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Geranylfarnesyl diphosphate synthase from *Methanosarcina mazei*: Different role, different evolution

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ABSTRACT

The gene of (all-*E*) geranylfarnesyl diphosphate synthase that is responsible for the biosynthesis of methanophenazine, an electron carrier utilized for methanogenesis, was cloned from a methanogenic archaeon *Methanosarcina mazei* Gö1. The properties of the recombinant enzyme and the results of phylogenetic analysis suggest that the enzyme is closely related to (all-*E*) prenyl diphosphate synthases that are responsible for the biosynthesis of respiratory quinones, rather than to the enzymes involved in the biosynthesis of archaeal membrane lipids, including (all-*E*) geranylfarnesyl diphosphate synthase from a thermophilic archaeon.

Introduction

Methanophenazine {2-[2′,3′-dihydro-(all-*E*)-geranylfarnesyloxy]phenazine} is a hydrophobic compound utilized for methanogenesis in *Methanosarcina* species [1]. It acts as an electron carrier for membranous hdrED-type heterodisulfide reductase, which catalyzes the oxidation of reduced methanophenazine (dihydromethanophenazine) and the reduction of CoB-S-S-CoM, the common terminal electron accepter in the energy-conserving electron transport chain of methanogens [1,2]. In *Methanosarcina* species, the reduction of methanophenazine is catalyzed by $F_{420}H_2$ dehydrogenase or F_{420}-non-reducing hydrogenase [2–4]. As an electron donor for the reduction of methanophenazine, the former enzyme utilizes cofactor $F_{420}H_2$ formed by the action of F_{420}-reducing hydrogenase, while the latter directly uses hydrogen. The structure and function of methanophenazine are analogous to those of respiratory quinones [4,5], which have thus far not been found from methanogens. Respiratory quinones, e.g., ubiquinone, menaquinone, and plastoquinone, also have a polyprenyl side-chain connected to a redox-active aromatic moiety, and act in the electron transport chain of aerobic or anaerobic respiration. It is interesting that methanogens other than *Methanosarcina* species (and probably their close relatives [6]) are known to lack methanophenazine. They have a distinct, soluble HdrABC-type heterodisulfide reductase, which requires electron donors other than methanophenazine. HdrABC-type heterodisulfide reductases from *Methanothermobacter thermautotrophicus* [7] and *M. marburgensis* [8] were reported to be in a complex with F_{420}-non-reducing hydrogenase, suggesting that electrons used for the reduction of CoB-S-S-CoM are donated from hydrogen through the enzyme complex. *Methanosarcina* species also possess HdrABC-type heterodisulfide reductase that can accept electrons from ferredoxin, although the enzyme is likely to be used solely in methylotrophic methanogenesis [9].

The biosynthetic pathway of methanophenazine is unclear, but is thought to resemble those of respiratory quinones. The simplest hypothesis is that the polyprenyl side-chain of methanophenazine is transferred from the donor of a C_{25} prenyl group, i.e., (all-*E*) geranylfarnesyl diphosphate (GFPP), to the 2-hydroxyphenazine moiety or its precursor, followed by the selective reduction of its double bond at the position 2. On the other hand, *Methanosarcina* species have been reported to produce archaea-specific C_{20}-C_{20} diether membrane lipids, which are synthesized from (all-*E*) geranylgeranyl diphosphate (GGPP) [10]. It is, therefore, an interesting question whether GFPP and GGPP are produced by the action of a single, bifunctional (all-*E*) prenyl diphosphate synthase or by two distinct enzymes, i.e., GFPP synthase (GFPS) and GGPP synthase (GGPS), respectively.

In the present study, we found two homologues of (all-*E*) prenyl diphosphate synthase encoded in the genome of *Methanosarcina mazei* Gö1. One of them was shown to be GFPS, which may be responsible for methanophenazine biosynthesis. Interestingly, the phylogenetic analysis on (all-*E*) prenyl diphosphate synthases sug-

Abbreviations: CoB-S-S-CoM, heterodisulfide of coenzyme B and coenzyme M; DMAPP, dimethylallyl diphosphate; F_{420}, cofactor F_{420}; FARM, the first aspartate rich motif; FPP, (all-*E*) farnesyl diphosphate; FPS, FPP synthase; GGPP, (all-*E*) geranylgeranyl diphosphate; GGPS, GGPP synthase; GFPP, (all-*E*) geranylfarnesyl diphosphate; GFPS, GFPP synthase; GPP, geranyl diphosphate; IPP, isopentenyl diphosphate; TLC, thin-layer chromatography.

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gested that the evolutionary route of *M. mazei* GFPS is very different from that of a previously found archaeal isozyme, GFPS from a hyperthermophilic archaeon *Aeropyrum pernix* [11].

Materials and methods

Materials. Precoated reversed-phase thin-layer chromatography plates LKC-18F were purchased from Whatman International Ltd., United Kingdom. Geranyl diphosphate (GPP) was donated by Drs. Kyozo Ogura and Tanetoshi Koyama, Tohoku University, Japan. Dimethylally diphosphate (DMAPP), (all-*E*) farnesyl diphosphate (FPP), and non-labeled isopentenyl diphosphate (IPP) were donated by Dr. Chikara Ohto, Toyota Motor Co. Japan. [1-^{14}C]IPP was purchased from GE healthcare, USA. GGPP was purchased from Larodan fine chemicals, Sweden. All other chemicals were of analytical grade.

Cultivation of the microorganism. *M. mazei* Gö1 was cultured in a DSMZ120 *Methanosarcina* medium at 30 °C and harvested at the late log phase.

Cloning, expression and purification of M. mazei GFPS. Homologues of (all-*E*) prenyl diphosphate synthase encoded in the genome sequence of *M. mazei* were searched against the MicroBial Genome Database (http://mbgd.genome.ad.jp/) using *Sulfolobus acidocaldarius* GGPS as a query sequence. The ORF, MM_0789, encoding one of the searched homologues, was amplified using the genome of *M. mazei* Gö1 as a template and primers 5′-CATGTACATATGAATATTGAAGAATGGGAAGAA-3′ and 5′-ATTCAACTCGAGATTCAATCAGAGGTTCTCAAGCAT-3′. The newly introduced restriction sites for *Nde*I and *Xho*I in the amplified gene (underlined) were excised and then ligated into a pET-15b vector (Novagen, United States). The resultant plasmid was introduced into *Escherichia coli* BL21 (DE3) and the transformant was grown at 37 °C in 500 ml LB medium, supplemented with 100 mg/liter ampicillin. When the optical density at 660 nm of the culture reached 0.4, 1.0 mM IPTG was added for induction. After additional overnight cultivation, the cells were harvested and disrupted by sonication in 20 mM sodium phosphate buffer, pH 7.4 (termed buffer A). The homogenate was centrifuged at 15,000 *g* for 30 min, and the supernatant was recovered as a crude extract. The pellet precipitated by the addition of 40–60% ammonium sulfate in the crude extract was collected by centrifugation at 15,000 *g* for 30 min and resuspended in 10 ml of buffer A containing 35% $(NH_4)_2SO_4$. The resuspended solution was loaded onto a Hiprep 16/10 Butyl FF column (GE Healthcare, USA) and then eluted with a gradient of 35 to 0% $(NH_4)_2SO_4$ in buffer A. The active fractions were gathered, dialyzed against buffer A, and loaded onto a Mono Q 5/50 GL column (GE Healthcare) and eluted with a gradient of 0 to 1.0 M NaCl in buffer A. The active fractions were gathered, concentrated, and loaded onto a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare) and eluted with buffer A containing 0.15 M NaCl. Active fractions were collected and used for characterization. The level of purification was confirmed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The N-terminal sequence of the purified enzyme was determined with a Procise HT sequencer (Applied Biosystems, USA).

Prenyltransferase assay. In a final volume of 200 μl, the assay mixture contained 0.5 nmol of [1-^{14}C]IPP (2.04 GBq/mmol), 0.5 nmol of allylic diphosphate (DMAPP, GPP, FPP, or GGPP), 0.2 μmol of $MgCl_2$, 20 μmol of MES buffer, pH 7.0, and a suitable amount of enzyme, and was incubated at 37 °C for 10 min. To determine the pH preference of the enzyme, the buffer was changed to succinate-NaOH, pH 5.0 and 6.0, MES, pH 6.0, or HEPES, pH 7.0 and 8.0. To elucidate the metal ion dependency, $MgCl_2$ was replaced with $CaCl_2$, $MnCl_2$, and $ZnCl_2$. After the reaction was stopped by chilling in an ice bath, the mixture was extracted with 600 μl of 1-butanol saturated with H_2O, and the butanol layer was washed with water saturated with NaCl. The radioactivity in 10% of the butanol layer was measured with a LSC-5100 liquid scintillation counter (Aloka, Japan). The rest of the butanol layer was treated with potato acid phosphatase (Sigma, USA) according to the method of Fujii et al. [12], and the hydrolysates were then extracted with *n*-pentane to be analyzed by reversed-phase thin-layer chromatography (TLC) using a precoated plate, LKC-18F, developed with acetone-H_2O (9:1). The distribution of radioactivity was detected using a BAS2000 bioimaging analyzer (Fujifilm, Japan). The authentic C_{20}, C_{25}, and C_{30} prenyl alcohols used for the TLC analysis were synthesized by hydrolyzing the reaction products of GGPS from *S. acidocaldarius* [13], its F77S mutant [14], and hexaprenyl diphosphate synthases from *Sulfolobus solfataricus* [15], respectively.

Phylogenetic analysis. Amino acid sequences of (all-*E*) prenyl diphosphate synthases obtained from public databases were aligned using the CLUSTAL X 2.0 program [16]. The phylogenetic tree was constructed with NJplot software based on the neighbor-joining method [17]. All parameters used in these programs were set at default.

Results and discussion

Cloning of the novel (all-E) prenyl diphosphate synthase gene from M. mazei

Previous studies of (all-*E*) prenyl diphosphate synthases enabled us to estimate the chain-length of their conclusive products based on the amino acid sequences of conserved regions, and to classify them using these estimates. The sequence around the first aspartate rich motif (FARM) is an especially important signature for distinguishing short-chain (all-*E*) prenyl diphosphate synthase, which is a traditionally-classified group of enzymes that yield products with a C_{10-25} hydrocarbon chain, from the enzymes that yield longer products [18]. FARM, with a typical sequence of DDXX(XX)D, is highly conserved through (all-*E*) prenyl diphosphate synthases and is involved in substrate binding. The crystal structures of the enzymes revealed that the hydrocarbon chain-length of the final product is determined by the size of the reaction pocket of the enzyme, which accepts the hydrocarbon chain elongated from FARM [19,20]. In most short-chain (all-*E*) prenyl diphosphate synthases, with some exceptions [21,22], a bulky amino acid such as phenylalanine or tyrosine at the fifth position upstream from FARM plays a large role in regulation of the pocket size [14,20,23,24].

Using the sequence of *S. acidocaldarius* GGPS [13] as the probe, a homology search was performed to find two ORFs, MM_1767 and MM_0789, that encode the homologues of (all-*E*) prenyl diphosphate synthase in the genome of *M. mazei* Gö1. The enzyme encoded in MM_1767, which is 40.4% identical to *S. acidocaldarius* GGPS and is annotated as a dimethylallyltranstransferase, has phenylalanine at the fifth position upstream from FARM, while that encoded in MM_0789, which is 37.2% identical and is annotated as a hypothetical geranyltranstransferase, has alanine (Fig. 1). Because all known archaeal GGPSs have an aromatic amino acid at the fifth position upstream from FARM, MM_1767 was considered to encode GGPS that is responsible for the biosynthesis of archaeal membrane lipids. On the other hand, the enzyme encoded in MM_0789 was expected to yield longer products that are probably utilized for the biosynthesis of methanophenazine. We therefore decided to isolate the latter gene to elucidate the role of the encoded protein.

We amplified the ORF, MM_0789, by PCR from the genome of *M. mazei*. The ORF is 888 bp long and encodes a protein of 295 amino

Fig. 1. Alignment of amino acid sequences around FARM. Partial sequences of *S. acidocaldarius* GGPS (sacGGPS) and its two homologues encoded in the genome of *M. mazei*, MM_1767 and MM_0789, were aligned. The fifth position upstream from FARM is emphasized by bold letters and an asterisk.

acids. The amplified fragment was subcloned into an expression vector, pET-15b. *E. coli* strain BL21 (DE3) was then transformed with the construct pET-MM_0789. The assay, using crude extracts from the transformant *E. coli*, showed an increase in prenyltransferase activity by induction with IPTG (data not shown), suggesting that the recombinant enzyme was expressed in *E. coli*. However, purification with a Ni-chelating affinity column was failed although the plasmid was constructed to yield the enzyme attached a polyhistidine-tag at its N-terminus.

Purification and characterization of recombinant M. mazei (all-E) prenyl diphosphate synthase

The crude extract from the transformant cells harvested from 500 ml of culture was provided for successive ammonium sulfate precipitation and a series of column chromatography: Butyl-Sepharose, Mono Q, and Superdex. SDS–PAGE demonstrated that the enzyme was purified to homogeneity (Fig. 2). The mass of the enzyme was estimated to be 33 kDa, which corresponded well with the molecular weight calculated from its amino acid sequence. The N-terminal sequence of the purified enzyme showed that the translation started from its second methionine codon (data not shown).

The products and substrate preference of the novel archaeal (all-*E*) prenyl diphosphate synthase were determined as described in Materials and methods. The main product of the enzyme was GFPP, while a considerable amount of GGPP as an intermediate was also observed when FPP was used as the substrate (Fig. 3A). This result strongly supports the hypothesis that the polyprenyl side-chain of methanophenazine is synthesized from GFPP, and that the enzyme encoded in MM_0789 is GFPS. GFPSs have been found from a haloalkaliphilic archaeon *Natronobacterium pharaonis* [25] and a hyperthermophilic archaeon *A. pernix* [11], but only the gene of the latter enzyme has been cloned. (It should be noted that the authors called the enzymes farnesylgeranyl diphosphate synthases.) As shown in Fig. 3B, *M. mazei* GFPS preferred GGPP and FPP as an allylic substrate, and showed only slight activity toward

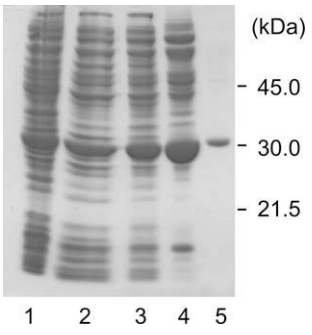

Fig. 2. SDS–PAGE of recombinant (all-*E*) prenyl diphosphate synthase from *M. mazei*. The purification procedure is described in the Materials and methods section. The gel was visualized by Coomassie Brilliant Blue staining. Lane 1, crude extract from *E. coli* BL21(DE3)/pET-MM_0789 induced with IPTG; lane 2, the active fraction after ammonium sulfate precipitation; lane 3, Butyl-Sepharose; lane 4, Mono Q; lane 5, Superdex.

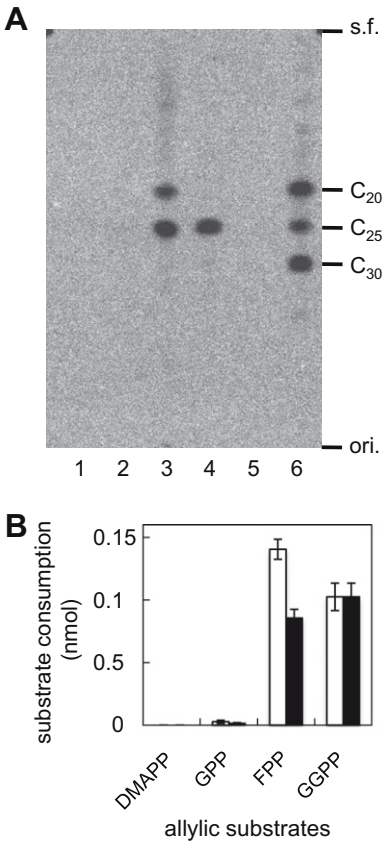

Fig. 3. Reaction products and substrate specificity of *M. mazei* (all-*E*) prenyl diphosphate synthase. (A) The TLC autoradiogram for the product analysis was obtained as described in the Materials and methods section. Lanes 1–5 represent the hydrolyzed products of purified recombinant *M. mazei* (all-*E*) prenyl diphosphate synthase using 0.5 nmol of [^{14}C]IPP (2.04 GBq/mmol) and the same amount of various allylic substrates as follows: lane 1, DMAPP; lane 2, GPP; lane 3, FPP; lane 4, GGPP; lane 5, without an allylic substrate. In each assay condition, less than 20% of each substrate reacted. Lane 6 represents the mixture of the authentic C_{20}, C_{25}, and C_{30} prenyl alcohols synthesized by the hydrolysis of GGPP, GFPP, and hexaprenyl diphosphate, respectively. ori., origin; s.f., solvent front. (B) Reactivity toward the allylic substrates is indicated as the consumption of the substrates in the enzyme reactions ($n = 3$ for each allylic substrate). Open and closed bars represent the amounts of IPP and the allylic substrates consumed, respectively. The amounts of the allylic substrates consumed were calculated from those of reacted IPP and from the product distribution determined from the TLC autoradiogram.

GPP. DMAPP was not accepted by the enzyme, suggesting that the enzyme utilizes the product of GGPS, which is probably encoded in MM_1767, as the primer substrate for prenyl elongation in the cells of *M. mazei*. Given the data on product distribution obtained from the radio-TLC analysis (for example, the ratio of GFPP to GGPP was 78% to 22% when FPP was used as the substrate), a larger amount of GGPP appeared to be consumed, compared with FPP. The substrate specificity resembled those of undecaprenyl diphosphate synthase from *S. acidocaldarius* [26] and hexaprenyl diphosphate synthase from *S. solfataricus* [15], both of which utilize GGPP as the primer substrate for prenyl elongation. The optimal reaction temperature and pH of the enzyme were 40 °C and 7.0. The enzyme showed activity with Mg^{2+} but not with other divalent cations such as Ca^{2+}, Mn^{2+}, and Zn^{2+}. Moreover, the effect of Mg^{2+} was highest around 5.0 mM, through the range from 1.0 to 50 mM.

Phylogenetic analysis of (all-E) prenyl diphosphate synthases

The phylogenetic tree constructed from the amino acid sequences of 23 (all-*E*) prenyl diphosphate synthases, including *M.*

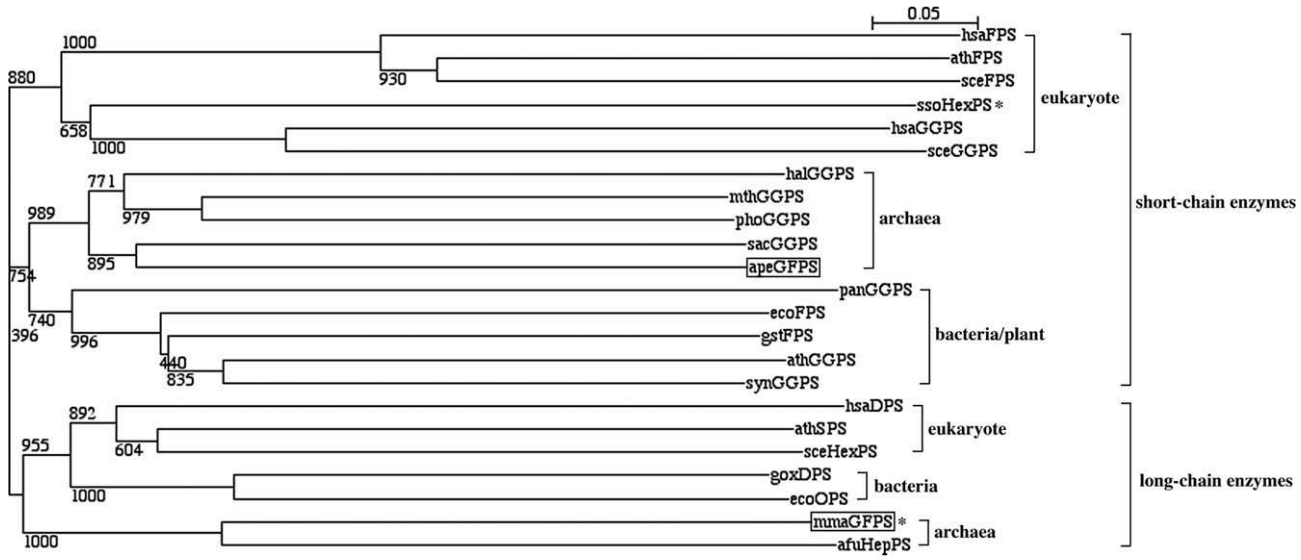

Fig. 4. Phylogenetic tree of (all-*E*) prenyl diphosphate synthases. Alignment of the amino acid sequences of (all-*E*) prenyl diphosphate synthases was performed by using the CLUSTAL X 2.0 program [16], and the phylogenetic tree was constructed with NJplot free software based on the neighbor-joining method [17]. The numbers at each node indicate the bootstrap values. The abbreviations and accession numbers for each protein are as follows: hsaFPS, *Homo sapiens* FPP synthase (BAA03523); athFPS, *Arabidopsis thaliana* FPP synthase (AAB07247); sceFPS, *Saccharomyces cerevisiae* FPP synthase (AAA34606); ssoHexPS, *S. solfataricus* hexaprenyl diphosphate synthase (AAK42496); hsaGGPS, *H. sapiens* GGPS (BAA75909); sceGGPS, *S. cerevisiae* GGPS (AAA83262); halGGPS, *Halobacterium* sp. NRC-1 GGPS (AAG19532); mthGGPS, *M. thermautotrophicus* GGPS (AAB84557); phoGGPS, *Pyrococcus horikoshii* GGPS (BAA30171); sacGGPS, *S. acidocaldarius* GGPS (AAY79519); apeGFPS, *A. pernix* GFPS (BAA80767); panGGPS, *Pantoea ananatis* GGPS (BAA14124); ecoFPS, *E. coli* FPP synthase (ABG68511); gstFPS, *Geobacillus stearothermophilus* FPP synthase (BAA02551); athGGPS, *A. thaliana* GGPS 2 (ACI22362); synGGPS, *Synechocystis* sp. GGPS (BAA16690); hsaDPS, *H. sapiens* decaprenyl diphosphate synthase, subunit 1 (BAE48216); athSPS, *A. thaliana* solanesyl diphosphate synthase 1 (BAD88533); sceHexPS, *S. cerevisiae* hexaprenyl diphosphate synthase (AAA34686); goxDPS, *Gluconobacter oxydans* decaprenyl diphosphate synthase (BAA32241); ecoOPS, *E. coli* octaprenyl diphosphate synthase (ABG71256); mmaGFPS, *M. mazei* GFPS (AAM30485); afuHepPS, *Archaeoglobus fulgidus* heptaprenyl diphosphate synthase (AAB89695). Bar = 0.05 amino acid substitution per site. Two GFPS, from *M. mazei* and *A. pernix*, are shown in boxes. Asterisks represent the enzymes that take anomalous positions in the tree, i.e., *S. solfataricus* HexPS is included in the branch of eukaryotic short-chain enzymes, and *M. mazei* GFPS is included in the branch of long-chain enzymes.

mazei GFPS, is basically compatible with those reported previously [11,15,18,27] (Fig. 4). In our tree, long-chain (all-*E*) prenyl diphosphate ($\geqslant C_{30}$) synthases formed a large group separate from the group of short-chain enzymes, and each group could be classified into several subgroups. With a few exceptions such as *S. solfataricus* hexaprenyl diphosphate synthase [15], the classification of the subgroups was in good agreement with the taxonomy of organisms based on the sequences of ribosome small subunit RNA. Tachibana et al. reported that GFPS from *A. pernix*, which is utilized to synthesize C_{25}-C_{25} diether lipids instead of the C_{20}–C_{20} lipids typical of an archaeal membrane, belongs to the subgroup of archaeal GGPSs [11]. Although *A. pernix* GFPS was classified correspondingly in our tree, *M. mazei* GFPS was, surprisingly, included in the branch of long-chain (all-*E*) prenyl diphosphate synthases, which have not been previously found in methanogens. Methanogens are not considered to possess this type of enzymes because they do not produce respiratory quinones. As mentioned above, the amino acid sequence around FARM of *M. mazei* GFPS also resembles those of long-chain (all-*E*) prenyl diphosphate synthases, rather than short-chain enzymes: *M. mazei* GFPS does not have an aromatic amino acid at the fifth position upstream from FARM, while *A. pernix* GFPS does, as do archaeal GGPSs. Moreover, the preference for allylic substrates of *M. mazei* GFPS was similar to that of long-chain enzymes, which usually catalyze prenyl elongation from (all-*E*) prenyl diphosphates produced by short-chain enzymes of the same organism. By contrast, *A. pernix* GFPS, as well as *N. pharaonis* GFPS, was reported to accept DMAPP and GPP as the substrate, at levels comparable with FPP and GGPP, like eukaryotic and bacterial FPSs and archaeal GGPSs [11,25].

These results strongly suggest that *M. mazei* GFPS and its isozyme from *A. pernix* have arisen from distinct routes of molecular evolution. Although they yield exactly the same final product, *M. mazei* GFPS and *A. pernix* GFPS are considered to have evolved from long-chain and short-chain (all-*E*) prenyl diphosphate synthases, respectively. It is interesting that the two GFPSs seem to have inherited their biological roles from their probable ancestors. *M. mazei* GFPS, which might be derived from long-chain (all-*E*) prenyl diphosphate synthases responsible for the biosynthesis of the side-chain of respiratory quinones, is utilized for the biosynthesis of methanophenazine, a structural analogue of respiratory quinones. On the other hand, *A. pernix* GFPS is responsible for the biosynthesis of archaeal membrane lipids, like GGPSs from other archaea. Conversion of *A. pernix* GFPS into GGPS by mutagenesis has been reported by Lee et al. [28] They showed that amino acid replacement in well-studied chain-length determination regions, i.e., the regions upstream from FARM [18] and upstream from the G(Q/E) motif [21,29], or in other regions, were responsible for the functional change of the mutants. It is conceivable that such mutational changes, in the reverse direction, might have occurred in the emergence of *A. pernix* GFPS. Although the route of evolution through which *M. mazei* GFPS might have arisen from ancestral long-chain enzymes is not known, its crystal structure, recently deposited in the Protein Data Bank (ID: 3IPI), will assist in identification of the unique features of *M. mazei* GFPS that give rise to the difference between the enzyme and long-chain (all-*E*) prenyl diphosphate synthases. Moreover, information about the molecular evolution of *M. mazei* GFPS might provide a clue to assist in understanding how methanophenazine, which is utilized in a limited fashion by a portion of methanogens, emerged in the evolutional history of methanogens.

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