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Characterization of solanesyl and decaprenyl diphosphate synthases in mice and humans

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Running title: mammalian prenyl diphosphate synthases

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Abbreviations: IPP, isopentenyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; SPS, solanesyl diphosphate synthase; DPS, decaprenyl diphosphate synthase; Q, ubiquinone; PCR, polymerase chain reaction; LB, Luria-Bertani; TLS, thin layer chromatography;

Abstract

The isoprenoid chain of ubiquinone (Q) is determined by *trans*-polyprenyl diphosphate synthase in microorganisms and presumably in mammals. As mice and humans produce Q-9 and Q-10, they are expected to posses solanesyl and decaprenyl diphosphate synthases as the determining enzyme for a type of ubiquinone. Here we show that murine and human solanesyl and decaprenyl diphosphate synthases are heterotetramers composed of newly characterized hDPS1 (mSPS1) and hDLP1 (mDLP1), which were identified as orthlogs of the *S.pombe* Dps1 and Dlp1, respectively. While *hDPS1* or *mSPS1* can complement the *S. pombe dps1* disruptant, neither *hDLP1* nor *mDLP1* could complement the *S. pombe dlp1* disruptant. Thus, only hDPS1 and mSPS1 are functional orthlogs of SpDps1. *Escherichia coli* was engineered to express the murine and human SpDps1 and/or SpDlp1 homologues and their ubiquinone types were determined. While transformants expressing a single component only produced Q-8 from *E. coli* origin, double transformants expressing mSPS1 and mDLP1 or hDPS1 and hDLP1 produced Q-9 or Q-10, respectively, and an *in vitro* activity of solanesyl or decaprenyl diphosphate synthase was verified. The complex size of the human and murine long-chain *trans*-prenyl diphosphate synthases, as estimated by gel filtration chromatography, indicates that they consist of heterotetramers. Expression in *E. coli* of heterologous combinations, namely, mSPS1 and hDLP1 or hDPS1 and mDLP1, generated both Q-9 and Q-10, indicating both components involves in determining the ubiquinone side chain. Thus, we identified the components of the enzymes that determine the side chain of ubiquinone in mammals and they resembles the *S. pombe*, but not plant or *S. cerevisiae*, type of enzyme.

Keywords: Ubiquinone; Coenzyme Q; prenyl transferase; isoprenoids

Introduction

Ubiquinone (Coenzyme Q) functions as an electron transporter in aerobic respiration and oxidative phosphorylation in the respiration chain [1]. In addition, many reports suggest that ubiquinone also functions as a lipid-soluble antioxidant in cellular biomembranes that scavenges reactive oxygen species (ROS) [2-5]. Indeed, several studies using yeast strains that do not produce ubiquinone suggest that an *in vitro* function of ubiquinone is to protect against oxidants [6, 7]. Another phenotype of such ubiquinone-less fission yeast is that they generate high levels of hydrogen sulfide [8-10]. As *Schizosaccharomyces pombe* and other eukaryotes are known to carry sulfide-ubiquinone reductase, an enzyme that oxidizes sulfide *via* ubiquinone [11], it was suggested that ubiquinone is linked to sulfide metabolism in many organisms. In addition, it was reported that ubiquinone (and menaquinone) function as an electron transporter in the DsbA-DsbB system of *Escherichia coli* to generate protein disulfide bonds [12]. Furthermore, the *clk-1*/*coq7* mutant that lacks to synthesize ubiquinone in *Caenorhabditis elegans* has a prolonged lifespan [13], which has introduced an interesting topic into the field of ubiquinone research. As well as the prolonged lifespan, the *clk-1* mutant shows developmental delay and low egg production, which suggests further novel roles of ubiquinone [14-16]. Thus, it appears that ubiquinone plays various roles in different organisms.

The ubiquinone molecule bears an isoprenoid side chain whose length varies between organisms. For example, the *Saccharomyces cerevisiae* and *E. coli* side chains are 6 and 8 isoprene units, respectively, while the side chain in mice and *C. elegans* is 9 and that in *S. pombe* and humans is 10 isoprene units [17]. The length of the side chain of ubiquinone is precisely defined by *trans*-polyprenyl diphosphate synthases rather than by the 4-hydroxybenzoate (PHB)-polyprenyl diphosphate transferases that catalyze the condensation of PHB and polyprenyl diphosphate [8, 18, 19]. The heterologous expression in *E. coli* and *S. cerevisiae* of *trans*-polyprenyl diphosphate synthase genes from other organisms generated the same type of ubiquinone as is expressed in the donor organisms

[20, 21]. These results also suggested that carrying a different type of ubiquinone (varying from Q-6 to Q-10) does not affect the survival of *S. cerevisiae* or *E. coli*. Recently, however, it was shown that the various ubiquinones do have type-specific biological effects, as exogenous Q-7 was not as efficient as Q-9 in restoring the growth of the *C. elegans clk-1* mutant [22]**.** Q-10 (CoQ10) has been used as a medicine for humans and has recently been employed as a food supplement [23]. Q-10 is a lipid antioxidant that is only biosynthesized in humans, therefore it will be important to know the biosynthetic pathway of Q-10 in humans. It is also important to know from a clinical aspect since the disease related to human muscle Q-10 deficiency was reported [24]. Despite its importance, so far only two genes for ubiquinone biosynthesis from mammals were reported [25].

The biosynthetic pathway that converts PHB into ubiquinone consists of nine steps. These steps include the condensation and transfer of the isoprenoid side chain to PHB [17] followed by methylation, decarboxylation and hydroxylation. The elucidation of this pathway has mostly come from studies of respiratory-deficient mutants of *E. coli* and *S. cerevisiae* [17, 26]. It is believed that the eukaryotic enzymes involved in ubiquinone biosynthesis are very similar to those in *E. coli* and *S. cerevisiae* except for the *trans*-polyprenyl diphosphate synthase that synthesizes the isoprenoid side chain.

Long-chain *trans*-polyprenyl diphosphate (C_{40}, C_{45}, C_{50}) synthases catalyze the condensation of farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP), which acts as a primer, and isopentenyl diphosphate (IPP) to produce the various prenyl diphosphates bearing varying chain lengths. These enzymes possess seven conserved regions, including two DDXXD motifs that are binding sites for the substrates in association with Mg^{2+} [27]. The structure of octaprenyl diphosphate synthase was recently solved and was found to be very similar to FPP synthase [28]. While short-chain polyprenyl diphosphate (C_{15}, C_{20}) synthases such as FPP synthase and GGPP synthase have been identified in organisms ranging from bacteria through to plants and mammals [27, 29-34], analysis of the long-chain *trans*-polyprenyl diphosphate synthases has been limited to those in several bacteria, two yeasts and one plant [9, 29, 35, 36]. Only the activity and

some characterization of solanesyl diphosphate synthase in rat was reported among animals [37, 38]. Analysis of the hexaprenyl diphosphate synthase from *S. cerevisiae* and the solanesyl diphosphate synthase from the plant *Arabidopsis thaliana* suggest that the long-chain *trans*-polyprenyl diphosphate synthases that synthesize the ubiquinone side chain tend to be monomeric enzymes [36, 39, 40]. However, the decaprenyl diphosphate synthase from *S. pombe* is a heterotetramer of two proteins, SpDps1 (*S. pombe* Decaprenyl diphosphate synthase) and SpDlp1 (*S. pombe D*-less polyprenyl diphosphate synthase) [9]. Given this disparity, it is of interest to investigate mammalian long-chain *trans*-polyprenyl diphosphate synthases.

Here we describe the identification and characterization of solanesyl and decaprenyl diphosphate synthases in mice and humans. We show that these enzymes are heteromers, like the decaprenyl diphosphate synthase from *S. pombe*. The murine enzyme is a solanesyl diphosphate synthase made up of mSPS1 (mouse Solanesyl diPhosphate Synthase) and mDLP1 (mouse *D*-less polyprenyl pyrophosphate synthase), while the human enzyme is a decaprenyl diphosphate synthase composed of hDPS1 (human Decaprenyl diPhosphate Synthase) and hDLP1 (human *D*-less polyprenyl diphosphate synthase). We found that mSPS1 and hDPS1 bear all the conserved regions found in the homodimeric prenyl diphosphate synthases and SpDps1, while mDLP1 and hDLP1 are homologues of SpDlp1. We also showed both components involved in determination of the isoprenoid chain length of ubiquinone.

Materials and methods

Materials

Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Shuzo Co. Ltd. and New England Biolabs, Inc. [1-^{14}C]IPP (1.96 TBq/mol) was purchased from Amersham Biosciences. IPP, all-*E*-farnesyl diphosphate (FPP), geranylgeraniol (GGOH), solanesol (all-*E*-nonaprenol), and polyprenols ($\mathbf{C_{40}}$- $\mathbf{C_{60}}$) from *Ailanthus altissima*

were purchased from Sigma Chemical Co. Kiesel gel 60 F_{254} thin-layer chromatography (TLC) plates were purchased from Merck. Reverse-phase LKC-18 TLC plates were purchased from Whatman Chemical Separation. The *mSPS1* clone (GenBank accession number BF180140) from mouse muscles, the *mDLP1* clone (GenBank accession number BD182639) from mouse liver, the *hDPS1* clone (GenBank accession number AI261617) from human kidney and the *hDLP1* clone (GenBank accession number BD182638) from human brain were purchased from Genome Systems Inc.

Strains and plasmids

E. coli strains DH10B, DH5α and BL21(DE3) were used for general plasmid construction [41]. The pBluescript II SK+/-, pBluescript KS+/-, pT7Blue-T (Novagen), pSTV28 (Takara Shuzo), pSTVK28 (Km^r marker harboring pSTV28), pQE31 (Qiagen), pGEX-KG (Amersham Biosciences), pET-28c (Novagen), pREP1 [42] and pREP2 (the *LEU2* marker of pREP1 was exchanged with the *ura4* marker) plasmids served as vectors. pRDPS1, pBSDPS1 and pSTVDLP1 were described before [9]. The *S. pombe* strain SP870 (*h*^{90} *leu1-32 ade6-M210 ura4-D18*) [43] served as the homothallic haploid wild-type. KS10 (*h*$^+$ *leu1-32 ade6-M210 ura4-D18* Δ*dps1::ura4*) and RS312 (*h*$^+$ *leu1-32 ade6-M210 ura4-D18* Δ*dlp1::ura4*) were used as host strains to express human or murine long-chain *trans*-prenyl diphosphate synthase components [7, 9]. Yeast cells were grown in YE (0.5% yeast extract, 3% glucose) or PM minimal medium with appropriate supplements as described [44]. YEA and PMA consist of YE and PM, respectively, that contain 75 μg of adenine per ml. The concentration of the supplemented amino acids was 100 μg/ml.

DNA manipulations

Cloning, restriction enzyme analysis, and preparation of plasmid DNAs were performed essentially as described previously [41]. The *mSPS1* gene was cloned and expressed in *E. coli* and *S. pombe* as follows. Two oligonucleotides were used to amplify a fragment containing the *mSPS1* gene: mouseSPS1-N, 5'-GCCATATGGCGAATTCGA TGCGCTGGTCGT-3' (creates *Nde*I and *Eco*RI sites) and mouseSPS1-C, 5'-

GCGTCGACTCA TTTATCTCTGGTG-3' (creates a *Sal*I site). The resulting 1.2 kb fragment was digested with *Eco*RI and *Sal*I and cloned into the same sites of pBluescript II SK+ to yield pBmSPS1. The amplified *mSPS1* fragment was also digested with *Nde*I and *Sal*I and cloned into the same sites of pREP1 to yield pRmSPS1. To construct pGEX-mSPS1, two other oligonucleotides were used to amplify the *mSPS1* gene: mSPS1-Ntag, 5'-GCGGATCCATGGCTATGCGCTGGTCGT G-3' (creates a *Bam*HI site) and mSPS1-Ctag, 5'-TATCTAGATCATTTATCTCTGGTGAGCA C-3' (creates an *Xba*I site). The amplified fragment was cloned into the *Bam*HI and *Xba*I sites of pGEX-KG to yield pGEX-mSPS1.

To clone and construct expression plasmids bearing the *mDLP1* gene, the oligonucleotides mDLP1-N (5'-GCGTCGACG AATTCTATGAGCCTCCGGCAG-3'), which creates *Sal*I and *Eco*RI sites, and mDLP1-C (5'-CCGGATCCTCAAGAAAATCTGGTCACAGC-3'), which creates a *Bam*HI site, were used to amplify a fragment containing the *mDLP1* gene. The resulting 1.2 kb fragment was digested with *Eco*RI and *Bam*HI and cloned into the same sites of pSTVK28 to yield pSTVmDLP1. The amplified *mDLP1* fragment was also digested with *Sal*I and *Bam*HI and cloned into the same sites of pREP2 and pBluescript KS+ to yield pRmDLP1 and pBmDLP1, respectively. To make pET-mDLP1, pBmDLP1 was digested with *Sal*I and *Not*I and then cloned into the same sites of pET-28c. To construct pGEX-mSPS1-mDLP1, pET-mDLP1 was digested with *Xba*I-*Xho*I and cloned into the same sites of pGEX-mSPS1.

The *hDPS1* gene was cloned and expressed in *E. coli* and *S. pombe* as follows. Two oligonucleotides were used to amplify the 1.2 kb fragment containing the *hDPS1* gene: hDPS1-N, 5'-AAGTCGACAATGGCCTCGCG CTGGTGGCGGTG-3' (creates a *Sal*I site) and hDPS1-C, 5'-GGCGGATCCTCATTTATCTCT TGTGAGTACAATTTC-3' (creates a *Bam*HI site). The amplified fragment was cloned into *Sal*I and *Bam*HI sites of pUC119 and pREP1 to yield pUhDPS1 and pRhDPS1, respectively. Two other oligonucleotides were used to amplify the *hDPS1* gene: hDPS1-N2tag, 5'-ACAAAGCTTATGGCCTCGCGCTGGTGGCGGTG-3' (creates a *Hin*dIII site) and

hDPS1-C2tag, 5'-CCGGAATTCTCATTTATCTCTTGT GAGTACAATTTC-3' (creates a *Eco*RI site). The amplified fragment was cloned into *Hin*dIII and *Eco*RI sites of pFLAG-ATS to yield pFhDPS1. To construct pGEX-hDPS1, two oligonucleotides were used to amplify the *hDPS1* gene: hDPS1-Ntag, 5'- GCGGAT CCATGGCCTCGCGCTGGTGGCGGTG-3' (creates a *Bam*HI site) and hDPS1-Ctag, 5'-TATCTAGATCATTTATCTCTTGTGAGTACAATTTC-3' (creates an *Xba*I site). The amplified fragment was cloned into the *Bam*HI and *Xba*I sites of pGEX-KG to yield pGEX-hDPS1.

To clone and construct expression plasmids containing the *hDLP1* gene, the oligonucleotides hDLP1-N (5'-CTGGATCCAT GAACTTTCGGCAGCTGCTGT-3', which creates a *Bam*HI site, and hDLP1-C (5'-TTCCCGGGTCATGAAAATCTGGTCACAGC-3'), which creates a *Sma*I site, were used to amplify a 1.2 kb fragment containing the *hDLP1* gene. The amplified gene was cloned into the *Bam*HI and *Sma*I sites of pREP2 and pBluescript SK+ to yield pRhDLP1 and pBhDLP1, respectively. To make pSTVhDLP1, pBhDLP1 was digested with *Bam*HI and *Hin*dIII and then cloned into the same sites of pSTVK28. pSTVhDLP1 was digested with *Bam*HI and *Hin*dIII and cloned into the same sites of pQE31 to yield pQhDLP1, which was in turn digested with *Xho*I and *Hin*dIII and cloned into the *Sal*I and *Hin*dIII sites of pSTV28 to yield pSTVHIShDLP1. To make pET-hDLP1, hDLP1-Ntag (5'-GCGTCGACAATGAACTTT CGGCAGCTGCTG-3'), which creates a *Sal*I site, and hDLP1-Ctag (5'-GTGCGGCCGC TCATGAAAATCTGGTCACAGC-3'), which creates a *Not*I site, were used to amplify the hDLP1 gene. The amplified fragment was cloned into *Sal*I and *Not*I sites of pET-28c to yield pET-hDLP1. To construct pGEX-hDLPS1-hDLP1, pET-hDLP1 was digested with *Xba*I-*Xho*I and then cloned into the same sites of pGEX-hDPS1 to yield pGEX-hDPS1-hDLP1.

Ubiquinone extraction and measurement

Ubiquinone was extracted as described previously [8]. The crude ubiquinone extract

was analyzed by normal-phase TLC using authentic Q-10 as the standard. Normal-phase TLC was carried out on a Kieselgel 60 F_{254} plate with benzene / acetone (97:3, v/v). The ubiquinone band was collected from the TLC plate following UV visualization and extracted with chloroform/methanol (1:1, v/v). Samples were dried and redissolved in ethanol. The purified ubiquinone was further analyzed by high-pressure liquid chromatography (HPLC) using ethanol as the solvent.

Prenyl-diphosphate synthase assay and product analysis

Prenyl diphosphate synthase activity was measured as described previously [45]. BL21 (DE3) cells harboring plasmids expressing mammalian long-chain *trans*-polyprenyl diphosphate synthases were ruptured by sonication with an ultrasonic disintegrator as described previously [46]. The incubation mixture consisted of 2.0 μmol $MgCl_2$, 0.2% (v/v) Triton X-100, 50 mM potassium phosphate buffer (pH 7.5), 5 mM KF, 10 mM iodoacetamide, 20 μmol [1-^{14}C]IPP (specific activity 0.92 MBq/mol), 100 μmol FPP and 1.5 mg/ml of the enzyme-bearing extract in a final volume of 0.5 ml. The sample mixtures were incubated for 30 min at 30°C. Reaction products, such as prenyl diphosphates, were extracted with water saturated 1-butanol and hydrolyzed with acid phosphatases [47]. The hydrolysis products were extracted with hexane and analyzed by reversed phase thin-layer chromatography with acetone/water (19:1, v/v). Radioactivity on the plate was detected with a BAS1500-Mac imaging analyzer (Fuji Film Co.). The plate was exposed to iodine vapor to detect the spots of the marker prenols.

Determination of mSPS1-mDLP1 and hDPS1-hDLP1 complex sizes by gel filtration chromatography

To measure the size of the mSPS1-mDLP1 complex by gel filtration chromatography, *E. coli* strain JM109 was transformed with pGEX-mSPS1-mDLP1, which expresses glutathione-*S*-transferase (GST)-fused mSPS1 protein and His-fused mDLP1. Similarly, pGEX-hDPS1-hDLP1 was used to estimate the size of the hDPS1-hDLP1 complex. The transformants were grown to the stationary phase in LB medium containing ampicillin, and 0.5 ml of the culture was then inoculated into 50ml of the same medium. A crude protein extract containing GST-

mSPS1 and His-mDLP1 (or hDPS1 and hDLP1) was then prepared from the transformant. The sample protein was then subjected to gel filtration chromatography by being loaded onto a packed and calibrated Superdex 200 pg column (Amersham Biosciences) and being allowed to flow at 0.4 ml/min while monitoring at A_{280} with 50 mM sodium phosphate buffer (pH 7.0) containing 300mM NaCl, 5% sucrose, 1% glycerol and 0.3% TritonX-100. Column equilibrium, gel filtration chromatography of the prepared samples, and distribution of the total fractions were performed by employing the BioLogic LP system (BIO-RAD) at 4°C. The separated fractions were subjected to Western blotting by using anti-GST antibodies (Santa Cruz Biotechnology) and anti-His antibodies (Qiagen), and the fraction containing the prenyl diphosphate synthase fusion proteins was determined. Molecular weight was calculated by comparison with the absorbance pattern of the Gel Filtration Standard (BIO-RAD).

Results

Isolation and sequence analysis of genes encoding murine and human long-chain trans-prenyl diphosphate synthases

The only eukaryotic *trans*-prenyl diphosphate synthases that synthesize the ubiquinone side chains that have been studied to date are those from *S. cerevisiae, S. pombe* and *A. thaliana* [9, 36, 39]. Solanesyl diphosphate synthase in rat liver was studied enzymatically but its primary structure and protein composition were not known [37]. While the *S. cerevisiae* and *A. thaliana* enzymes are monomeric, the decaprenyl diphosphate synthase of *S. pombe* is a heterotetramer consisting of SpDps1 and SpDlp1 [9]. To determine which enzyme structure predominates in eukaryotes, we analyzed mammalian long-chain *trans*-prenyl diphosphate synthases. The BLAST program was used to search for SpDps1 and SpDlp1 homologues in the EST database collected at the National Center for Biotechnology Information (NCBI). Many highly homologous sequences were found in both the murine and human EST databases. We purchased many of the candidate clones

from Genome Systems Inc. and sequenced them. Eventually, the murine and human cDNA clones that showed the greatest homology to SpDps1 (accession numbers BF180140 for the murine homologue and AI590245 and AI261617 for the human homologue) were selected. We also cloned the cDNAs with the highest homology to SpDlp1 (accession numbers BE283879 and AI097731 for the murine homologue and AI742294 and BI551760 for the human homologue). In the cases where the full length cDNA was not included in a single clone, we combined two cDNA clones into one and determined the resulting complete cDNA sequence. The murine and human *SpDps1* homologues were denoted as *mSPS1* and *hDPS1*, respectively. The murine and human *SpDlp1* homologues were denoted as *mDLP1* and *hDLP1*, respectively. The open reading frames (ORFs) of *mSPS1* and *hDPS1* were 1230 bp and 1245 bp, respectively, while those of *mDLP1* and *hDLP1* were 1206 bp and 1200 bp, respectively. The *mSPS1* and *hDPS1* genes were 83.0% identical while their translated products were 82.1% identical. The *mDLP1* and *hDLP1* genes were 87.2% while their translated products were 88.3% identical. The mSPS1 and hDPS1 proteins were also highly similar to the *S. pombe* homologue SpDps1 (48.7% and 46.0%, respectively), but mDLP1 and hDLP1 showed considerably less similarity to the *S. pombe* homologue SpDlp1 (31.3% and 27.4%, respectively). mSPS1 and hDPS1 also displayed higher similarity to the *A. thaliana* homologue At-SPS1 (35.8% and 36%, respectively) [36] than to the *E. coli* homologue IspB (30.0% and 30.7%, respectively) [46].

Both mSPS1 and hDPS1 possess the conserved domains I to VII and contain DDXXD sequence motifs that are typically found in all known *trans*-prenyl diphosphate synthases (Fig. 1A). In mDLP1 and hDLP1, domains I to VII are also conserved but neither protein contains the typical aspartate-rich DDXXD motifs normally found in domains II and VI (Fig. 1B). As a result, *mDLP1* and *hDLP1* were given the name *DLP* (D[Aspartate)-less polyprenyl pyrophosphate synthase). *hDPS1* and *hDLP1* are located at the 10p12.1 locus in chromosome 10 and at the 6q21 locus in chromosome 6 and have the tentative gene names TPRT and C6orf210, respectively. We could find SpDlp1 homologues in the rat, *Xenopus* and *Drosophila* but not in *C. elegans* (Fig. 1B). There is also another *dlp1*-like

transcript in humans and mice that we called *hDLP2* and *mDLP2*, respectively, as they are splicing variants of *hDLP1* and *mDLP*1. The *hDLP1* gene is split into eight exons while the *hDLP2* gene is split into four exons. The first three exons of *hDLP1* and *hDLP2* are equivalent but the latter exons differ. The same is true for the *mDLP2* murine gene.

Expression of human and murine long-chain trans-prenyl diphosphate synthases in *E. coli*

We expressed the murine or human homologues of *SpDps1* and *SpDlp1* in *E. coli* to determine whether both genes are needed to form a functional prenyl diphosphate synthase. To do so, we constructed the pBmSPS1, pSTVmDLP1, pUhDPS1 and pSTVhDLP1 plasmids that express the *mSPS1*, *mDLP1*, *hDPS1* and *hDLP1* genes, respectively (Fig. 2). *E. coli* DH5α cells expressing both mSPS1 and mDLP1 synthesized Q-9, while the same strain carrying hDPS1 and hDLP1 produced Q-10 (Fig. 3E, H). In contrast, when the host strain bore only one of the four plasmids, it produced only Q-8, which is the product of the endogenous *E. coli* octaprenyl diphosphate synthase (Fig. 3C, D, F and G). Thus, both of the murine or human genes (i.e. *mSPS1* and *mDLP1*, or *hDPS1* and *hDLP1*) are necessary and sufficient for producing an extra ubiquinone type in *E. coli*. When *hDLP2* was co-expressed with *hDPS1* in *E. coli*, Q10 was not produced (data not shown). Thus, *hDLP2* cannot partner *hDPS1* in producing a long-chain *trans*-prenyl diphosphate synthase.

We further tested whether the *E. coli* cells that co-express mSPS1 and mDLP1 or hDPS1 and hDLP1 possess solanesyl and decaprenyl diphosphate synthase activity by measuring the *in vitro* activity of these enzymes. Consistent with the above observations, cells that expressed both *mSPS1* and *mDLP1* could produce solanesol; in contrast, cells transformed with only pGEX-mSPS1 or pET-mDLP1 did not possess solanesyl diphosphate synthase activity (Fig. 4 A). Similarly, cells harboring both pFhDPS1 and pSTVHIShDLP1 could produce decaprenol, unlike cells harboring either plasmid on its own (Fig. 4B). These observations further support the notion that the long-chain *trans*-prenyl diphosphate synthases in mice and humans need two proteins (i.e. both mSPS1 and

mDLP1 or both hDPS1 and hDLP1, respectively) to be active.

Heteromeric complex formation by the murine and human homologues of SpDps1 and SpDlp1

The above results suggest that, like the decaprenyl diphosphate synthase of *S. pombe*, mSPS1 and mDLP1 form a heteromeric complex that can then act as a long-chain *trans*-prenyl diphosphate synthase. The same appears to be true for hDPS1 and hDLP1. To test this notion, we determined the sizes of the murine and human long-chain *trans*- prenyl diphosphate synthases produced by *E. coli* JM109 expressing mSPS1 plus mDLP1 or hDPS1 plus hDLP1. The plasmids used for this were pGEX-mSPS1-mDLP1 and pGEX-hDPS1-hDLP1 (Fig. 2), which express both the *SpDps1* and *SpDlp1* homologues under the same promoter, thus enhancing the efficiency and evenness of expression. The *SpDps1* homologue is expressed as a GST-fusion protein, while the *SpDlp1* homologue is expressed as a His-fusion protein. The *E. coli ispB* disruptant KO229 harboring pKA3(*ispB*) [48] was successfully swapped with pGEX-hDPS1-hDLP1 or pGEX-mSPS1-mDLP1, to generate only Q-10 or Q-9, respectively, without *E. coli* originated Q-8 (data not shown). The success of swapping indicates the enzymatic activity is sufficiently high and heterologous SpDps1 and SpDlp1 proteins are together sufficient to produce their own ubiquinone type in *E. coli* KO229 (*ispB*$^-$) harboring pGEX-hDPS1-hDLP1 or pGEX-mSPS1-mDLP1.

We extracted the crude proteins from the pGEX-hDPS1-hDLP1- or pGEX-mSPS1-mDLP1-recombinant *E. coli* JM109 cells and measured the size of the solanesyl/decaprenyl diphosphate synthases in the extracts. To do this, we first performed gel filtration chromatography with the crude proteins and obtained a number of fractions containing GST-mSPS1 and His-mDLP1 or GST-hDPS1 and His-hDLP1. We then analyzed the separated fractions by Western blot analysis using both GST- and His-specific antibodies. The murine solanesyl diphosphate synthase detected at fractions 3-4 in Fig. 5 was estimated to be around 230 kDa in size. This corresponds to the calculated complex size of the postulated murine heterotetramer since GST-mSPS1 and His-mDLP1 are 73kDa and 45

kDa in size, respectively. The postulated heterotetrameric human decaprenyl diphosphate synthase was also of the appropriate size relative to calculations. To ensure that the chromatography was operating properly, we loaded an extract containing homodimeric His-IspB and purified monomeric GST-mSPS1: both were detected at around 70 kDa (fraction 7, Fig. 5) under the same conditions, as expected from their calculated molecular sizes. Thus, we conclude that the solanesyl and decaprenyl diphosphate synthase from mice and humans form a heterotetramer, like the enzyme from *S. pombe*.

Effect of coexpressing long-chain trans- prenyl diphosphate synthase components from different eukaryotic species

The observations above indicate that the long-chain *trans*- prenyl diphosphate synthase of mice and humans, like that from *S. pombe*, consists of two heterologous components. We next asked whether the components from the three species are interchangeable by expressing (i) *mSPS1* or *hDPS1* in the KS10 *S. pombe dps1* disruptant (Δ*dps1::ura4*) or (ii) *mDLP1* or *hDLP1* in the *S. pombe dlp1* disruptant (Δ*dlp1::ura4*). We assessed whether these heterologous proteins caused the disruptants to produce ubiquinone and to grow on minimal medium, as the two disruptants cannot grow on minimal medium without the supplementation of cysteine or glutathione [9]. The expression of *mSPS1* in KS10 (Δ*dps1::ura4*) caused its growth on minimal medium to recover, as did the expression of *hDPS1*; moreover, the former generated small amounts of Q-9 and Q-10 while the latter generated small amounts of Q-10 (Fig. 6). These cells, not like typical ubiquinone less fission yeast [8-10], did not produce sulfide and were not oxidative stress sensitive (data not shown), indicating that small amounts of Q-10 are sufficient for preventing sulfide production and stress sensitivity. In contrast, the expression of either *mDLP1* or *hDLP1* failed to restore the growth of the RS312 *dlp1* disruptant (Δ*dlp1::ura4*) on minimal medium (data not shown). Thus, while mSPS1 and hDPS1 can form functional complexes with SpDlp1 in *S. pombe*, mDLP1 and hDLP1 cannot form functional complexes with SpDps1.

Since we identified the components of the solanesyl/decaprenyl diphosphate synthases in mice and humans, we can ask which component is more important in determining the chain length of ubiquinone by exchanging either component with homologues from other species and analyzing the type of ubiquinone that is produced. Thus, the murine, human and *S. pombe* homologues were coexpressed in heterologous combinations in *E. coli* (Fig. 7). The combination of *mDPS1-hDLP1* and *hDPS1-mDLP1* generated both Q-9 and Q-10 (Fig. 7D, F). Thus, both components of the mammalian long-chain diphosphate synthases contribute to determining the side chain of ubiquinone. However, the combination of *SpDps1-hDLP1* or *SpDps1-mDLP1* did not produce an extra ubiquinone type. (Fig. 7C, E). Thus, the *S. pombe* SpDps1 protein cannot form a complex with the SpDlp1 homologues from mice and humans. This is consistent with the fact that the expression of *mDLP1* or *hDLP1* in the *dlp1* disruptant RS312 failed to restore growth on minimal medium whereas *mSPS1* or *hDPS1* expression in the *dsp1* disruptant KS10 enabled growth on minimal medium (Fig. 6 and data not shown). Table 1 summarized the result obtained by heterologous expression of prenyl diphosphate synthase in *E. coli* and *S. pombe* and it will be discussed later.

Discussion

In this study, we characterized the solanesyl and decaprenyl diphosphate synthase responsible for the side chain of the ubiquinone in mice and humans, respectively. Both are heterotetrameric enzymes composed of SpDps1 and SpDlp1 homologues. This heterotetrameric composition has only been found in *S. pombe* previously [9] as the long-chain *trans*- prenyl diphosphate synthases from other organisms, including bacteria, plants and an another yeast (*S. cerevisiae*) are composed of only one type of protein. The murine and human homologues of SpDps1 (mSPS1 and hDPS1, respectively) show high similarity (30% to 49.0%) to the typical long-chain *trans*- prenyl diphosphate synthases from other organisms such as IspB [46], SdsA [20], DdsA [35], and AtSPS1 [36]. They also possess all

seven conserved regions (domains I to VII) found in the long-chain *trans*- prenyl diphosphate synthases from other organisms. In contrast, the murine and human SpDlp1 homologues (mDLP1 and hDLP1, respectively) show limited similarity to SpDps1 (23%) and lack the aspartate-rich motifs located in domains II and VI that are found in the mSPS1, hDPS1, and SpDps1 proteins and the long-chain *trans*- prenyl diphosphate synthases from other organisms. Despite the marked similarities of mSPS1 and hDPS1 to the homodimer-type of prenyl diphosphate synthases of bacteria, *S. cerevisiae*, and *A. thaliana*, these proteins are not functional enzymes without mDLP1 or hDLP1. This is an important example of the fact an enzymatic activity cannot be assigned to a particular protein on the basis of sequence information alone; its putative function should always be verified experimentally. We showed that humans also carry another Dlp1-like protein that we denoted as hDLP2. However, we found that hDLP2 cannot form a functional decaprenyl diphosphate synthase with hDPS1. It is possible that hDLP2 may function as a regulator or associate with another partner.

Our observations and those of others [35, 36] suggest the long-chain *trans*- prenyl diphosphate synthases that are responsible for ubiquinone biosynthesis are either homodimers or heterotetramers (Fig. 8). Intriguingly, since the hexaprenyl diphosphate synthase from *S. cerevisiae* and the solanesyl diphosphate synthase from the plant *A. thaliana* are homomeric enzymes, it appears that the eukaryotic enzymes are not always heteromeric. Moreover, heteromeric enzymes have been observed in prokaryotes. For example, heteromeric enzymes have been found in *B. subtilis* and *M. luteus* [49, 50], although these enzymes are responsible for the side chain of menaquinone. In addition, GPP synthase from spearmint, which synthesizes short chain isoprenoids, is a heterotetramer [51]. Moreover, we could detect a Dlp1 homolog in the rat, *Drosophila* and *Xenopus*, but not in *C. elegans*. Thus, the *trans*- prenyl diphosphate synthases cannot be classified according to the different kingdoms with regard to their composition. Rather, it appears that the composition of the enzyme in each species is variable and has evolved in this way for unknown reasons.

We asked whether the two components of the heteromeric long-chain *trans*- prenyl diphosphate synthases in *S. pombe*, mice and humans are interchangeable in forming a functional enzyme. To address this question, we first sought to restore long-chain *trans*-prenyl diphosphate synthase activity in *S. pombe* cells with a disrupted *SpDps1* or *SpDlp1* gene by introducing the murine or human homologue of the disrupted gene (Table 1). The mutant phenotypes of the *dps1* mutant were complemented by mSPS1 or hDPS1, but mDLP1 or hDLP1 failed to suppress the mutant phenotypes of the *dlp1* mutant. Moreover, when we expressed heterologous combinations of the murine, human and *S. pombe* enzyme components in *E. coli* and examined the ubiquinone types generated (Table 1), we found again that *hDLP1* and *mDLP1* could not complex with SpDps1 to produce a functional enzyme. In contrast, *E. coli* cells expressing the *mSPS1-hDLP1* or *hDPS1-mDLP1* heterologous combinations produced both Q-10 and Q-9. These observations together indicate two points. First, although *S. pombe* SpDlp1 cannot be substituted by murine or human homologues, the remaining combinations produce viable enzymes. Second, it appears that both components contribute to determining the side chain of ubiquinone, although the hDPS1 or mSPS1 protein appears to have a stronger effect since more Q9 is produced when *mSPS1-hDLP1* is expressed than when *hDPS1-mDLP1* is expressed.

We found that even when the complemented *S. pombe dps1* or *dlp1* disruptants described above produced just one-thirtieth of the wild type levels of ubiquinone, these levels were sufficient to induce growth recovery, prevent sulfide production, and stress sensitivity in fission yeast. This suggests that wild type cells generally contain considerably more ubiquinone than is required for basic cell metabolism.

Why did a heteromeric structure of *trans*- prenyl diphosphate synthase evolve in mammals? It is possible that these heteromeric prenyl diphosphate synthases may have evolved from the homodimeric prokaryotic synthases. Supporting this notion is our previous study with the homodimeric octaprenyl diphosphate synthase from *E. coli*. We previously showed that when *E. coli* is transformed with a construct encoding a octaprenyl diphosphate synthase molecule that is functionally inactive due to a mutation, an active

enzyme is nonetheless formed when the mutant is paired with the wild type enzyme [52]. This observation suggests that the components of the homodimeric enzyme could be subjected to evolutionary alteration wherein they act in a heteromeric form with another molecule.

The finding that the mammalian long-chain *trans*- prenyl diphosphate synthases are heterotetrameric enzymes like that in *S. pombe* suggests that the ubiquinone biosynthetic pathway may not be as conserved as is currently believed. It may thus be necessary to examine this possibility by further characterizing the *S. pombe* genes and to compare with mammalian genes responsible for ubiquinone biosynthesis. It would be also important to take these approaches for understanding the genetic diseases caused by Q-10 deficiency in human [24].

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FIGURE LEGENDS

Fig. 1. Alignment of the amino acid sequences of known long chain *trans*- prenyl diphosphate synthases. (A) Alignment of the amino acid sequences of known long chain-

producing *trans*- prenyl diphosphate synthases. (B) Alignment of the amino acid sequences of a partner protein present in the long chain *trans*- prenyl diphosphate synthases of some organisms. Residues conserved in more than three sequences are boxed. Conserved regions (I-VII) are underlined. The typical aspartate-rich DDXXD motifs in regions II and VI were present in (A) but absent in (B). Numbers on the right indicate amino acid residue positions. (1) One of the two components of solanesyl diphosphate synthase in the mouse, encoded by *mSPS1* (NCBI accession no. AB210838). (2) One of the two components of decaprenyl diphosphate synthase in humans, encoded by *hDPS1* (accession no. AB210839). (3) One of the two components of decaprenyl diphosphate synthase in *S. pombe,* encoded by *SpDps1* (accession no. D84311). (4) The octaprenyl diphosphate synthase in *E. coli* encoded by *ispB* (accession no. U18997). (5) The solanesyl diphosphate synthase in *A. thaliana* encoded by *AtSPS1* (accession no. AB188497). (6) The other component of solanesyl diphosphate synthase in the mouse, the *SpDlp1* homologue *mDLP1* (accession no. AB210840). (7) The other component of decaprenyl diphosphate synthase in humans, the *SpDlp1* homologue *hDLP1* (accession no. AB210841). (8) A splicing variant of *hDLP1, hDLP2* (accession no. AI742294). (9) The *Drosophila SpDlp1* homologue *dDLP1* (accession no.AY05159). (10) The *S. pombe SpDlp1* gene (accession no. AB118853).

Fig. 2. Plasmid constructs used in this study. pBmSPS1, pSTVmDLP1, pUhDPS1 and pSTVhDLP1 express the entire length of the *mSPS1*, *mDLP1*, *hDPS1* and *hDLP1* genes, respectively, under the control of the *lac* promoter. pRmSPS1, pRmDLP1, pRhDPS1 and pRhDLP1 contain the same full-length genes, respectively, under the control of the strong *nmt1* promoter for expression in *S. pombe*. pGEX-mSPS1 contains the full-length *mSPS1* gene

fused to the *GST* gene while pGEX-mSPS1-mDLP1 contains the full-length *mSPS1* and *mDLP1* genes fused to the GST-tag and His6-tag, respectively. The latter was used to express the GST-mSPS1 and His-mDLP1 fusion proteins in *E. coli*. pGEX-hDPS1-hDLP1 contains the full-length *hDPS1* and *hDLP1* genes fused with the GST-tag and His6-tag, respectively, and was used to express the GST-hDPS1 and His-hDLP1 fusion proteins in *E. coli*. B, *Bam*H I; EI, *Eco*R I; H, *Hin*d III; Nd, *Nde* I; No, *Not* I; Sa, *Sal* I; Sm, *Sma* I; Xb, *Xba* I; Xh, *Xho* I.

Fig. 3. HPLC analysis of the ubiquinone extracted from *E. coli* expressing murine or human long chain *trans*- prenyl diphosphate synthase genes. Ubiquinone was extracted from wild type DH5α and DH5α expressing the *SpDps1* homologue and/or the *SpDlp1* homologue from mice or humans, as follows: A, Q-10 standard; B, wild-type (W. T.) *E. coli*; C-H, *E. coli* harboring pBmSPS1 (C), pSTVmDLP1 (D), pBmSPS1 and pSTVmDLP1 (E), pUhDPS1 (F), pSTVhDLP1 (G), pUhDPS1 and pSTVhDLP1 (H). Ubiquinone was first separated from cell extracts by TLC and further analyzed by HPLC.

Fig. 4. Thin-layer chromatogram of the product of the solanesyl diphosphate synthase or decaprenyl diphosphate synthase produced by recombinant *E. coli*. (Panel A) Solanesyl diphosphate synthase activity in BL21 (wild-type, lane 1) and BL21 harboring pGEX-mSPS1 (lane 2), pET-mDLP1 (lane 3), or pGEX-mSPS1-mDLP1 (lane 4) was measured by using [1-^{14}C]IPP and FPP as substrates. (Panel B) Decaprenyl diphosphate synthase activity in BL21 harboring pFhDPS1 (lane 5), pSTVHIShDLP1 (lane 6), or pFhDPS1 and pSTVHIShDLP1 (lane 7) was measured by using the same substrates in panel A. The products were hydrolyzed with phosphatase and the resulting alcohols were analyzed by reverse-phase TLC. Equivalent

amounts of the radiolabeled products (5000 d.p.m) were applied onto the TLC plate. The arrowhead indicates the position of the synthesized decaprenols. The standard alcohols, whose positions are indicated on the right, are GGOH (*all-E*-geranylgeraniol) and SOH (*all-E*-solanesol). Ori., origin; S.F., solvent front.

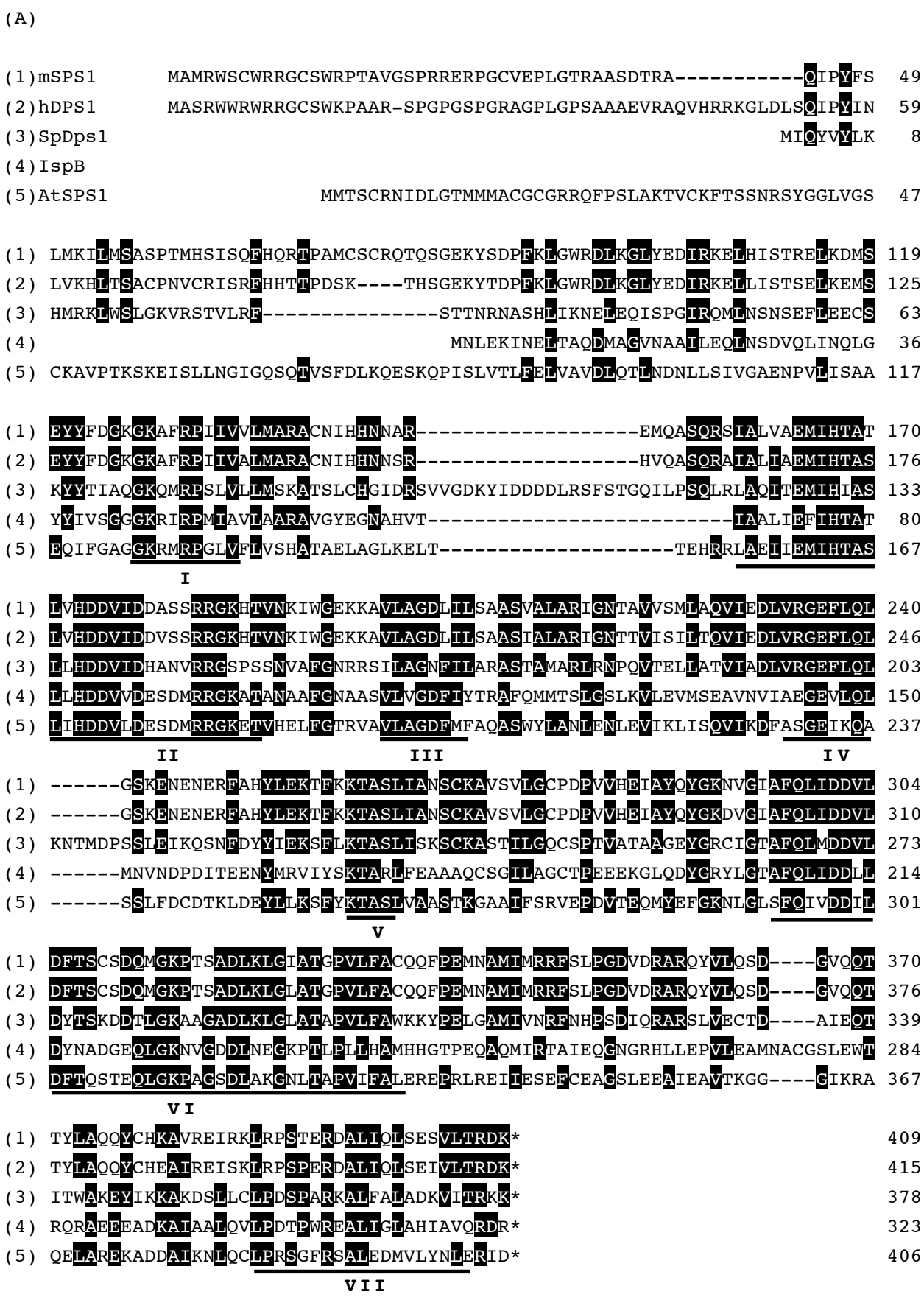
Fig. 5. Size determination of the long-chain *trans*- prenyl diphosphate synthases from mice and humans by gel filtration chromatography and Western blot analysis. Crude proteins from *E. coli* harboring pGEX-mSPS1-mDLP1 or pGEX-hDPS1-hDLP1 were partially separated by gel filtration chromatography on Superdex 200. (Upper panel) Elution behavior of the thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B12 (1.35 kDa) standards. (Lower panel) Elution behavior from the same column of (1) GST-mSPS1 and (2) His-mDLP1, or (3) GST-hDPS1 and (4) His-hDLP1, or (5) GST-mSPS1 purified by Glutathione Sepharose 4B and (6) His-IspB as determined by Western blot analysis of the corresponding column fraction.

Fig. 6. Effect of expressing *mSPS1* or *hDPS1* in the *dps1* disruptant KS10 on its growth on minimal medium and ubiquinone production. (A) The KS10 *(Δdps1::ura4*) disruptant harboring pREP1 (*LEU2* marker) together with pRDPS1, pRmSPS1, or pRhDPS1 were grown on PM medium supplemented with 75 μg/ml adenine. (B) The same strains were grown on PM medium supplemented with adenine and 200 μg/ml cysteine. KS10 harboring pRmSPS1 or pRhDPS1 grow on PM medium lacking cysteine (A). (C) Ubiquinone was extracted from untransfected KS10 cells and KS10 cells harboring pREP1, pRDPS1, pRmSPS1 and pRhDPS1. Ubiquinone was first separated by TLC and then further analyzed

by HPLC.

Fig. 7. Effect on ubiquinone type of expressing heterologous combinations of SpDps1 and SpDlp1 homologues from various eukaryotes in *E. coli*. Ubiquinone was extracted from wild-type DH5α and DH5α harboring various heterologous combinations of the SpDps1 and SpDlp1 homologues from mice, humans and *S. pombe*. A, Q-10 standard; B, wild-type (W. T.); C, *E. coli* harboring pBSDPS1 and pSTVhDLP1; D, pBmSPS1 and pSTVhDLP1; E, pBSDPS1 and pSTVmDLP1; F, pUhDPS1 and pSTVmDLP1; G, pBmSPS1 and pSTVDLP1; H, pUhDPS1 and pSTVDLP1.

Fig. 8. Classification of *trans*- polyprenyl diphosphate synthases. The various types of *trans*- polyprenyl diphospahte synthases are schematically depicted. The *trans*-polyprenyl diphosphate synthases used to construct ubiquinone in bacteria and plants are homodimeric, while bacterial *trans*-polyprenyl diphosphate synthases used to construct menaquinone are heterodimeric. The *trans*-polyprenyl diphosphate synthases used to construct ubiquinone in *S. pombe*, mice and humans are heterotetrameric, while the equivalent enzyme in *S. cerevisiae* is believed to be homomeric. The circular components show the primary structures of the typical prenyl diphosphate synthases.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(3)SpDps1 MIQYVYLK 8
(4)IspB
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(2) LVKHLTSACPNVCRISREHHTTPDSK----THSGEKYTDPFKLGWRDLKGLYEDIRKELLISTSELKEMS 125
(3) HMRKLWSLGKVRSTVLRF---------------STTNRNASHLIKNELEQISPGIRQMLNSNSEFLEECS 63
(4) MNLEKINELTAQDMAGVNAAILEQLNSDVQLINQLG 36
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(4) YYIVSGGGKRIRPMIAVLAARAVGYEGNAHVT----------IAALIEFIHTAT 80
(5) EQIFGAGGKRMRPGLVFLVSHATAELAGLKELT----------TEHRRLAEIIEMIHTAS 167
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(3) KNTMDPSSLEIKQSNFDYYIEKSFLKTASLISKSCKASTILGQCSPTVATAAGEYGRCIGTAFQLMDDVL 273
(4) ------MNVNDPDITEENYMRVIYSKTARLFEAAAQCSGILAGCTPEEEKGLQDYGRYLGTAFQLIDDLL 214
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VI
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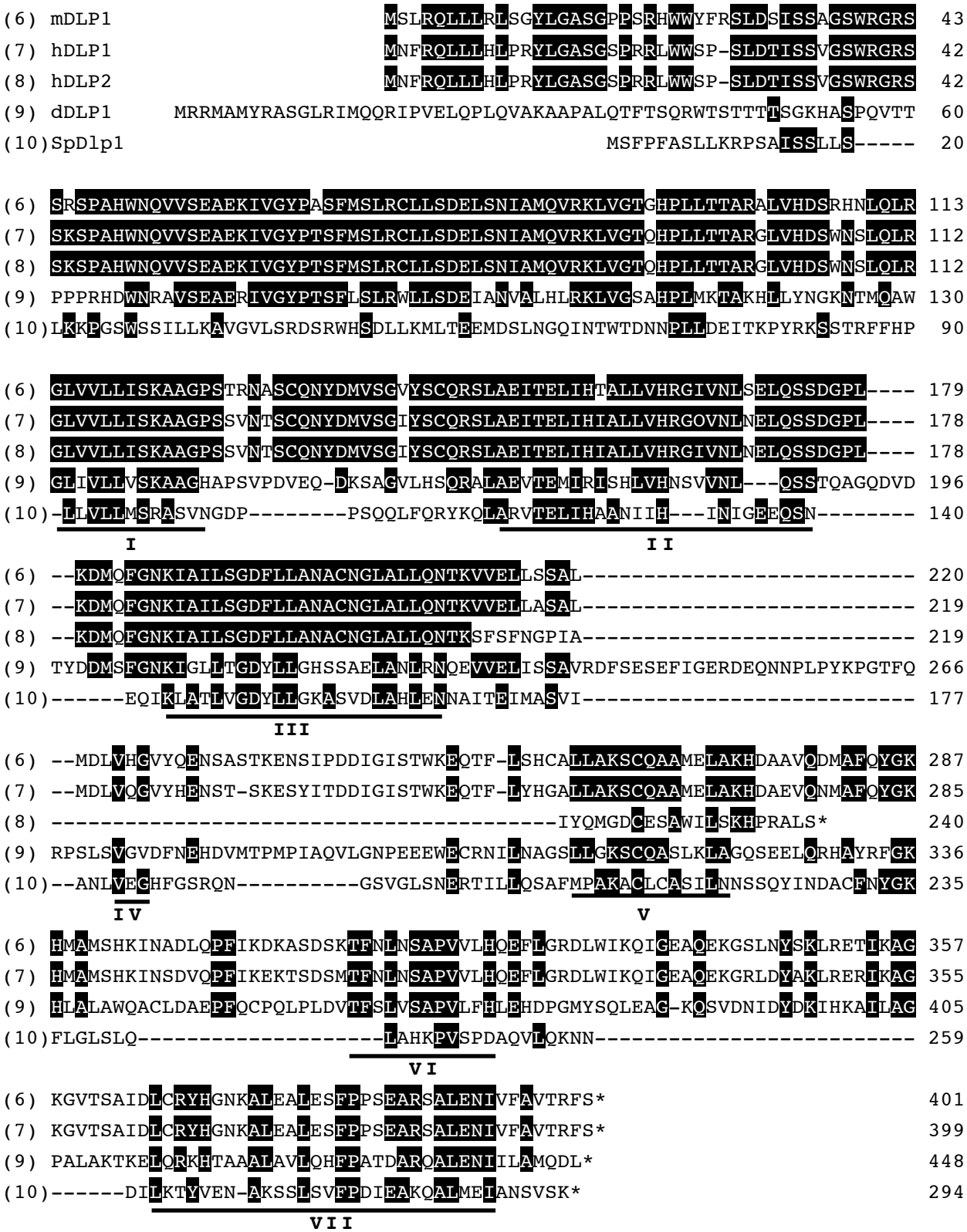
VII

(B)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III
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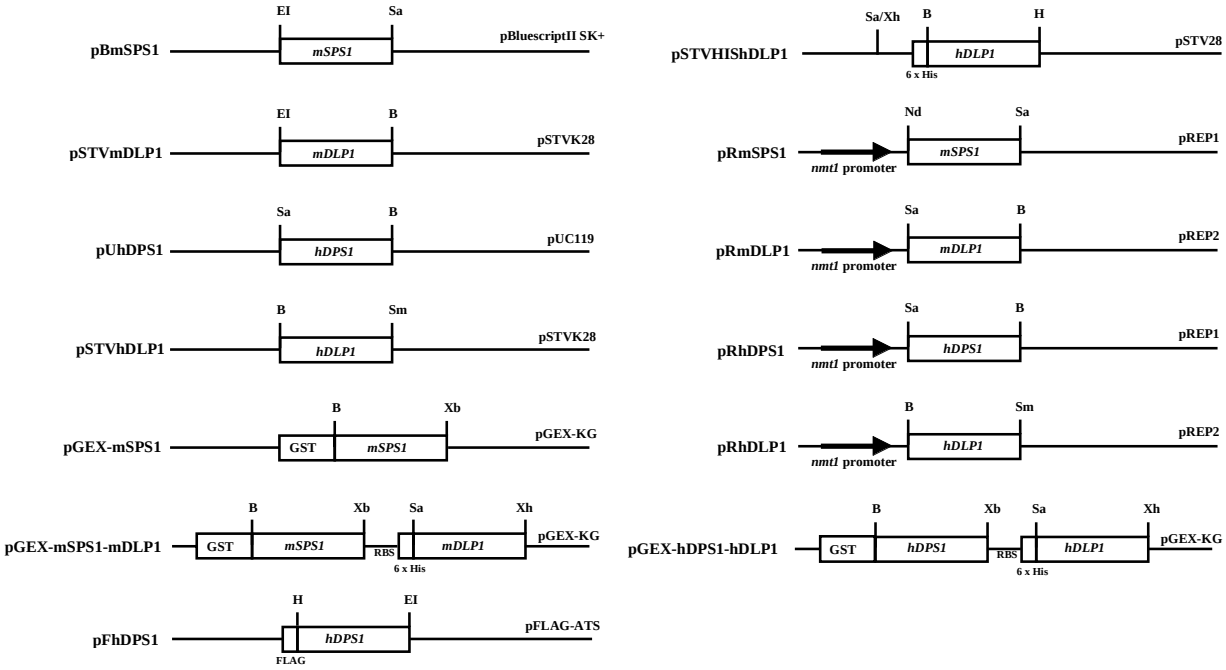
VII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RBS
mDLP1
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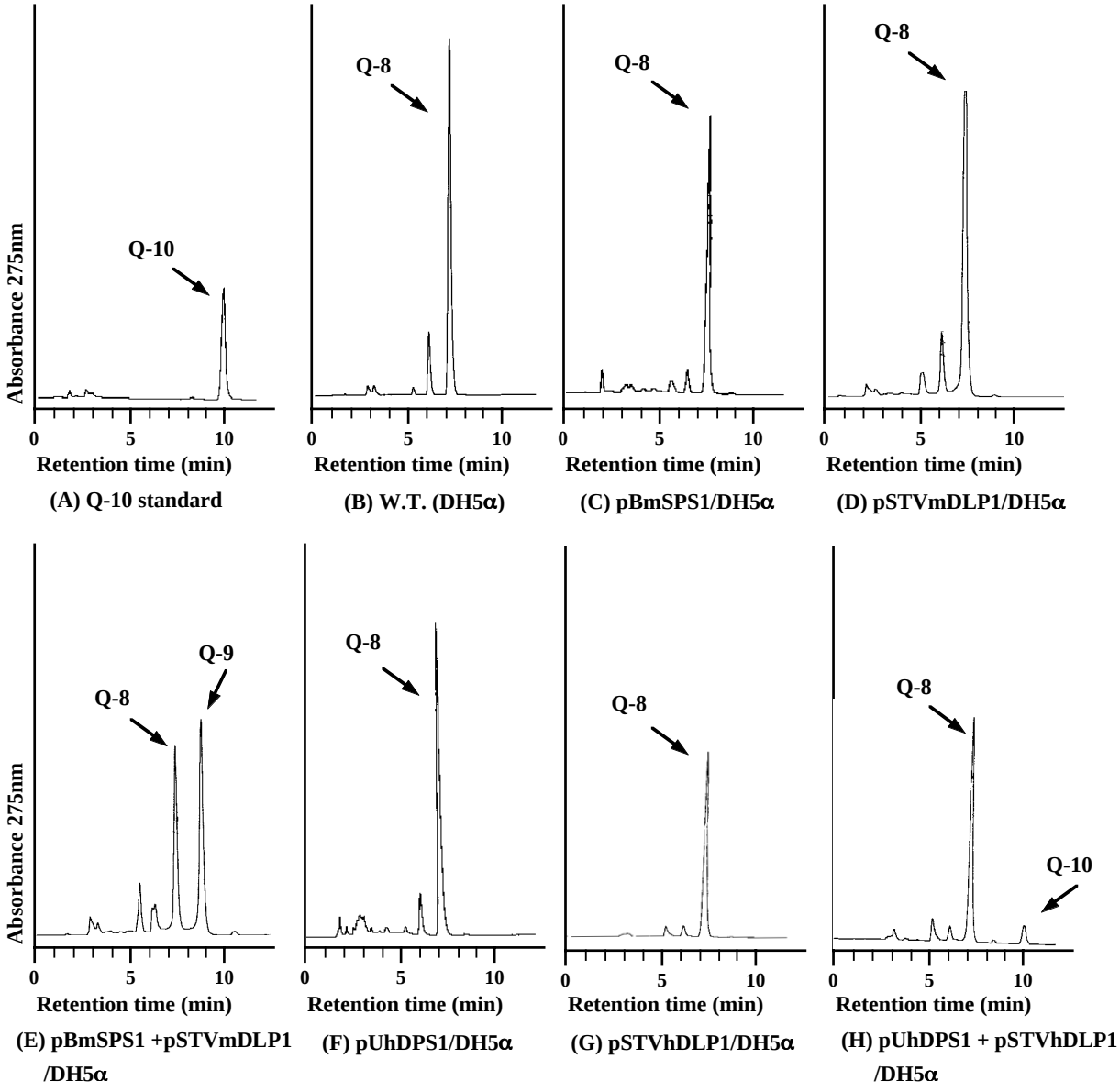
Fig. 2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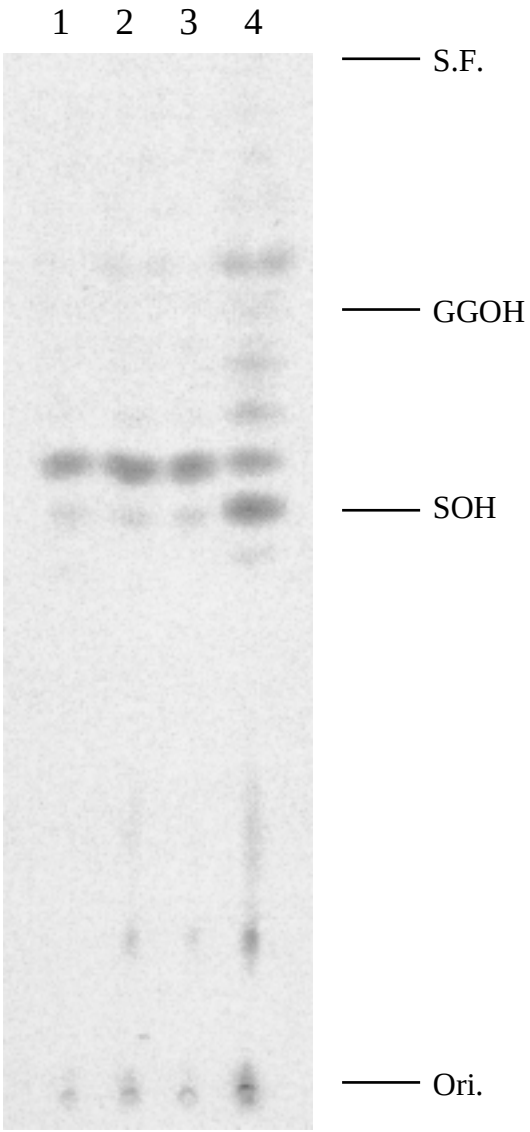
5
10
Retention time (min)
(A) Q-10 standard
(B) W.T. (DH5α)
(C) pBmSPS1/DH5α
(D) pSTVmDLP1/DH5α
(E) pBmSPS1 +pSTVmDLP1 /DH5α
(F) pUhDPS1/DH5α
(G) pSTVhDLP1/DH5α
(H) pUhDPS1 + pSTVhDLP1 /DH5α

Fig. 3

(A)



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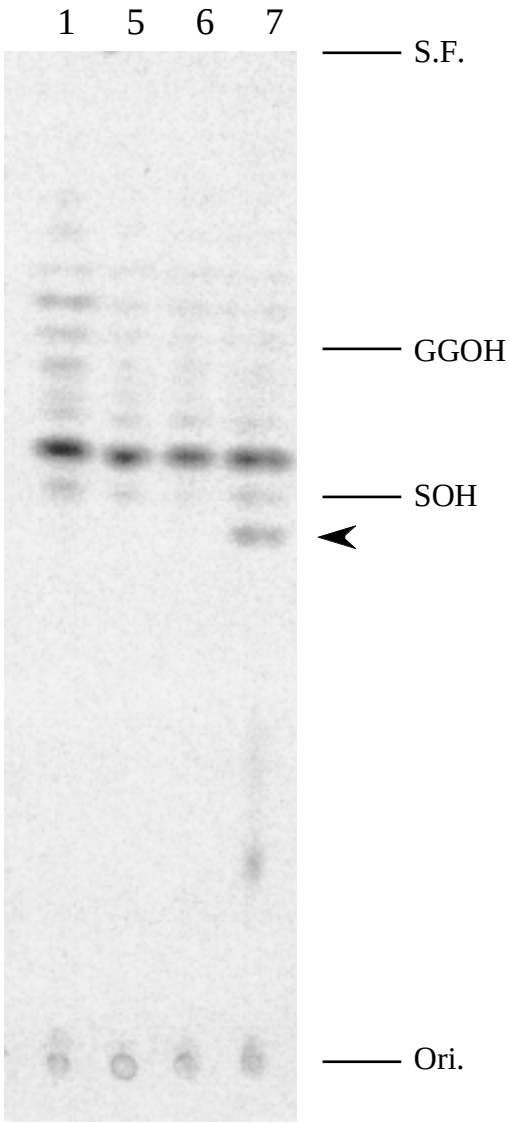
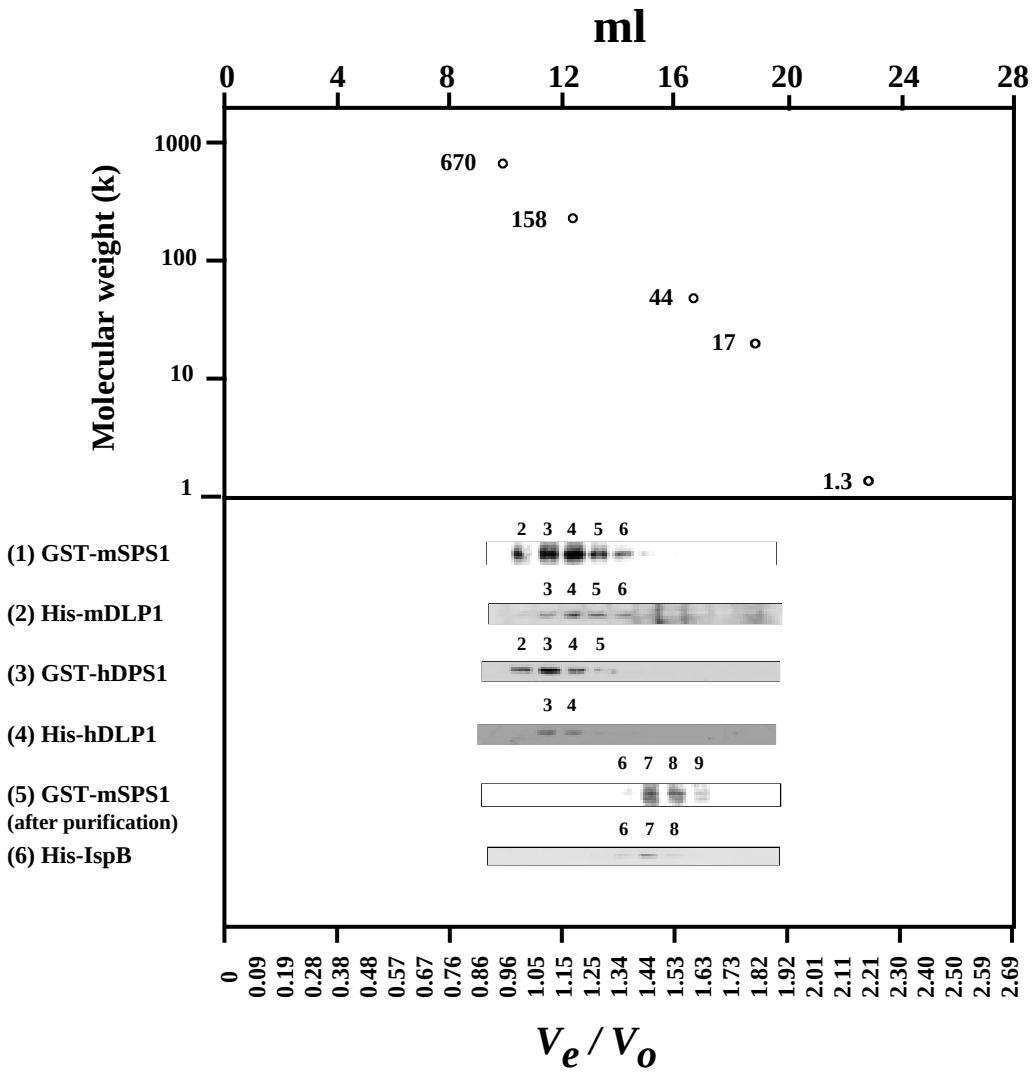


Fig. 4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12
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20
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28
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100
10
1
670
158
44
17
1.3
(1) GST-mSPS1
(2) His-mDLP1
(3) GST-hDPS1
(4) His-hDLP1
(5) GST-mSPS1
(after purification)
(6) His-IspB
2 3 4 5 6
3 4 5 6
2 3 4 5
3 4
6 7 8 9
6 7 8
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0.09
0.19
0.28
0.38
0.48
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0.76
0.86
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1.73
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2.69
Ve / Vo

Fig. 5

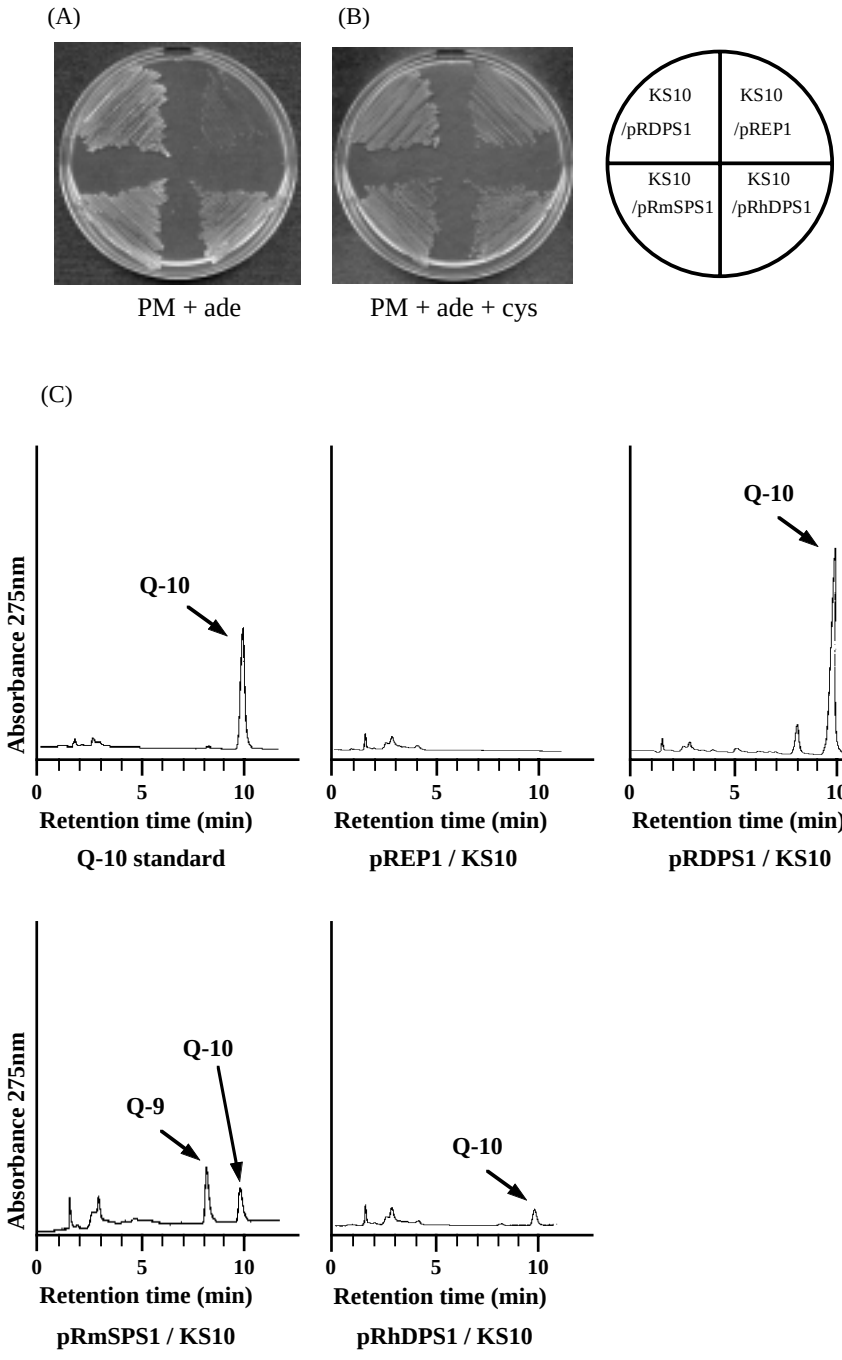
(A)
(B)
KS10 /pRDPS1
KS10 /pREP1
KS10 /pRmSPS1
KS10 /pRhDPS1
PM + ade
PM + ade + cys
(C)
Absorbance 275nm
Q-10
Retention time (min)
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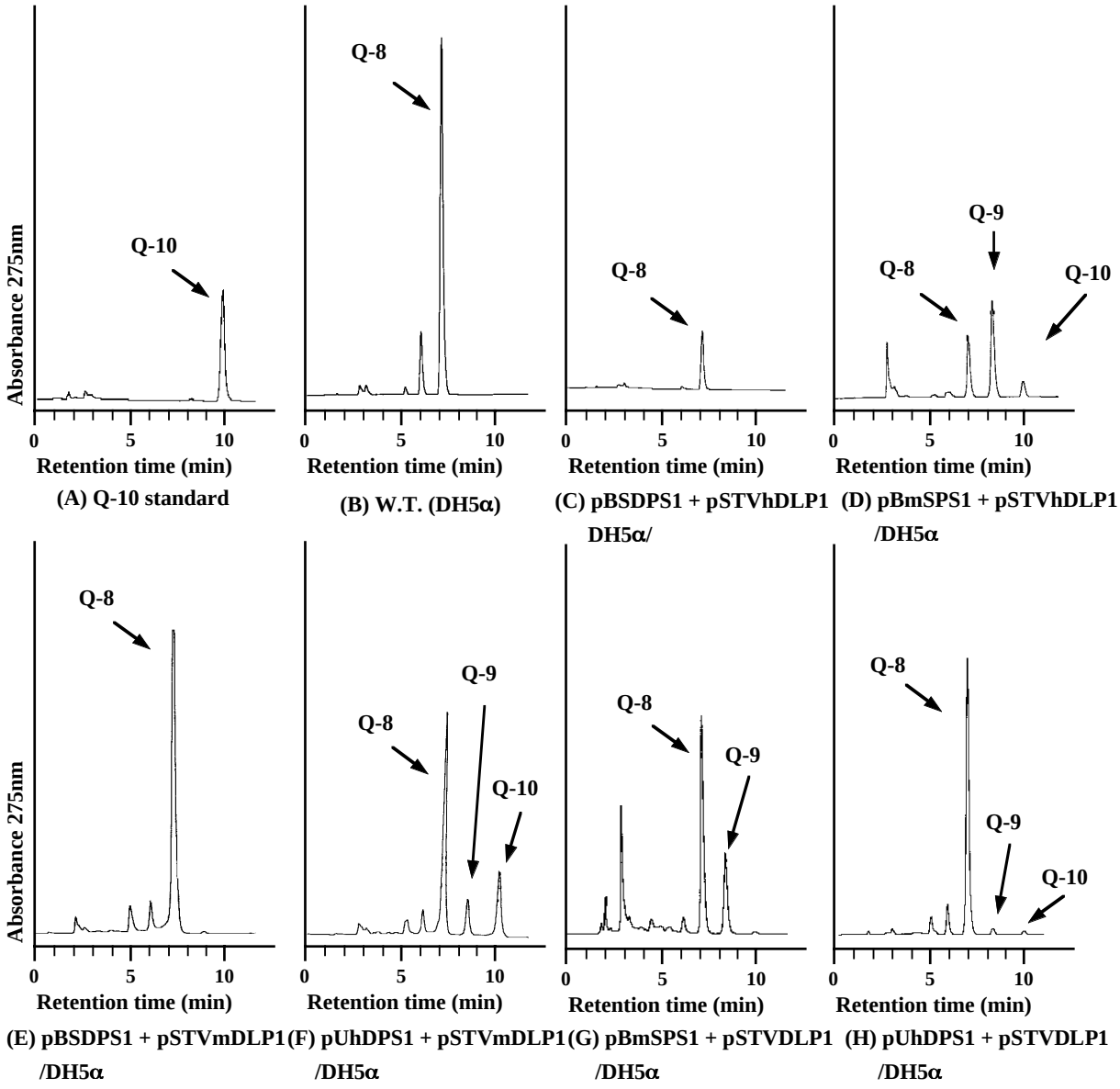
Fig. 6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Q-10
Q-8
Q-9
Q-9
Q-10
0
5
10
Retention time (min)
(A) Q-10 standard
(B) W.T. (DH5α)
(C) pBSDPS1 + pSTVhDLP1 DH5α/
(D) pBmSPS1 + pSTVhDLP1 /DH5α
(E) pBSDPS1 + pSTVmDLP1 /DH5α
(F) pUhDPS1 + pSTVmDLP1 /DH5α
(G) pBmSPS1 + pSTVDLP1 /DH5α
(H) pUhDPS1 + pSTVDLP1 /DH5α

Fig. 7

Classification of long-chain prenyl diphosphate synthases

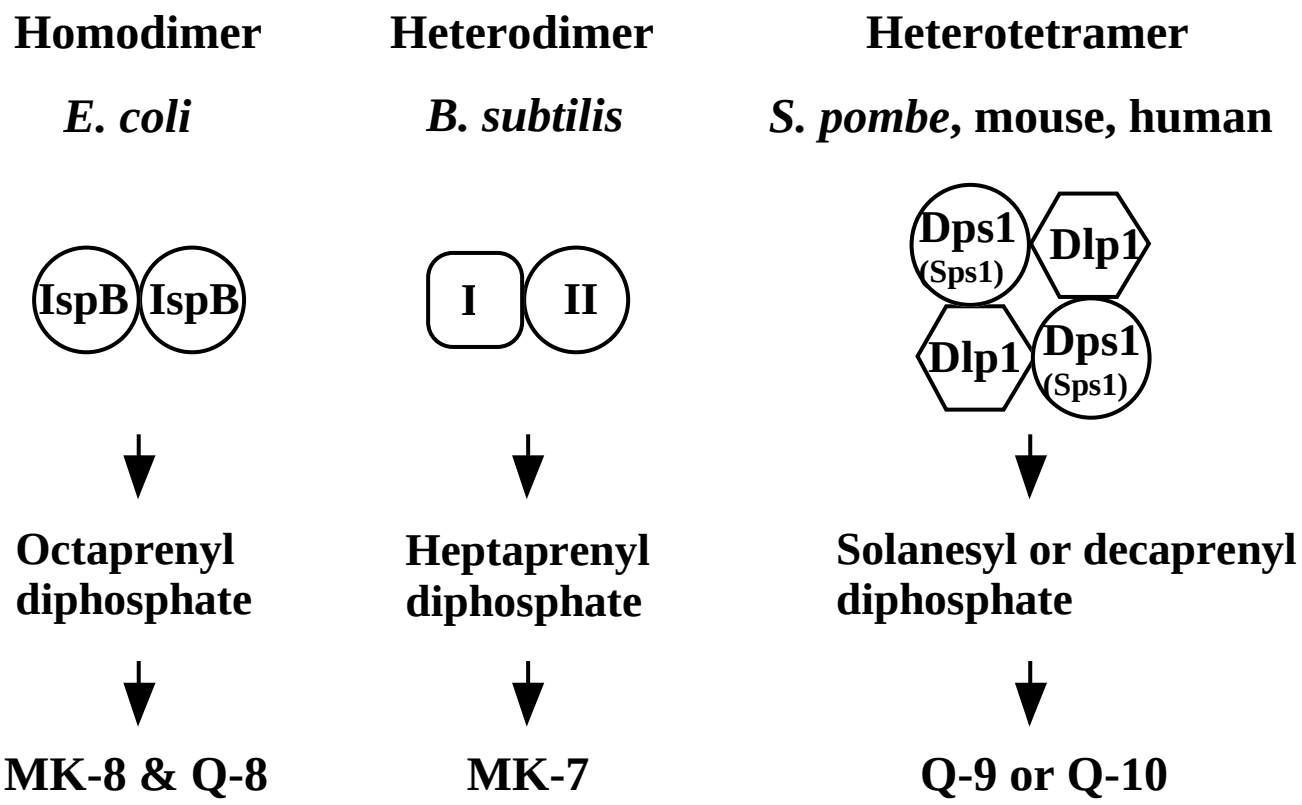

Fig. 8

Table 1. Heterologous combination of polyprenyl diphosphate synthases

combinations	*E. coli* (W.T.)	*S. pombe*	
		Δ*dps1*	Δ*dlp1*
mSPS1	Q-8	Q-9+Q-10	N. D.
mDLP1	Q-8	N. D.	N. D.
hDPS1	Q-8	Q-10	N. D.
hDLP1	Q-8	N. D.	N. D.
Dps1	Q-8	Q-10	N. D.
Dlp1	Q-8	N. D.	Q-10
mSPS1+mDLP1	Q-8+Q-9		
hDPS1+hDLP1	Q-8+Q-10		
Dps1+Dlp1	Q-8+Q-9+Q-10		
Dps1+hDLP1	Q-8		
mSPS1+hDLP1	Q-8+Q-9+Q-10		
Dps1+mDlp1	Q-8		
hDPS1+mDLP1	Q-8+Q-9+Q-10		
mSPS1+Dlp1	Q-8+Q-9		
hDPS1+Dlp1	Q-8+Q-9+Q-10		

* Underline indicates the major type of ubiquinone. N.D.; Ubiquinone was not detected.