TaKaRa Bio, Japan) to construct pBAD-*crtEb*. Then the region containing carotenogenic genes from *Pantoea ananatis*, i.e., *crtE* (geranylgeranyl diphosphate

synthase gene), *crtB* (phytoene synthase gene), and *crtI* (phytoene desaturase gene), was amplified from the plasmid pACYC-IBE[14] using primers 5'-ttcatcagatataagttataaggacagcccgaatgac-3' and 5'-ctagaggatccccggtacctagagcgggcgctgc-3'. The amplified fragment was inserted in a *Kpn*I-treated pBAD-*crtEb* using an InFusion HD Cloning Kit to construct pBAD-*crtEbEIB*.

The *E. coli ispH* gene encoding HMBPP reductase was amplified from *E. coli* genome using origonucleotide primers 5'-cagcgcccgctctaggaaggaagattataatgcagatcctgttggcc-3' and 5'-tctagaggatccccggtaccttaatcgacttcacgaatatcgac-3'. The amplified fragment was inserted in a *Kpn*I-treated pBAD-*crtEbEIB* using an InFusion HD Cloning Kit to construct pBAD-*crtEbEIB-ispH*.

Extraction of carotenoids produced in E. coli – The *E. coli* Top10 strain was transformed using the pBAD-*crtEbEIB* plasmid. The Top10/pBAD-*crtEbEIB* cells were cultivated overnight at 37°C in 1 L of a LB medium supplemented with 100 mg/L ampicillin and 0.02% L-arabinose. Harvested cells were dissolved with 10 mL of methanol/0.3% NaCl (9:1) solution and disrupted by sonication. Lipid containing carotenoids was extracted from the solution with 10 mL *n*-pentane. The solvent was evaporated under a N_2 stream, and the residual was dissolved with 10 mL 2-propanol. The sample was analyzed by normal-phase TLC using a silica gel 60 F_{254} plate (Merck Millipore). Carotenoid extraction from Top10/pBAD-*crtEbEIB-ispH* cells was performed with the same procedure.

FAB-MS analysis of C_{50} carotenoid– The solution of carotenoid extracted by *n*-pentane

from Top10/pBAD-*crtEbEIB* was concentrated under a N_2 stream and dissolved with an excess volume of acetone. The acetone-soluble fraction was spotted on a normal-phased TLC using a partisil LK6 plate (Whatmann), and the plate was developed with hexane/acetone (2:1). An orange-colored spot with a R_f of ~0.5 was scraped from the plate, and the carotenoid that it contained was eluted with acetone. After the solvent was removed by evaporation, the residual was subjected to fast-atom bombardment (FAB)-MS analysis, which was performed with a MStation JMS-700 mass spectrometry system (JEOL, Japan) using a 6 keV Xe beam at a 10 kV accelerating voltage. The positive FAB-MS spectrum was obtained using 3-nitrobenzylalcohol as a matrix.

HPLC analysis of carotenoids– One hundred μL of the carotenoid sample dissolved in 2-propanol was analyzed with a Shimadzu Prominence HPLC system equipped with a SPD-20A UV-visible detector and a SPD-M20A photodiode array (PDA) detector. A COSMOSIL $5C_{18}$-PAQ column (4.6 ID×250 mm, Nacalai Tesque, Japan) was connected, and samples were eluted with an isocratic methanol/2-propanol (1:1) mixture with flow rate of 0.5 mL/min.

Carotenoid production under the MEP pathway inhibition – Top10/pBAD-*crtEbEIB* was transformed using a pJBEI-2999 plasmid [15], which contained MVA pathway-related genes from *S. cerevisiae* and was purchased from Addgene, MA. The cells were cultivated at 37°C in 100 mL of a LB medium supplemented with 100 mg/L ampicillin, 30 mg/L chloramphenicol, 2 g/L lithium acetate, and 0.02% arabinose. When OD_{600} reached ~1.0, 5 mg of FR-900098 monosodium salt (Sigma Aldrich) was added into the medium, and the cells were harvested after an additional 24 h of cultivation.

Construction of plasmids for the expression of (all-E)-prenyl diphosphate synthases– The gene of a hypothetical (all-*E*)-geranylgeranyl diphosphate synthase (GGPS) from *F. johnsoniae*, *fjoh_1263*, was amplified from the *F. johnsoniae* genome using oligonucleotide primers 5'-ggtgccgcgcggcagccatatgcacgatattagccagtacc-3' and 5'-cagccggatcctcgagcatacttaaacttttctacccattaaattttc-3'. The gene of *C. glutamicum* GGPS, *idsA*,[16] was amplified from the *C. glutamicum* genome using oligonucleotide primers 5'-ggtgccgcgcggcagccatatgaaggacgtctccttgagc-3' and 5'-cagccggatcctcgagcatactacatccgacgttcggttg-3'. The gene of (all-*E*)-farnesyl diphosphate synthase (FPS) from *L. brevis*,[17] *lvis_0975*, was amplified from the *L. brevis* genome using oligonucleotide primers 5'-ggtgccgcgcggcagccatatgccgattaatgcacgg-3' and 5'-cagccggatcctcgagcatacttagttcacccgttccgtatc-3'. The gene of *S. cerevisiae* FPS, *erg20*,[18] was amplified from the *S. cerevisiae* genome using oligonucleotide primers 5'-ggtgccgcgcggcagccatatggcttcagaaaaagaaattagg-3' and 5'-cagccggatcctcgagcatacctatttgcttctcttgtaaactttg-3'. By electroporation, the *Nde*I-treated pET-15b plasmid (Novagen) and each of the amplified DNA fragments were simultaneously introduced into the *E. coli AQ3625* strain, which was provided by the National BioResource Project and enabled *in vivo E. coli* cloning (iVEC, https://shigen.nig.ac.jp/ecoli/strain/). A portion of the colonies selected with ampicillin-containing plates contained plasmids with each gene appropriately inserted. The plasmids for the expression of *F. johnsoniae* hypothetical GGPS, *C. glutamicum* GGPS, *L. brevis* FPS, and *S. cerevisiae* FPS were designated as pET15b-*fjggps*, pET15b-*cgggps*, pET15b-*lbfps*, and pET15b-*scfps*, respectively.

Recombinant expression and purification of (all-E)-prenyl diphosphate synthases– Each of the plasmids for the expression of (all-*E*)-prenyl diphosphate synthases were introduced into the *E. coli* BL21(DE3) strain. The transformed *E. coli* cells were cultivated at 37°C in a 200 mL LB medium supplemented with 100 mg/L ampicillin. When OD_{600} reached 0.4, 1 mM IPTG was added for induction. Then, the cells were cultivated overnight at 20°C. The enzymes from the harvested cells was purified using a 1 mL HisTrap FF crude affinity column (GE Healthcare) following the manufacturer's protocol, using 20 and 500 mM imidazole for the binding and elution buffers, respectively. Recombinant expression and purification of *Geobacillus stearothermophilus* FPS, *Sulfolobus acidocaldarius* GGPS, and *Aeropyrus pernix* (all-*E*)-geranylfarnesyl diphosphate synthase (GFPS) were performed as described elsewhere.[19-21)]

Assay of (all-E)-prenyl diphosphate synthases– Each of the purified enzymes was reacted with 5 nmol DMAPP or HMBPP (Sigma Aldrich) and 5 nmol [1-^{14}C]IPP (2.2 Ci/mol, American Radiolabeled Chemicals Inc.) for 1 h, in a 200 μL volume of reaction mixture containing 250 μmol $MgCl_2$ and 20 μmol of a Tris-HCl buffer, pH8.5, for *G. stearothermophilus* FPS, 20 μmol of a MES-NaOH buffer, pH5.8, for *S. acidocaldarius* GGPS, or a MOPS-MaOH buffer, pH7.0, for the other enzymes. The reaction temperature was 55°C for *G. stearothermophilus* FPS, *S. acidocaldarius* GGPS, and *A. pernix* GFPS, and 30°C for the other enzymes. The amounts of the enzymes were controlled so that no more than 20% of the substrates reacted. After the reaction, 200 μL of saturated saline and 600 μL of 1-butanol saturated with saline were added, and

the reaction products were extracted into the 1-butanol layer. Radioactivity in an aliquot of the 1-butanol layer was measured with an LSC-5100 liquid scintillation counter (Aloka, Japan). The radioactivity extracted in the butanol layer was used to calculate the specific activity of the (all-*E*)-prenyl diphosphate synthases, which is expressed by an unusual unit: nmol IPP·min^{-1}·mg protein.

For the kinetic analysis of *S. acidocaldarius* GGPS, 33 ng of the enzyme was used for the reaction with 1-20 μM DMAPP against 25 μM IPP; 330 ng of the enzyme was used for the reaction with 5-80 μM HMBPP against 25 μM IPP; 6.3 ng of the enzyme was used for the reaction with 1-20 μM IPP against 25 μM DMAPP; and, 33 ng of the enzyme was used for the reaction with 1-20 μM IPP against 25 μM HMBPP. The enzyme amounts were set to assure that no more than 20% of the substrates would react. Kinetic parameters were calculated using a KaleidaGraph software (Synergy software) by fitting the Michaelis-Menten equation to the data.

Analysis of the products of (all-E)-prenyl diphosphate synthases– For the radio-TLC analysis of the reaction products, 0.2 nmol [1-^{14}C]IPP and either 0.2 nmol DMAPP or HMBPP were used for the reaction. Other conditions were the same as those used in the enzyme assay to obtain specific activity. When significant radioactivity was extracted with 1-butanol, the reaction products in the butanol layer were hydrolyzed with potato acid phosphatase (Sigma Aldrich) using a method developed by Fujii et al.[22] The resultant alcohols were analyzed by reversed-phase TLC using a precoated RP18 plate (Merck Millipore) developed with acetone/water (9:1).

The products of *S. acidocaldarius* GGPS were analyzed using an Esquire 3000 electron spray ionization (ESI)-ion trap MS (Bruker Daltonics) by direct infusion.

The butanol-extracted products from the reaction of 3.3 μg *S. acidocaldarius* GGPS with 10 nmol non-labeled IPP and either 10 nmol DMAPP or HMBPP were evaporated under a N_2 stream and then dissolved with acetonitrile/100 mM NH_4CO_3 (3:1). The samples were injected with a flow rate of 180 μL/h and analyzed using the parameters below: analysis mode, negative; sheathe gas, N_2 (10 psi); dry gas, N_2 (5.0 $L \cdot min^{-1}$, 300°C); scan range, 100-1500 *m/z*; scan speed, 13,000 $m/z \cdot sec^{-1}$; ion charge control target, 20,000; and, maximum accumulation time, 200 ms.

Results

Prenyl donor substrate used by lycopene elongase The *E. coli* strain producing a C_{50} carotenoid, flavuxanthin, was constructed by introducing *crtEb*, the gene of lycopene elongase from *C. glutamicum*, into *E. coli* with other carotenogenic genes, which was sufficient for lycopene production. For this purpose, the *C. glutamicum* *crtEb* gene was first inserted into the pBAD18 vector to construct pBAD-*crtEb*. Then, carotenogenic genes from *P. ananatis*, *crtE*, *crtB*, and *crtI*, encoding geranylgeranyl diphosphate synthase, phytoene synthase, and phytoene desaturase, respectively, were inserted into pBAD-*crtEb* to construct pBAD-*crtEbEBI*. The carotenoids produced in *E. coli* harboring the plasmid were separated by normal-phase TLC (Figure 2A). Two major spots, which were colored orange and thus expected to be derived from carotenoids, were observed. One of the spots co-migrated with the spot of authentic lycopene, with a R_f of ~0.95. In contrast, the other one had a R_f of ~0.63, which suggested a more hydrophilic property. Thus, a corresponding spot was scraped from a TLC plate, on which a larger amount of the sample had been loaded, in order to recover the probable carotenoid compound via elution with acetone. The recovered compound was analyzed via FAB-MS and gave a positive ion with an *m/z* of 705, which corresponded well with the $[M+H]^+$ ion of a dihydroxylated C_{50} carotenoid, flavuxanthin, which was the expected product of *C. glutamicum* lycopene elongase (Figure 2B).

To investigate the substrate preference of lycopene elongase, we constructed an *E. coli* strain in which both the C_{50} carotenoid biosynthetic pathway and the MVA pathway were introduced. The strain Top10/pBAD-*crtEbEBI*/pJBEI-2999 allowed us to inhibit the endogenous MEP pathway using a drug without killing the microorganism,

to evaluate the effect of the depletion of HMBPP on carotenogenesis. The carotenoids produced in the strain, which were grown in a medium supplied with lithium acetoacetate to enhance isoprenoid biosynthesis via the MVA pathway, were analyzed via HPLC equipped with UV-visible and PDA detectors. The elution profile of the sample at 472 nm gave two major peaks and one minor peak (Figure 2C). One of the major peaks eluted at ~8 min, which corresponded with the elution time of flavuxanthin recovered from TLC, and the other eluted at ~12 min, which corresponded with the peak of authentic lycopene. The UV-visible spectrum of the compound eluted at ~8 min, which was measured by the PDA detector, had maximum absorption peaks at 469 and 500 nm, which was in good agreement with the reported spectrum of flavuxanthin[5] (Figure 2D). The minor peak that eluted at ~10 min remains unidentified, but was likely derived from a C_{45} carotenoid, nonaflavuxanthin, which is an intermediate of C_{50} carotenoid biosynthesis. Inhibition of the MEP pathway was conducted using an inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase, FR-900098.[23] To avoid significant growth inhibition, the inhibitor was added when OD_{600} reached ~1.0, which enabled the same level of growth as that in the absence of the inhibitor. The elution profile of the carotenoids from the cells demonstrated that the inhibition of the MEP pathway caused nearly a complete loss of flavuxanthin, in contrast with the retention of about half of the lycopene production. A slight amount of flavuxanthin still formed, which could have accumulated prior to inhibition because a slight red coloration of the cells was observed when the inhibition began. There was no production of new carotenoids due to the inhibition. The selective decrease in flavuxanthin strongly suggested that HMBPP, which would be depleted by the inhibition of the MEP pathway, was utilized as the prenyl donor substrate by *C. glutamicum* lycopene elongase.

At that point, however, we could not exclude the possibility that the MEP-pathway inhibitor FR-900098 also inhibits lycopene elongase. To clarify this problem, *in vitro* assay of lycopene elongase was performed using a crude extract from the *E. coli* Top10/pBAD-crtEb strain, but the production of C_{50} (or C_{45}) carotenoids from lycopene was not detected when either HMBPP or DMAPP was used as the prenyl donor substrate (data not shown). We also conducted NMR analysis of flavuxanthin extracted from the Top10/pBAD-*crtEbEBI*/pJBEI-2999 strain fed with [1-^{13}C]glucose, to establish the labeling pattern of all C_5 units in the carotenoid in order to accurately determine if each of the isoprene units was derived from either the MEP or MVA pathways. However, the results were unclear because of an inadequate amount of purified flavuxanthin (data not shown). Finally, we tried to control the metabolic flow through the MEP pathway to see its effect on carotenogenesis. The overexpression of an HMBPP-metabolizing enzyme is considered to reduce the intracellular concentration of HMBPP. *E. coli ispH* gene encoding HMBPP reductase, which can convert HMBPP into IPP and DMAPP, was additionally inserted into the plasmid for flavuxanthin production. The comparison of carotenoids produced in *E. coli* cells transformed with the new plasmid pBAD-*crtEbEBI-ispH* and those with the original plasmid pBAD-*crtEbEBI* demonstrated that the overexpression of HMBPP reductase caused significant decrease in a flavuxanthin/lycopene ratio (Figure 3). This result also supported our hypothesis that *C. glutamicum* lycopene elongase uses HMBPP as the prenyl donor substrate.

Substrate specificities of (all-*E*)-prenyl diphosphate synthases The use of HMBPP as a prenyl donor substrate has been reported with *trans*-zeatin synthase from

Agrobacterium tumefaciens, which catalyzes the transfer of a C_5 prenyl group to AMP and prefers HMBPP to DMAPP as the donor substrate for the reaction.[24-26] The utilization of HMBPP by lycopene elongase is, however, still surprising because a few structural studies have proposed an S_N1-type carbocation mechanism for UbiA-family prenyltransferases,[27, 28] in contrast to trans-zeatin synthase that is classified in *p*-loop containing nucleoside triphosphate hydrolase superfamily and has been suggested to catalyze prenyltransfer reaction with an S_N2-type concerted mechanism[25] (Figure 4). If we can ignore the difference in the affinity with which a prenyltransferase recognizes a substrate, the preference for HMBPP to DMAPP through the S_N1-type reaction seems curious because the existence of an electron-withdrawing 4-hydroxyl group can destabilize the carbocation intermediate. Therefore, we attempted to evaluate the capability of such prenyltransferases to accept HMBPP as the donor substrate instead of DMAPP, their natural substrate. For this purpose, we prepared several short-chain (all-*E*)-prenyl diphosphate synthases: FPSs from *S. cerevisiae*[18], *L. brevis*[17], and *G. stearothermophilus*[29]; GGPSs from *S. acidocaldarius*[30] and *C. glutamicum*[16], GFPS from *A. pernix*[31]; and, hypothetical GGPS from *F. johnsoniae*. These synthases accept DMAPP as the primary donor substrate for producing (all-*E*)-prenyl diphosphates with various carbon chain lengths via consecutive condensation of IPP. Importantly, *trans*-prenyltransferase-family enzymes including (all-*E*)-prenyl diphosphate synthases are considered to employ the S_N1-type mechanism.[32-35] Among the prepared enzymes, *L. brevis* FPS, *G. stearothermophilus* FPS, and *C. glutamicum* GGPS showed no activity toward HMBPP, but they accepted DMAPP as the substrate (data not shown). Therefore, specific activities of the other enzymes toward 5 μM HMBPP or DMAPP and 5 μM IPP were measured to compare their preferences for the prenyl donor substrates (Table 1).

The ratios of the specific activities toward HMBPP and DMAPP were relatively high for the archaeal enzymes, at 0.58 for *A. pernix* GFPS and 0.16 for *S. acidocaldarius* GGPS, but lower for *F. johnsoniae* hypothetical GGPS and *S. cerevisiae* FPS, at 0.018 and 0.011, respectively. Kinetic studies were conducted only with *S. acidocaldarius* GGPS. As shown in Table 2, the K_M for HMBPP was about 3-fold larger than that for DMAPP, while the K_M for IPP against 25 μM HMBPP was 4-fold smaller than that against DMAPP. When HMBPP was used as the substrate, the value for k_{cat} was 6 to 16-fold smaller than that when DMAPP was used. Overall, the 4 to 18-fold lower k_{cat}/K_M of the reaction with HMBPP compared with that of the reaction with DMAPP was mainly caused by the difference in k_{cat}, rather than that in K_M.

The products of the HMBPP-accepting enzymes were analyzed by radio-TLC after phosphatase treatment. When DMAPP was used as the donor substrate, the chain lengths of the major products from *F. johnsoniae* hypothetical GGPS, *A. pernix* GFPS, *S. acidocaldarius* GGPS, and *S. cerevisiae* FPS were C_{30}, C_{25}, C_{20}, and C_{15}, respectively (Figure 5A). Contrary to our previous expectation, and also to annotations on public databases such as KEGG, the hypothetical GGPS from *F. johnsoniae* was shown to be (all-*E*)-hexaprenyl diphosphate synthase (HexPS), which might be involved in the biosynthesis of menaquinone-6.[36] When HMBPP was used, the major products of *F. johnsoniae* HexPS, *A. pernix* GFPS, and *S. acidocaldarius* GGPS seemed to get shorter in that order, probably by one isoprene unit (Figure 5B). The radioactive spots from the products of *S. cerevisiae* FPS from HMBPP were invisible, although a sufficient amount of radioactivity existed before pentane extraction, which was conducted to extract the alcohols obtained from the phosphatase treatment of the products. The disappearance of the spots from the products of *S. cerevisiae* FPS, and also the fact that spots from the

products of *S. acidocaldarius* GGPS were thinner than that expected from radioactivity existed before pentane extraction, was likely caused by the hydrophilic properties of the alcohols due to the additional hydroxyl groups, which prevented them from being efficiently extracted into the pentane layer. Only the products of *S. acidocaldarius* GGPS, in diphosphate forms, were analyzed by negative ESI-MS via the direct infusion technique. In the ion spectrum of the sample from the reaction with HMBPP, an ion peak with an m/z of 464.9, which corresponded well with the $[M-H]^-$ ion of hydroxylgeranylgeranyl diphosphate, was observed (Figure 5C). Other major ion peaks in the spectrum were also observed in the spectrum of the sample from the reaction with DMAPP, in which we could detect the ion with an m/z of 448.5 derived from geranylgeranyl diphosphate (data not shown). Moreover, the MS/MS analysis of the m/z 464.9 ion gave fragmentation ions with m/z values of 446.9 and 158.6, which corresponded with $[M-H_2O-H]^-$ and $[P_2O_6H]^-$, respectively (Figure 5D). These results suggest that the major product of *S. acidocaldarius* GGPS from HMBPP is hydroxylgeranylgeranyl diphosphate. We also tried to identify the product from HMBPP of *A. pernix* GFPS by ESI-MS and MS/MS analyses, but could not get reliable data due to unknown contaminants (data not shown).

Discussion

In this study, we tried to prove that HMBPP is the donor substrate of lycopene elongase from *C. glutamicum*. The results from the two different lines of experiments, i.e., the MEP pathway inhibition experiment and the HMBPP reductase overexpression experiment, support our hypothesis that HMBPP is used for the production of flavuxanthin. Based on these results, we reached the conclusion that the hydroxyl groups of bacterial C_{50} carotenoids such as flavuxanthin and decaprenoxanthin from *C. glutamicum* are derived from those of HMBPP. Hydroxylation of carotenoids is usually catalyzed by an hydratase, such as carotenoid 1,2-hydratases CrtC[37-39] and CruF[40, 41], or by a monoxygenase, such as cytochrome P450-type[42-44] and non-heme diiron-type carotenoid hydroxylases[45]. Hydratases, however, cannot be employed for hydroxylation at the terminal methyl groups of carotenoids because hydroxylation by the enzymes occurs selectively at the tertiary carbon. Monooxygenases can catalyze the terminal methyl hydroxylation of isoprenoid, as observed with geraniol 8-hydroxylase (CYP76B6) that is involved in iridoid biosynthesis,[8, 9] or with CYP735A1 and CYP735A2 that are involved in *trans*-zeatin biosynthesis by plant,[46] but the known carotenoid hydroxylases catalyze hydroxylation at the non-methyl positions. The use of HMBPP for the introduction of hydroxyl groups is a limited, but reasonable, path toward the biosynthesis of hydroxylated carotenoids. This approach could be applicable to the bio-production of valuable hydroxylated isoprenoids. For example, the product of *S. acidocaldarius* GGPS from HMBPP, hydroxygeranylgeranyl diphosphate, has an ω-hydroxyl group probably at position 16 and might be applied as the precursor for the production of important bioactive compounds such as the mating hormones of the plant pathogen *Phytophthora*.[47]

The acceptance of HMBPP by *C. glutamicum* lycopene elongase and also by some (all-*E*)-prenyl diphosphate synthases was surprising because these distinct types of prenyltransferases are thought to employ the carbocation-involving S_N1-type mechanism. From the results of the kinetic study on *S. acidocaldarius* GGPS, the low reactivity of the enzyme toward HMBPP compared with that toward DMAPP can be attributed mainly to the difference in k_{cat}, rather than that in K_M. This suggests that the difference in the stability of carbocations derived from HMBPP and DMAPP affects the reactivity of the donor substrates, supporting the previously proposed reaction mechanism. Notably, all the (all-*E*)-prenyl diphosphate synthases that were shown to accept HMBPP in this study are derived from organisms possessing only the MVA pathway. In contrast, the enzymes from organisms possessing the MEP pathway, i.e., *C. glutamicum* and *G. stearothermophilus*, showed no activity toward HMBPP, probably because HMBPP is a cellular component that the enzymes would encounter. If the enzymes that naturally utilize DMAPP accept HMBPP as the donor substrate, the resultant unnatural products might disturb downstream isoprenoid biosynthetic pathways or cellular processes involving isoprenoid compounds. *L. brevis* FPS also do not accept HMBPP even though the organism has only the MVA pathway; however, this fact might be explained by a hypothetical evolutionary scenario where the MVA pathway, which is now known to be rare in domain Bacteria, was acquired by some bacterial lineages via relatively recent horizontal gene transfer events and then replaced the MEP pathway inherited from the common bacterial ancestor. The strict preference toward DMAPP might be an overall trend for bacterial enzymes because of their (or their ancestors') coexistence with HMBPP. The results from the present study, however, also raise expectations for the existence of undiscovered prenyltransferases that

naturally utilize HMBPP as the preferable donor substrate, because the compound is potentially acceptable by a wide variety of prenyltransferases.

Author contribution

H.H. conceived and designed the experiments. Y.H., T.I., Y.T and H.H. performed the experiments and analyzed the data. Y.H. and H.H wrote the paper.

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Table 1 Specific activities of (all-*E*)-prenyl diphosphate synthases

These values were obtained from the reactions against 5 μM IPP and either 5 μM DMAPP or HMBPP.

enzyme	donor substrate	specific activity (nmol IPP·min^{-1}·mg protein)	ratio
A. pernix GFPS	DMAPP	12.2	
	HMBPP	7.04	0.58
S. acidocaldarius GGPS	DMAPP	15.6	
	HMBPP	2.47	0.16
F. johnsoniae hypothetical GGPS*	DMAPP	1,720	
	HMBPP	30.4	0.018
S. cerevisiae FPS	DMAPP	346	
	HMBPP	3.90	0.011

**F. johnsoniae* hypothetical GGPS turned out to be HexPS.

Table 2 Kinetic parameters of *S. acidocaldarius* GGPS

These parameters were all calculated based on the consumption of IPP.

donor	acceptor	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1} \cdot \mu M^{-1}$)
DMAPP	(25 μM IPP)	7.05	10.8	1.53
HMBPP	(25 μM IPP)	19.1	1.64	0.0859
(25 μM DMAPP)	IPP	13.5	21.0	1.55
(25 μM HMBPP)	IPP	3.38	1.28	0.379

Figure legends

Figure 1 Biosynthetic pathways of C_{50} carotenoids in *C. glutamicum* and *H. salinarum*. The prenyltransfer reactions catalyzed by lycopene elongases CrtEb and Lye are indicated by the broad arrows.

Figure 2 Analyses of carotenoids produced in recombinant *E. coli*. (A) Normal-phase TLC analysis of carotenoids extracted from Top10/pBAD-*crtEbEBI*. ori., origin; s.f., solvent front. (B) FAB-MS analysis of the relatively hydrophilic [R_f= ~0.6 in (A)] carotenoid recovered from a TLC plate. (C) HPLC elution profile at the A_{472} of carotenoids extracted from *E. coli* Top10/pBAD-*crtEbEBI*/pJBEI-2999 cultivated in the absence (solid line) and presence (dotted line) of FR-900098. (D) UV-visible spectra of the carotenoid eluted at ~8 min in (C) measured by the PDA detector attached to HPLC.

Figure 3 The effect of the overexpresson of HMBPP reductase on flavuxanthin formation. Shown are the HPLC elution profiles at the A_{472} of carotenoids extracted from *E. coli* Top10/pBAD-*crtEbEBI* (A) and Top10/pBAD-*crtEbEBI-ispH* (B).

Figure 4 Prenyltransfer reactions via the S_N1 and S_N2-type mechanisms.

Figure 5 Analyses of the products of (all-*E*)-prenyl diphosphate synthases. (A and B) Radio-TLC analysis of the hydrolyzed products that are synthesized from DMAPP (A) and HMBPP (B). After enzyme reaction, radiolabeled products, i.e., (all-*E*)-prenyl

diphosphates in (A) and probably ω-hydroxylated prenyl diphosphates in (B), were extracted with 1-butanol, and then hydrolyzed by acid phosphatase. Resultant alcohols were analyzed by reversed-phase TLC. ori., origin; s.f., solvent front. (C and D) Ion profiles from the negative-ESI-MS (C) and MS/MS (D) analyses of the products of *S. acidocaldarius* GGPS from HMBPP. Ions with asterisks were also detected in the analysis of a control sample from the reaction using DMAPP instead of HMBPP. The hypothetical structures of the molecular ion and fragment ions were represented.

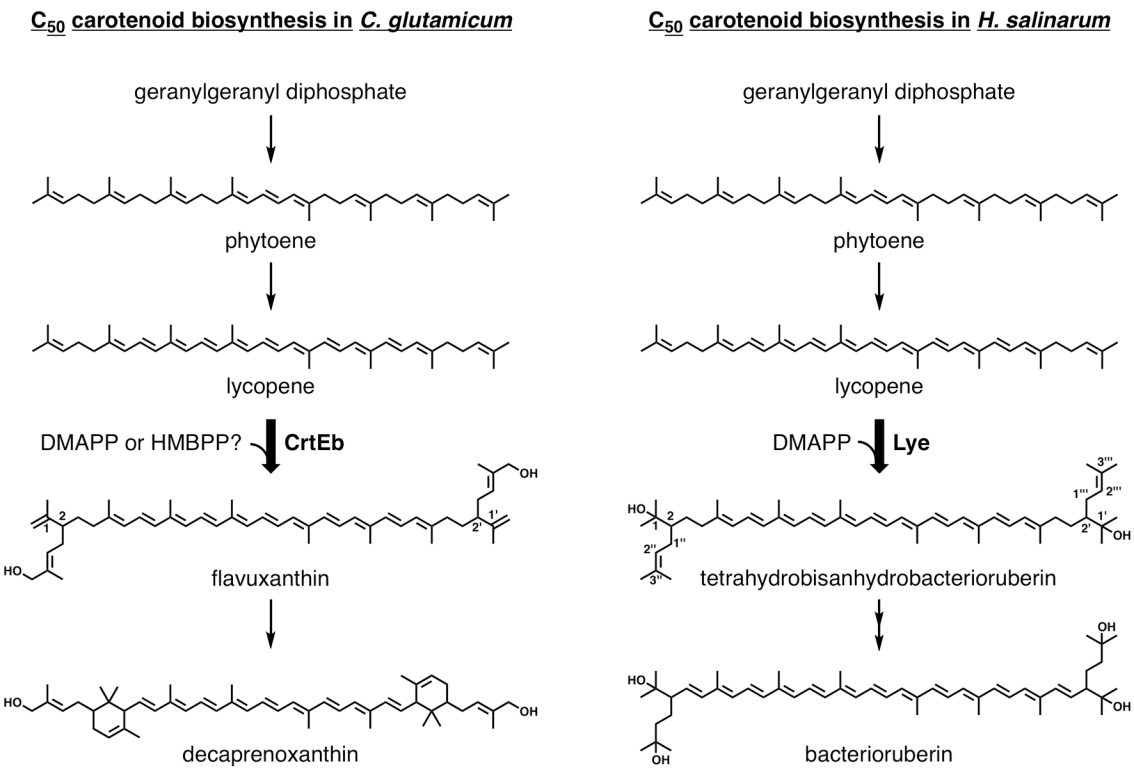
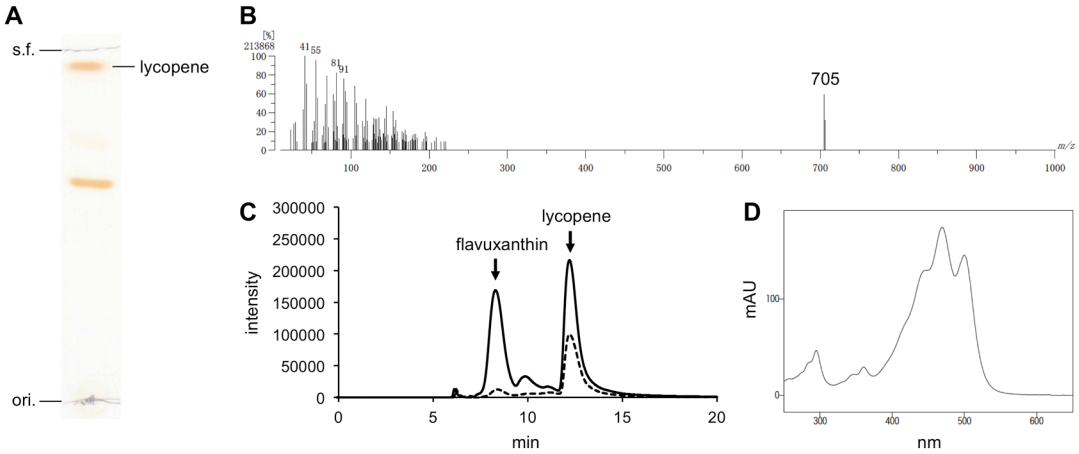

C50 carotenoid biosynthesis in C. glutamicum
geranylgeranyl diphosphate
phytoene
lycopene
DMAPP or HMBPP?
CrtEb
flavuxanthin
decaprenoxanthin
C50 carotenoid biosynthesis in H. salinarum
geranylgeranyl diphosphate
phytoene
lycopene
DMAPP
Lye
tetrahydrobisanhydrobacterioruberin
bacterioruberin

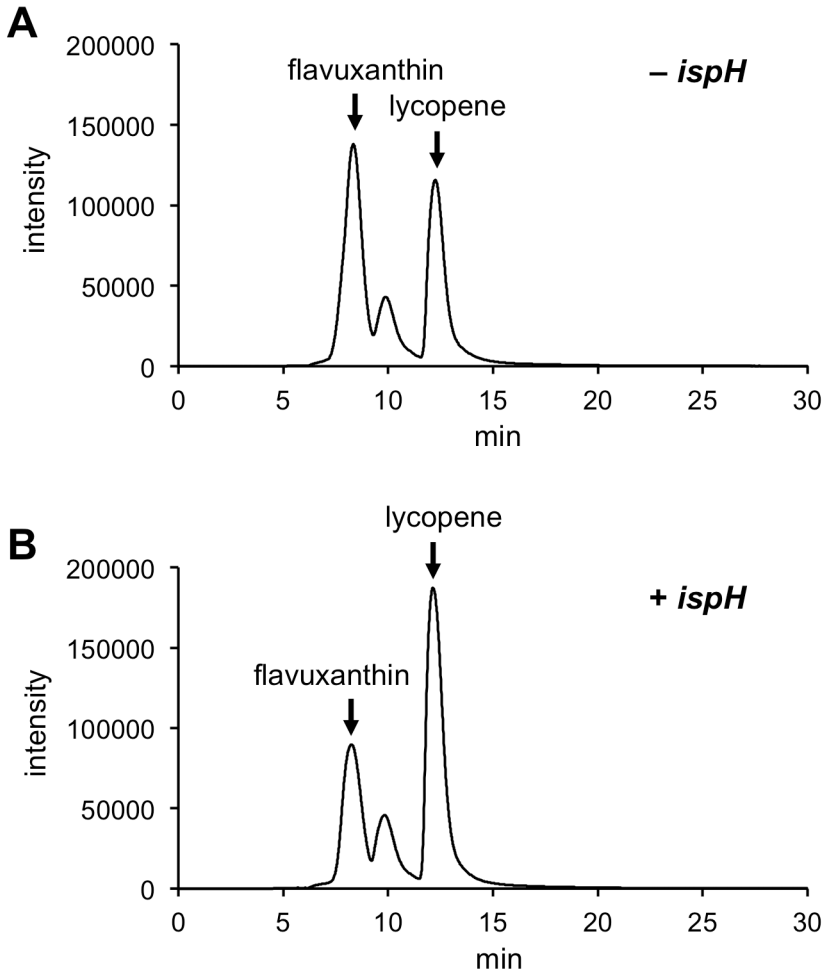
Figure 1


A
s.f.
lycopene
ori.
B
[%]
213868
41
55
81
91
705
m/z
100
200
300
400
500
600
700
800
900
1000
C
300000
250000
200000
150000
100000
50000
0
intensity
flavuxanthin
lycopene
0
5
10
15
20
min
D
mAU
100
0
300
400
500
600
nm

1

2 Figure 2

3


A
200000
150000
100000
50000
0
intensity
flavuxanthin
lycopene
– ispH
0
5
10
15
20
25
30
min
B
lycopene
200000
150000
100000
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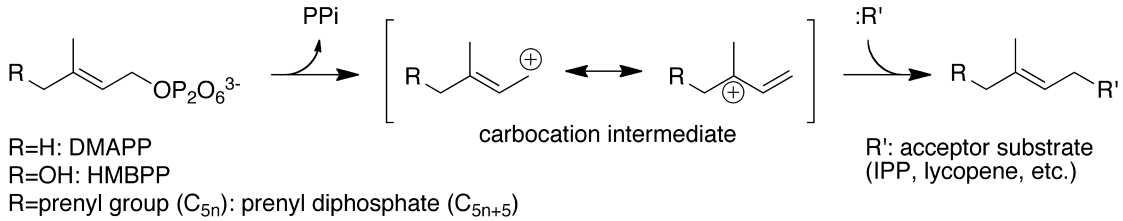
intensity
flavuxanthin
+ ispH
0
5
10
15
20
25
30
min

Figure 3

S_N1-type reaction



S_N2-type reaction

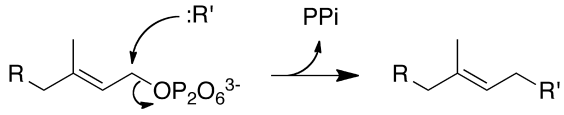


Figure 4

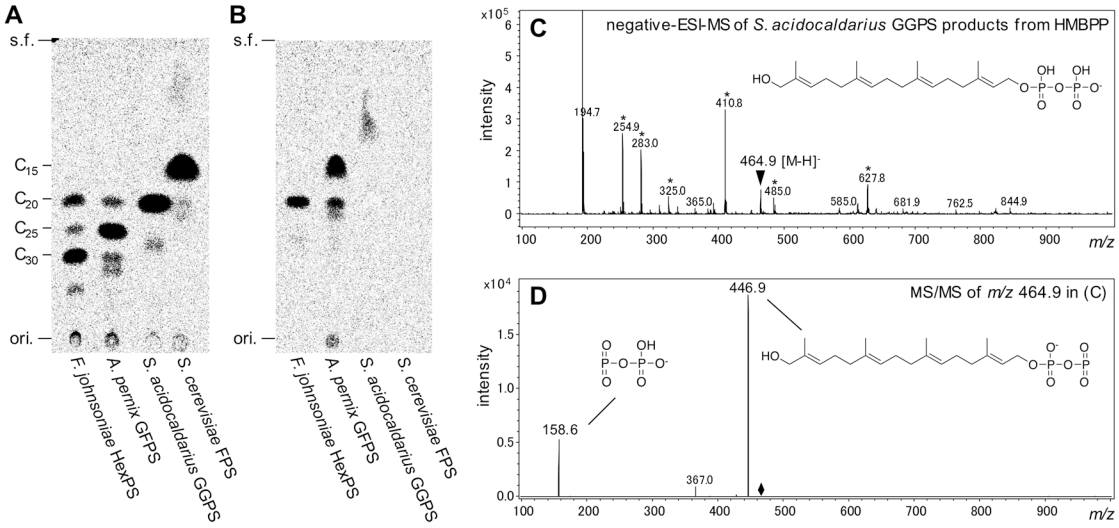

A
s.f.
C15
C20
C25
C30
ori.
F. johnsoniae HexPS
A. pernix GFPS
S. acidocaldarius GGPS
S. cerevisiae FPS
B
s.f.
ori.
F. johnsoniae HexPS
A. pernix GFPS
S. acidocaldarius GGPS
S. cerevisiae FPS
C
negative-ESI-MS of S. acidocaldarius GGPS products from HMBPP
intensity
x10^5
194.7
254.9
283.0
325.0
365.0
410.8
464.9 [M-H]-
485.0
585.0
627.8
681.9
762.5
844.9
m/z
D
MS/MS of m/z 464.9 in (C)
intensity
x10^4
158.6
367.0
446.9
m/z

Figure 5