

Novel Medium-Chain Prenyl Diphosphate Synthase from the Thermoacidophilic Archaeon *Sulfolobus solfataricus*

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Two open reading frames which encode the homologues of (all-*E*) prenyl diphosphate synthase are found in the whole-genome sequence of *Sulfolobus solfataricus*, a thermoacidophilic archaeon. It has been suggested that one is a geranylgeranyl diphosphate synthase gene, but the specificity and biological significance of the enzyme encoded by the other have remained unclear. Thus, we isolated the latter by the PCR method, expressed the enzyme in *Escherichia coli* cells, purified it, and characterized it. The archaeal enzyme, 281 amino acids long, is highly thermostable and requires Mg^{2+} and Triton X-100 for full activity. It catalyzes consecutive *E*-type condensations of isopentenyl diphosphate with an allylic substrate such as geranylgeranyl diphosphate and yields the medium-chain product hexaprenyl diphosphate. Despite such product specificity, phylogenetic analysis revealed that the archaeal medium-chain prenyl diphosphate synthase is distantly related to the other medium- and long-chain enzymes but is closely related to eucaryal short-chain enzymes.

Prenyl diphosphate synthases catalyze consecutive condensations of IPP with allylic primer substrates, and their products have various hydrocarbon chain lengths specific to each enzyme. Based on the stereochemistry of the products, they are divided into two types: *E*-type and *Z*-type enzymes. Ogura and Koyama further classified the former, which yields (all-*E*) prenyl diphosphates, into three groups based on their quaternary structure and the chain length of the conclusive products (8, 11). The first group, short-chain prenyl diphosphate synthases, is composed of the enzymes which yield C_{15} to C_{25} products, the precursors of terpenes, sterols, carotenoids, prenylated proteins, and archaeal membranes. The products are also utilized as the substrates of prenyl diphosphate synthases in the other groups. Most of the enzymes are homodimers which consist of two subunits tightly bound together. The second group, medium-chain prenyl diphosphate synthases, yields C_{30} to C_{35} products as the precursors of respiratory quinones. Although only a few enzymes of this group have been studied in detail, they are known to have a unique quaternary structure. They form a dissociable heterodimer consisting of a large subunit that has a similarity to other *E*-type enzymes and a small, dissimilar subunit. The third group, long-chain enzymes, produces C_{40} to C_{50} prenyl diphosphates as the precursors of respiratory quinones. They also have a tightly associated homodimeric structure but require other proteins as activators. Although current information about their structures and products seems to be insufficient to classify (all-*E*) prenyl diphosphate synthases from whole organisms, we used the above designation for the assortment of the enzymes in this work.

Because their amino acid sequences have high similarity, the enzymes of the three groups are considered to have similar structures and use the same mechanism to elongate the prenyl

chain. The mechanism that determines the chain length of their products has been studied. Through our mutational studies, a few typical features of the chain-length determination of the short-chain enzymes have been found (12–16). However, little is known about the mechanism of chain length determination in medium- and long-chain enzymes. From where their difference arises and whether the unique quaternary structure of medium-chain enzymes is involved in the mechanism remain uncertain.

In this study, we isolated a gene of the homologue of (all-*E*) prenyl diphosphate synthase from a thermoacidophilic archaeon, *Sulfolobus solfataricus*, based on the information obtained from genome analysis. From among archaeal (all-*E*) prenyl diphosphate synthases, several short-chain enzymes, which are utilized for the biosynthesis of archaeal membrane lipids, have been investigated (2, 17, 20–22), but no information about the other enzymes has been published. Therefore, in this study, the archaeal enzyme was expressed in *Escherichia coli*, purified, and characterized. Our data show that the archaeal enzyme yields the medium-chain product hexaprenyl diphosphate in the absence of other components. In addition, phylogenetic analysis revealed the anomalous position of the archaeal medium-chain enzyme in (all-*E*) prenyl diphosphate synthases, providing an interesting hypothesis about their evolution.

S.a.GGPS	71:LEVLTHTFLVHDDIMDQDNIRRG:93
S.s.bac10_016	75:VELLHNEFLVHDDIMDNDGLRRG:97
S.s.bac19_042	70:IEILHSASIALDDIVLDATRRG:92

* FARM

FIG. 1. Alignment of amino acid sequences around FARM of (all-*E*) prenyl diphosphate synthases from *Sulfolobus*. S.a. and S.s. indicate *S. acidocaldarius* and *S. solfataricus*, respectively. The fifth position before FARM is emphasized by an asterisk.

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TABLE 1. Purification of recombinant prenyl diphosphate synthase of *S. solfataricus*

Fraction	Total protein (mg)	Total activity (nmol/min)	Sp act ^a (nmol/mg/min)	Purification (fold)	Recovery (%)
Crude extract	124	2.16	0.0175	1.00	100
Heat treatment ^b	2.27	2.15	0.948	54.2	99.5
DEAE-Toyopearl	0.572	1.05	1.84	105	48.6

^a Enzymatic activities were determined by measuring the amount of [¹⁴C]IPP incorporated into 1-butanol-extractable materials when GGPP was used as an allylic substrate.

^b The second heat treatment after acetone precipitation.

MATERIALS AND METHODS

Abbreviations. IPP, isopentenyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPS, geranylgeranyl diphosphate synthase; FPS, farnesyl diphosphate synthase; HexPS, hexaprenyl diphosphate synthase; FARM, the first aspartate rich motif; ORF, open reading frame; IPTG, isopropyl 1-thio-β-D-galactoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Materials. Precoated reversed-phase thin-layer chromatography plates, LKC-18F were purchased from Whatman Chemical Separation, Inc. (All-*E*) GGPP, (all-*E*) farnesyl diphosphate, geranyl diphosphate, and dimethylallyl diphosphate were donated by Kyozo Ogura and Tanetoshi Koyama, Tohoku University. [¹⁴C]IPP was purchased from Amersham. All other chemicals were of analytical grade.

General procedures. Restriction enzyme digestions, transformations, and other standard molecular biology techniques were carried out as described by Sambrook et al. (19).

Cultivation of archaea. *S. solfataricus* was cultured in an ATCC 1304 broth at 70°C and harvested at the late log phase.

Isolation of the gene encoding novel archaeal prenyl diphosphate synthase. A homology search from the database of the *Sofolobus solfataricus* genome was done by using the BLAST program. The amino acid sequence of *S. acidocaldarius* GGPS was used as the probe. The ORF encoding one of the searched homologues was amplified by PCR by using the primers specific to the 5' and 3' ends: 5'-TTAAGCTATCATGAGTATTATAGAG-3' and 5'-TGAATTTAGGATCCCTTAAATCTTATCTATG-3', respectively. The genome of *S. solfataricus*, as the template, and KOD DNA polymerase (TOYOBO) were used for the

reaction. The restriction sites newly introduced in the primers, the *Bsp*HI and *Bam*HI sites, are indicated by underlines. The amplified fragment, extracted from 0.8% agarose gel after electrophoresis, was digested with *Bsp*HI and *Bam*HI and then ligated into the *Nco*I-*Bam*HI sites of the pET-3d vector (Amersham-Pharmacia). The resultant plasmid was designated pET-PTH.

Expression and purification of recombinant enzyme. *E. coli* BL21 (DE3) transformed with pET-PTH was cultivated into 2 liters of M9YG broth supplemented with ampicillin (50 mg per liter). When the optical density at 600 nm of the culture reached 0.5, the transformed bacteria were induced with 1.0 mM IPTG. After additional overnight cultivation, the cells were harvested and disrupted by sonication in 50 mM Tris-Cl buffer, pH 7.0, containing 10 mM 2-mercaptoethanol, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 4,000 × *g* for 15 min, and the supernatant was recovered as a crude extract. The crude extract was heated at 55°C for 30 min, and the denatured proteins were removed by centrifugation at 20,000 × *g* for 10 min. To the supernatant fraction, an equal volume of ice-cold acetone was added, and the mixture was placed at -80°C for 5 min to precipitate proteins. After centrifugation at 15,000 × *g* for 10 min, the supernatant was discarded. These steps of acetone precipitation were repeated once. The precipitate was dissolved with 10 ml of 10 mM Tris-Cl buffer, pH 7.7, containing 10 mM 2-mercaptoethanol and 1 mM EDTA (termed buffer A). The enzyme solution was heated at 80°C for 2 h and then centrifuged at 15,000 × *g* for 10 min. The supernatant was recovered as a heat-treated enzyme. The enzyme solution was subjected to DEAE-Toyopearl column chromatography, which was developed with a gradient of 0 to 0.85 M NaCl in buffer A. Active fractions were collected and used for characterization. The level of purification was determined by SDS-15% PAGE.

Preparation of crude extract of *S. solfataricus*. The cells of *S. solfataricus* harvested from 4 liters of culture were harvested and disrupted by sonication in buffer A containing 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 4,000 × *g* for 15 min, and the supernatant was used as the native crude enzyme.

Measurement of prenyltransferase activity. The assay mixture contained, in a final volume of 200 μl, 1 nmol of [¹⁴C]IPP (0.185 GBq/mmol), 0.25 nmol of (all-*E*) GGPP, 1 μmol of MgCl₂, 2 μmol of phosphate buffer (pH 6.3), 0.1% Triton X-100, and a suitable amount of enzyme. This mixture was incubated at 55°C for 15 min, and the reaction was stopped by chilling the reaction mixture in an ice bath. The mixture was shaken with 600 μl of 1-butanol saturated with H₂O. The butanol layer was washed with water saturated with NaCl, and radioactivity in the butanol layer was measured with a TRI-CARB 1600 liquid scintillation counter (Packard).

Product analysis. After an enzymatic reaction using various concentrations of [¹⁴C]IPP (0.185 or 2 GBq/mmol) and 2 nmol of (all-*E*) GGPP, the prenyl diphosphates were extracted with 1-butanol and were then treated with acid phosphatase according to the method of Fujii et al. (5). The hydrolysates were extracted with pentane and analyzed by reversed-phase thin-layer chromatography using a precoated plate, LKC-18F, developed with acetone-H₂O (9:1). Authentic standard alcohols were visualized with iodine vapor, and the distribution of radioactivity was detected by a molecular imager (Bio-Rad).

Phylogenetic analysis. Amino acid sequences of (all-*E*) prenyl diphosphate synthases obtained from public databases were aligned by using the CLUSTAL W 1.8 program. The phylogenetic tree was constructed with GENETYX software based on the neighbor-joining method. All parameters used in these programs were set at default.

RESULTS

Isolation of the novel prenyl diphosphate synthase gene of *S. solfataricus*. Because the results of genome analysis in progress for a thermoacidophilic archaeon, *S. solfataricus*, have been

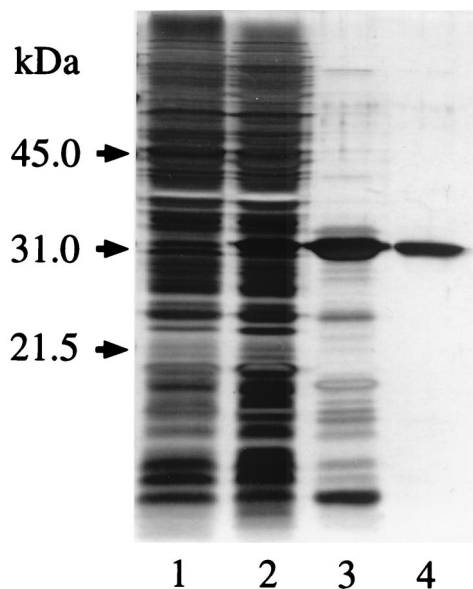


FIG. 2. SDS-PAGE of purified recombinant prenyl diphosphate synthase from *S. solfataricus*. The purification procedure is described in Materials and Methods. The gel was visualized by silver staining. Lanes 1 and 2 contain crude extracts from BL21(DE3)/pET-3d and BL21(DE3)/pET-PTH, respectively. Both transformants were induced by IPTG. Lane 3, a heat-treated enzyme; lane 4, an active fraction from DEAE-Toyopearl column chromatography.

disclosed (<http://niji.imb.nrc.ca>, recently moved to <http://www-archbac.u-psud.fr/projects/sulfolobus/sulfolobus.html>), now we can search for genes encoding the homologues of various known enzymes from the list of ORFs found in the genome. By using the sequence of *S. acidocaldarius* GGPS as the probe for the homology search, we found two ORFs, designated bac10_016 and bac19_042, encoding putative (all-*E*) prenyl diphosphate synthase from the database (Fig. 1).

Research into the chain-length determination of (all-*E*) prenyl diphosphate synthases has been done, enabling us to estimate the chain length of their conclusive products based on the amino acid sequences of conserved regions (10, 25). Specifically, the sequence around FARM is important for distinguishing short-chain prenyl diphosphate synthase from other enzymes. FARM, with a typical sequence of DDXX(XX)D, is highly conserved through all *E*-type enzymes and is thought to bind the allylic substrate via the bridge of Mg^{2+} . Based on the crystallographic analysis of avian FPS, the hydrocarbon chain of a prenyl diphosphate is considered to elongate along the α -helix which includes FARM, and the elongation is thought to be blocked by bulky amino acids existing before FARM on the α -helix (23). In general, when the amino acid at the fifth position before FARM is a bulky one like phenylalanine or tyrosine, the enzyme is expected to be a short-chain prenyl diphosphate synthase. Concerning the putative (all-*E*) prenyl diphosphate synthases of *S. solfataricus*, the one encoded by bac10_016, which is 62% identical to *S. acidocaldarius* GGPS, has phenylalanine at the fifth position before FARM, and the other, which is 28% identical and encoded by bac19_042, has alanine. These results suggest that the former, whose accession number is AAK40423, should be GGPS and that the latter, AAK42496, would yield longer products. Thus, we decided to isolate the gene encoding the latter to characterize its function.

We amplified the ORF, bac19_042, by PCR from the genome of *S. solfataricus*. The ORF is 846 bp long and encodes a protein of 281 amino acids. The amplified fragment was digested with restriction enzymes at the specific sites created through the amplification, and the restricted fragment was cloned into an expression vector, pET3d. *E. coli* strain BL21 (DE3) was then transformed by the construct pET-PTH. The assay using crude extracts showed that thermostable prenyl-transferase activity was expressed in the transformant which was induced with IPTG (data not shown). This datum suggests that the archaeal enzyme is active in the absence of other proteinaceous components which are required for activity of bacterial medium-chain enzymes.

Purification and characterization of recombinant *S. solfataricus* prenyl diphosphate synthase. The crude extract from 2 liters of culture of the transformant was provided for two rounds of heat treatment interposed by acetone precipitation. As shown in Table 1, the enzyme was proved to be highly thermostable and completely active after heat treatment at 80°C for 2 h. The supernatant fraction of centrifugation after heat treatment was subjected to DEAE-Toyopearl column chromatography, and active fractions were collected and used for characterization. SDS-PAGE visualized by silver staining demonstrated that the enzyme was purified to homogeneity (Fig. 2). Its molecular mass was estimated to be ca. 32 kDa, which corresponds well with the molecular weight calculated from the amino acid sequence: 32,274.

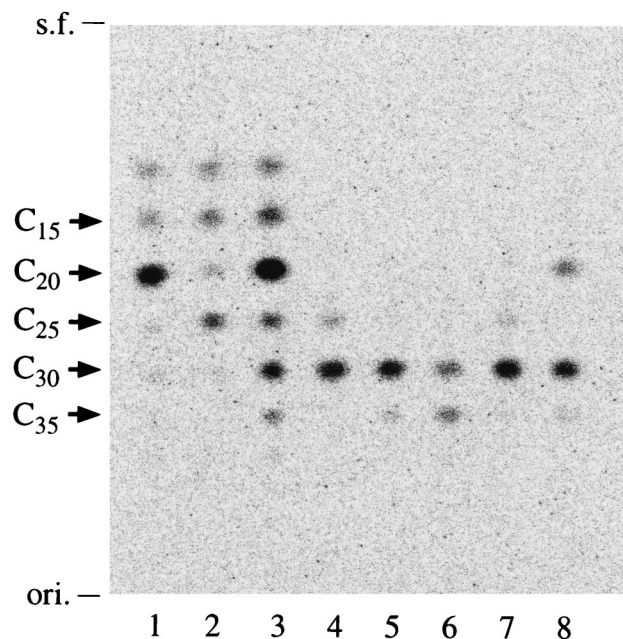


FIG. 3. Thin-layer chromatography autoradiogram of reaction products of *S. solfataricus* prenyl diphosphate synthase. The products were analyzed as described in Materials and Methods. Lanes 1 to 3 show the products from the assays using wild-type and mutant *S. acidocaldarius* GGPSs as authentic standards. These assays were carried out as described in reference 12, using 25 nmol of [^{14}C]IPP (0.185 MBq/nmol) and 25 nmol of dimethylallyl diphosphate as substrates. Lane 1, wild-type GGPS prepared as described in reference 12; lane 2, GGPS mutant 3 (reference 12); lane 3, GGPS mutant 15 (reference 14). Lanes 4 to 6 represent the products of purified recombinant *S. solfataricus* prenyl diphosphate synthase using 2 nmol of GGPP and various concentrations of [^{14}C]IPP as follows: lane 4, 0.2 nmol of [^{14}C]IPP (2 MBq/nmol); lane 5, 2 nmol of [^{14}C]IPP (0.185 MBq/nmol); lane 6, 20 nmol of [^{14}C]IPP (0.185 MBq/nmol). Lanes 7 and 8 show the products of purified recombinant *S. solfataricus* prenyl diphosphate synthase and a crude extract of *S. solfataricus*, respectively, using 2.5 nmol of [^{14}C]IPP (2 MBq/nmol) and 2.5 nmol of GGPP as substrates. In every assay condition, less than 20% of each substrate reacted. Abbreviations: ori., origin; s.f., solvent front.

The optimal reaction pH of the enzyme was 6.0, which agrees with those of other prenyl diphosphate synthases from *Sulfolobus* sp. (7, 17). The enzyme did not show activity without a divalent cation and preferred Mg^{2+} to other cations such as Mn^{2+} and Ca^{2+} . However, at concentrations ranging from 2.5 to 50 mM, the effect of Mg^{2+} did not change greatly. Triton X-100 approximately increased the activity of the enzyme two-fold and retained the effect at a similar level through various concentrations from 0.01 to 1%. The enzyme preferred GGPP to farnesyl diphosphate as an allylic substrate and did not show activity for geranyl diphosphate and dimethylallyl diphosphate. The substrate specificity resembles that of undecaprenyl diphosphate synthase from *S. acidocaldarius* and strongly suggests that GGPP is a branch point of the isoprenoid biosynthesis in the genus *Sulfolobus* (7).

Under the established optimal reaction conditions described in Materials and Methods, the products of the novel archaeal (all-*E*) prenyl diphosphate synthase were determined. As previously supposed based on the amino acid sequence around FARM, the enzyme yielded products longer than those of

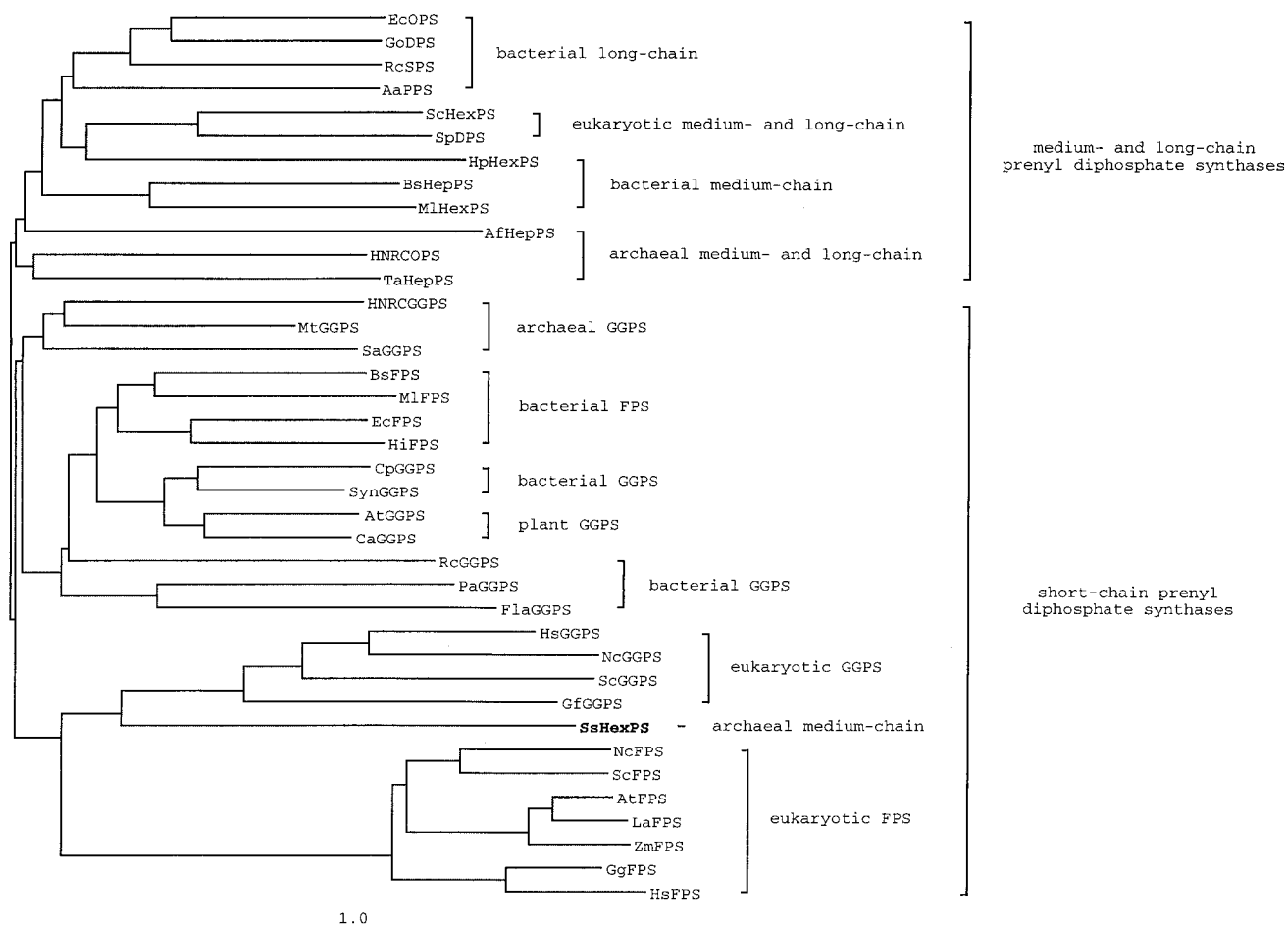


FIG. 4. Phylogenetic tree of (all-*E*) prenyl diphosphate synthases. Alignment of the amino acid sequences of (all-*E*) prenyl diphosphate synthases was performed by using the CLUSTAL W 1.8 program, and the phylogenetic tree was constructed with GENETYX software based on the neighbor-joining method. The abbreviation and accession number of each protein are as follows: EcOPS, *E. coli* octaprenyl diphosphate synthase (AAA57988); GoDPS, *Gluconobacter oxydans* decaprenyl diphosphate synthase (BAA32241); RcSPS, *Rhodobacter capsulatus* solanesyl diphosphate synthase (BAA22867); AaPPS, *Aquifex aeolicus* polyprenyl diphosphate synthase (AAC06998); ScHexPS, *Saccharomyces cerevisiae* HexPS (AAA34686); SpDPS, *Schizosaccharomyces pombe* decaprenyl diphosphate synthase (BAA12314); HpHexPS, *Helicobacter pylori* putative HexPS (AAD07307); BsHepPS, *Bacillus subtilis* heptaprenyl diphosphate synthase (AAA20856); MlHexPS, *Micrococcus luteus* HexPS (BAA25268); AfHepPS, *A. fulgidus* putative heptaprenyl diphosphate synthase (AAB89695); HNRCOPS, *Halobacterium* sp. NRC-1 putative octaprenyl diphosphate synthase (AAG20290); TaHepPS, *T. acidophilum* putative heptaprenyl diphosphate synthase (CAC11578); HNRCGGPS, *Halobacterium* sp. NRC-1 GGPS (AAG19532); MtGGPS, *Methanobacterium thermoautotrophicum* GGPS (AAB32421); SaGGPS, *S. acidocaldarius* GGPS (BAA43200); BsFPS, *Bacillus stearothermophilus* FPS (BAA02551); MlFPS, *M. luteus* FPS (BAA25265); EcFPS, *E. coli* FPS (AAC73524); HiFPS, *Haemophilus influenzae* FPS (AAC23087); CpGGPS, *Cyanophora paradoxa* GGPS (AAA81312); SynGGPS, *Synechocystis* sp. GGPS (BAA16690); AtGGPS, *Arabidopsis thaliana* GGPS (BAB02589); CaGGPS, *Capsicum annuum* GGPS (CAA56554); RcGGPS, *R. capsulatus* GGPS (CAA36538); PaGGPS, *Pantoea ananatis* GGPS (BAA14124); FlaGGPS, *Flavobacterium* sp. GGPS (AAC44848); HsGGPS, *Homo sapiens* GGPS (BAA75909); NcGGPS, *Neurospora crassa* GGPS (AAC13867); ScGGPS, *S. cerevisiae* GGPS (AAA83262); GfGGPS, *Gibberella fujikuroi* GGPS (CAA75568); SsHexPS, *S. solfataricus* HexPS (AAK42496); NcFPS, *N. crassa* FPS (CAA65645); ScFPS, *S. cerevisiae* FPS (AAA34606); AtFPS, *A. thaliana* FPS (AAB07247); LaFPS, *Lupinus albus* FPS (AAA87729); ZmFPS, *Zea mays* FPS (AAB39276); GgFPS, *Gallus gallus* FPS (P08836); HsFPS, *Homo sapiens* FPS (BAA03523). Because the consecutive products of several medium- and long-chain enzymes found through genome sequencing projects could not be predicted from their amino acid sequences, the enzymes were named based on the length of the side chain of respiratory quinones produced by the organisms and were prefixed by the word "putative" (see references 9, 24, and 4 for HpHexPS, AfHepPS, and the others, respectively). Exceptionally, the enzyme from *A. aeolicus* was designated polyprenyl diphosphate synthase because we found no reports on the quinones produced by the organism. Bar = 1.0 amino acid substitution per site.

short-chain enzymes. As shown in Fig. 3, the main product was hexaprenyl diphosphate (C_{30}) when the concentrations of IPP and GGPP were the same. We obtained a similar result from the assay using a crude extract of *S. solfataricus*. A 10-fold increase in the concentration of IPP resulted in the increased chain length of the products: a significant amount of hepta-

prenyl diphosphate (C_{35}) was produced, although hexaprenyl diphosphate remained the main product. Thus, we concluded that the enzyme is a HexPS, providing the first example of an archaeal enzyme producing medium-chain products.

Phylogenetic analysis of prenyl diphosphate synthases. The phylogenetic tree constructed using 38 (all-*E*) prenyl diphos-

phate synthase sequences, shown in Fig. 4, is compatible with the trees previously proposed (1, 22, 25). In our tree, medium- and long-chain enzymes, some of them obtained from databases as putative enzymes, formed a large group isolated from the other group of short-chain enzymes. Both of them could be classified into several subgroups in good agreement with the phylogenetic classification of organisms based on the sequences of ribosome small subunit RNA. The subgroups shown in the group of short-chain prenyl diphosphate synthases were similar to those proposed by Tachibana et al. (22) and Wang and Ohnuma (25). Concerning the group of medium- and long-chain enzymes, bacterial enzymes were clearly divided into two subgroups, long-chain enzymes and medium-chain enzymes, excepting the putative HexPS from *Helicobacter pylori*. Two eucaryal enzymes which produce C₃₀ and C₅₀ products, respectively, existed in the same branch. The putative medium- and long-chain enzymes from several archaea, whose products could be predicted based on the length of the side chain of their respiratory quinones, were separated from other subgroups at the point near the root of the tree, indicating they are far from all other prenyl diphosphate synthases. However, *S. solfataricus* HexPS lies far from other archaeal medium- and long-chain enzymes. Surprisingly, it was clearly assigned as a member of the subgroup of eucaryal short-chain enzymes and seemed to be more closely related to GGPSs than to FPSs of eucarya.

DISCUSSION

The ORF we isolated from the thermophilic archaeon *S. solfataricus* was shown to encode HexPS. Because *Sulfolobus* sp. is known to produce the specific respiratory quinone, caldariellaquinone, which has a fully saturated C₃₀ isoprenyl side chain, the enzyme is expected to be involved in its biosynthesis (Fig. 5) (3, 26). Recombinant expression of the enzyme indicated that only one polypeptide is sufficient for its activity, unlike the well-studied bacterial medium-chain enzymes which require two components. Indeed, heptaprenyl diphosphate synthase from *Haemophilus influenzae* was also reported to be active under the recombinant expression of a single gene in *E. coli* (18). These facts suggest that the two medium-chain enzymes have the same structural property with short- and long-chain prenyl diphosphate synthases and that the heterodimeric bacterial medium-chain enzymes might be exceptional in whole (all-*E*) prenyl diphosphate synthases. The unique heterodimeric structure was proved not to be necessary to yield medium-chain products. The mechanism to determine product chain length in the archaeal enzyme is our next area of interest.

The phylogenetic analysis of (all-*E*) prenyl diphosphate synthases revealed the anomalous position of *S. solfataricus* HexPS. In our phylogenetic tree, it existed far from the large branch of medium- and long-chain enzymes. Although probable medium- and long-chain enzymes from the other archaea—i.e., *Archaeoglobus fulgidus*, *Halobacterium* sp. NRC-1, and *Thermoplasma acidophilum*—also seemed to be separated from those from bacteria and eucarya, *S. solfataricus* HexPS is exceptional because it is apparently included within the subgroup of eucaryal short-chain enzymes. This exception might come from the fact that *Sulfolobus* is a member of the *Crenarchaeota*, whereas the other archaea mentioned above are

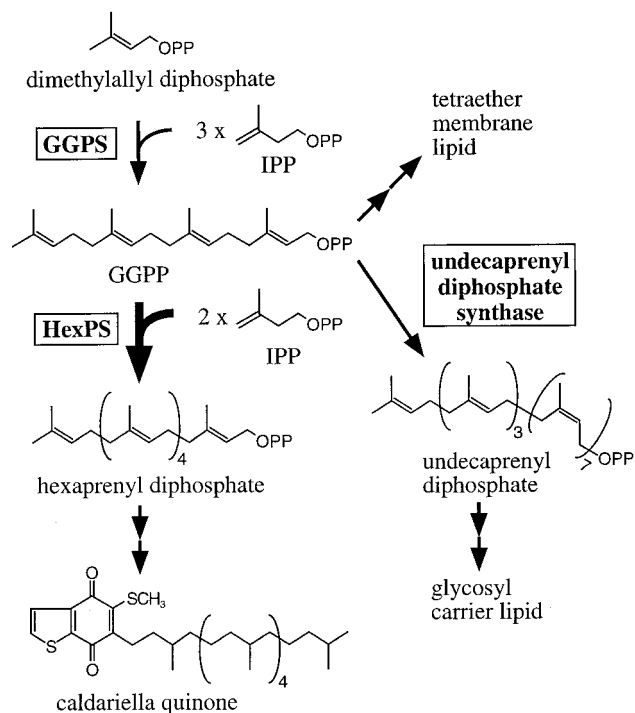


FIG. 5. Hypothetical pathway of isoprenoid biosynthesis in genus *Sulfolobus*. Prenyl diphosphate synthases so far discovered in genus *Sulfolobus* are shown in boxes. OPP, $\text{OP}_2\text{O}_5^{3-}$.

included in the *Euryarchaeota*. The molecular cloning of medium- and long-chain enzymes from other crenarchaeotes will be required for a more precise classification of (all-*E*) prenyl diphosphate synthases.

Moreover, *S. solfataricus* HexPS seemed to be remarkably related with eucaryal GGPSs in our phylogenetic tree. Ohnuma et al. referred to the exceptional nature of eucaryal GGPSs in their investigation into the mechanism determining the product of (all-*E*) prenyl diphosphate synthases (10, 25). They discussed the relation between the chain length of the products and the sequences around FARM and concluded that the products of the enzymes are determined by several features around FARM: bulky amino acids at the fourth and fifth positions before FARM and amino acid insertion into FARM. However, eucaryal GGPSs do not obey this rule. Their amino acid sequences around FARM have the same characteristics as those of medium- and long-chain prenyl diphosphate synthases, including *S. solfataricus* HexPS. These facts led us to propose the novel hypothesis that eucaryal GGPSs evolved from medium- or long-chain enzymes, probably derived from crenarchaeotes, by obtaining properties different from those of the other short-chain enzymes. This idea reminds us of hypotheses which postulate that eucarya have arisen through symbiotic association of an archaeon and a bacterium (6). The "eocyte" archaeon might be related to the *Crenarchaeota*. However, we cannot deny the possibility that evolution took place in the counterdirection after the horizontal genetic transfer from eucarya to *Sulfolobus*. Such a scenario is implausible, though, because these organisms inhabit distinct environments.

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