Cloning, Expression, and Characterization of *cis*-Polyprenyl Diphosphate Synthase from the Thermoacidophilic Archaeon *Sulfolobus acidocaldarius*

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***cis*-polyprenyl diphosphate synthases are involved in the biosynthesis of the glycosyl carrier lipid in most organisms. However, only little is known about this enzyme of archaea. In this report, we isolated the gene of *cis*-polyprenyl diphosphate synthase from a thermoacidophilic archaeon, *Sulfolobus acidocaldarius*, and characterized the recombinant enzyme.**

In bacteria and eucarya, a specific prenyltransferase plays a key role during some of the glycosylation processes. The enyme, *cis*-polyprenyl diphosphate synthase (CPDS), catalyzes consecutive *Z*-type condensations of isopentenyl diphosphate (IPP) with allylic primer substrates, and the product diphosphates are utilized as the precursor of glycosyl carrier lipids, i.e., undecaprenyl monophosphate in bacteria and dolichyl monophosphate in eucarya. The genes of both bacterial and eucaryal CPDSs were recently cloned, and the high homology among them was revealed (1, 14, 15).

Glycosylation of proteins and membrane lipids also occurs ubiquitously in archaea (10, 11). Based on the sensitivity of the antibiotics such as bacitracin, the archaeal glycosylation process is thought to involve a glycosyl carrier lipid (11, 16). Previous studies using halophilic archaea revealed that the lipid has a polyprenyl chain of 11 or 12 isoprene units (8, 10). However, little is known about the CPDS involved in the biosynthesis of the archaeal glycosyl carrier lipid. To elucidate properties of the enzyme and obtain information on insights into its biological role in archaea, we isolated the gene encoding CPDS from a thermophilic archaeon, *Sulfolobus acidocaldarius*, and characterized the enzyme expressed in *Escherichia coli* cells.

From the DNA databases in which whole genome sequences of several archaea, e.g., *Archaeoglobus fulgidus*, *Methanobacterium thermoautotrophicum*, and *Pyrococcus horikoshii*, have been registered, we found the genes that encode the homologue of the known CPDSs by using BLAST, a homology search program. The alignment of amino acid sequences of these archaeal homologues shows the existence of several highly conserved regions that could be observed also in bacterial and eucaryal CPDSs (Fig. 1). Based on the nucleotide sequences of the regions, we designed degenerated PCR primers: FW1, 5′-GC(A/C/G)AT(A/C)AT(A/C/T)ATGGA(C/T)GG(A/T)AA-3′, and RV2, 5′-(C/G)(A/C)A(A/G)TT(C/T)TC(A/G/T)(C/G)T(A/T)(C/G)(A/T)(A/G)AA(G/T)GC-3′. As a consequence of PCR using these primers, KOD DNA polymerase (TOYOBO), and *S. acidocaldarius* genome as a template, a 200-bp fragment was amplified. Colony hybridization using this fragment as a probe yielded two positive clones from ca. 7,500 colonies of *S. acidocaldarius* genomic library. The plasmid p3d, which was one of the positive clones, was sequenced and proved to contain an open reading frame of 786 bp (here termed *cpds*) which encodes a protein of 262 amino acids (DDBJ/GenBank/EMBL accesion number AB048249). The encoded protein shows high sequence similarity to known CPDSs, 36% identity with both undecaprenyl diphosphate synthase of *Micrococcus luteus* B-P 26 and dehydrodolichyl diphosphate synthase of *Saccharomyces cerevisiae*, and shares the highly conserved regions proposed by Koyama et al. (7) and Apfel et al. (1)

Because we could not detect thermostable prenyltransferase activity in the crude extract of the transformant cells harboring the plasmid p3d, the full-length *cpds* gene was amplified using the PCR primers 5′-TGAGA<u>CCATGG</u>CCAAAGATGTGAT AACTAG-3′ and 5′-AGAAT<u>GGATCC</u>TTAAGCCCCAAA GTTTC-3′. The restriction sites newly introduced in the primers, the *Nco*I site and *Bam*HI site, are indicated by underlines. The amplified fragment was digested with *Nco*I and *Bam*HI and then ligated into *Nco*I-*Bam*HI sites of pET22b(+) vector. The *E. coli* BL21(DE3) cells were transformed with the resultant plasmid, designated pET-CPDS, and the transformant cells were grown in M9 minimal medium supplemented with yeast extract (2 g per liter), glycerol (2 g per liter), and ampicillin (50 mg per liter). When the optical density at 600 nm of the culture reached 0.5, 1.0 mM (final concentration) isopropyl 1-thio-β-D-galactoside was added to the medium and this was followed by overnight cultivation. The cells were harvested and disrupted by sonication in 50 mM Tris-Cl buffer, pH 7.7, containing 10 mM 2-mercaptoethanol and 1 mM EDTA. The homogenate was centrifuged at 3,000 × *g* for 20 min, and the supernatant was recovered as a crude extract. Prenyltransferase activity was assayed as follows. The assay mixture contained, in a final volume of 200 µl, 0.1 nmol of [1-^{14}C]IPP (2.0 GBq/mmol), 1 nmol of the indicated allylic substrate (dimeth-

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M.l. UPS       1:-------------------------MFPIKKRKAIKNNNINAAQIPKHIAIIMDGNG  32

A.f. (a.a.)    1:-------------------------MIFHKIYENKLLRSVRKGAIPHHIAIIMDGNR  32
M.t. (a.a.)    1:---------------------MSPLKPLYKLYEWYISRNLRRDRMPRHVAIIMDGNR  36
P.h. (a.a.)    1:-------------MIYRIISHIPKIFFKPAYDLYERYLIEKVKSGVLPKHVAIIMDGNR  46
                                                             Region1

M.l. UPS      33:R-WAKQKKMPRIKGHYEGMQTVKKITRYASDLGVKYLTLYAFSTENWSRPKDEVNYLMKL  91

A.f. (a.a.)   33:R-FARKKGLEPHEGHFFGSKKAEEVLEWCWDLGVKMLTLYAFSTENFRRSEKEKKNIFQL  91
M.t. (a.a.)   37:RYSKLQGSMNPIEGHKKGIETLEKVLDWCVDLGIEIVTAYAFSTENFKRPEKEVKGLMKL  96
P.h. (a.a.)   47:R-WARKHEKPPWYGHLFGSKKLEEILEWCHELGIRILTVYAFSTENFKRSKEEVDRLMKL 105
                                                Region2

M.l. UPS      92:--PGDFLNTFLPELIEKNVKVETIGFIDDLPDHTKKAVLEAKEKTKHNTGLTLVFALNYG 149

A.f. (a.a.)   92:LESELRRLLKDRRTYERELRVKVVGKRELLPENLRETIKEVEERTKKHRRHYLNVAVAYG 151
M.t. (a.a.)   97:FRENFEAIASNEKIHKNRVRVRAVGKLELLPEDVRRAIEIAEKSTEQYSDRLVNIAIGYD 156
P.h. (a.a.)  106:FEEKFRELVTDKRVHEYGVRVNVIGRKELLPKSVRDAVEEAERATRKYNNYILNVALAYG 165

M.l. UPS     150:GRKEIISAVQLIAERYKSGEISLDEISETHFNEYLFT--ANMPDPELLIRTSGEERLSNF 207

A.f. (a.a.)  152:GRQEIIDAVRAILRKVRKGEVRPEEIDEKMLEEHLY-GEGRYSKVDLIIRTGGEQRLSNF 210
M.t. (a.a.)  157:GRQEIVDATRKIAEDVKAGLIDPEDIDEDMINRNLYTAGL--EDPHLIIRTSGEERLSGF 214
P.h. (a.a.)  166:GRSEIVDAVKDIARDVISGKLRIEEIDEFLLRRYLY-VPNM-PDPDIVIRTGGEVRISNF 223

M.l. UPS     208:LIWQCSYSEFVFIDEFWPDFNEESLAQCTSIYQNRHRRFGGL               249

A.f. (a.a.)  211:LPWQAANSVAYFCDVYWPEFRKIDLLRAIRAWQYRKSHEVV-               251
M.t. (a.a.)  215:LLWQSSYSELYFCDSLWPELRKVDFLRAIRSYQQRERRFGV-               255
P.h. (a.a.)  224:LLYQIAYSELFFVDVYFPEFRKIDFLRIIREFQKRERRFGR-               264
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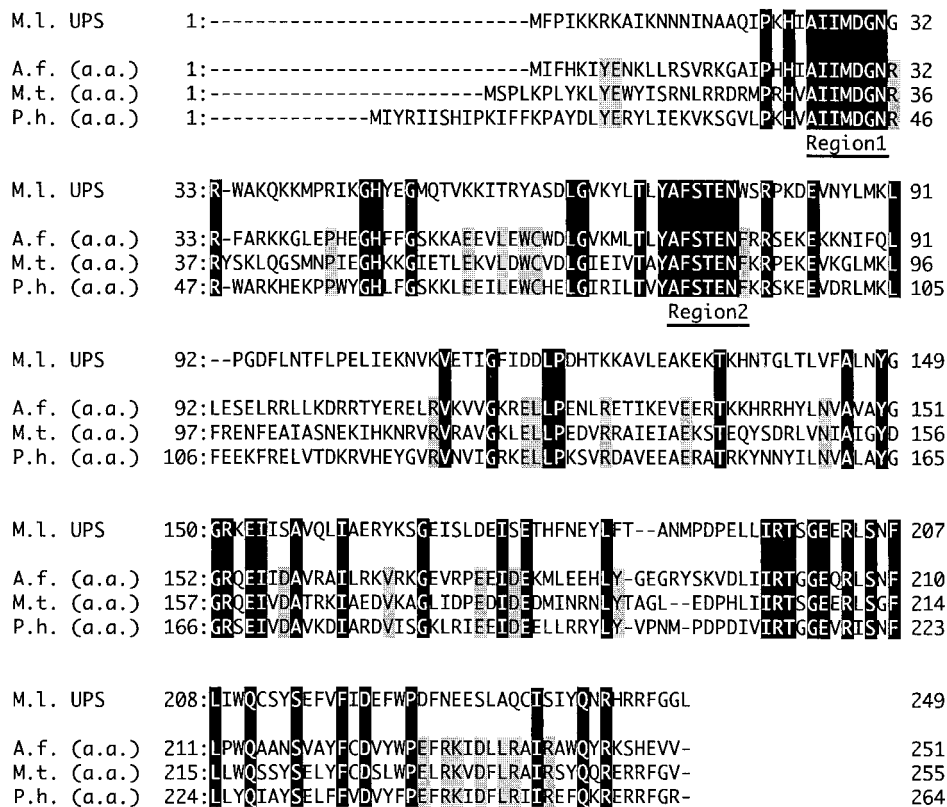
FIG. 1. Conserved regions in archaeal putative CPDSs. The hypothetical open reading frames which show high homology to previously cloned CPDSs, e.g., *M. luteus* B-P 26 undecaprenyl diphosphate synthase (M.l. UPS), were searched for among the whole genome sequences of three archaea, *A. fulgidus* (A.f.), *M. thermoautotrophicum* (M.t.), and *P. horikoshii* (P.h.), and their amino acid (a.a.) sequences are multiply aligned. Residues conserved among the bacterial enzyme and the three archaeal putative homologues are shown in black boxes, while shadowed areas denote the residues conserved only among the archaeal enzymes. Two highly conserved regions utilized for the design of degenerated PCR primers are underlined (regions 1 and 2).

ylallyl diphosphate [DMAPP], geranyl diphosphate [GPP], [all-*E*] farnesyl diphosphate [FPP], and [all-*E*] geranylgeranyl diphosphate [GGPP]), 0.1 μmol of MgCl$_2$, 2 μmol of phosphate buffer (pH 6.0), 0.1% Triton X-100, and a suitable amount of enzyme. This mixture was incubated at 55°C for 1 h, 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 H$_2$O. The butanol layer was washed with water saturated with NaCl, and radioactivity in the butanol layer was measured.

The crude extract of the BL21(DE3) strain carrying pET-CPDS showed much higher prenyltransferase activity than that of *E. coli* carrying the parental plasmid (data not shown). To confirm the expression of the archaeal thermostable CPDS, the crude extract was heated at 55°C for 7 h and then centrifuged. However, the enzyme activity was found to be coprecipitated with denatured proteins of *E. coli* during the heat treatment. This problem was circumvented by the addition of 1.5% Triton X-100 (final concentration) to the crude extract before the heat treatment, which allowed the enzyme activity to remain soluble in the supernatant. The solubilized activity was subjected to DEAE-Toyopearl column chromatography and was eluted as a single peak with a gradient of 0 to 0.85 M NaCl. The active fractions were collected and used for further characterization.

The partially purified enzyme was characterized in terms of its substrate and product specificities, stability, metal ion requirement, and effects of detergents on activity. As shown in Fig. 2, the archaeal CPDS prefers GGPP to FPP as the allylic substrate. On the other hand, DMAPP and GPP scarcely reacted. Thus, we used GGPP as the allylic substrate for subsequent characterization. The optimal reaction pH was 6.0, and the optimal reaction temperature was about 60°C, although more than 90% of the enzyme activity was retained after heat treatment at 70°C for 1 h. The Mg^{2+} ion was required for the enzyme activity, and its optimal concentration was 0.5 to 2 mM. Other divalent cations, such as Mn^{2+} and Ca^{2+}, showed no ability to substitute for Mg^{2+}. Triton X-100 activated the enzyme (sevenfold) at concentrations of 0.05 to 0.1%; however, at high concentrations, an inhibitory effect reminiscent of a surface dilution effect was observed (3, 4). To determine the conclusive product of the archaeal CPDS, enzyme reactions were made according to the assay conditions described above, except that 5 μM GGPP and various concentrations of IPP were added to the reaction mixture. The products were extracted with 1-butanol and then treated with acid phosphatase
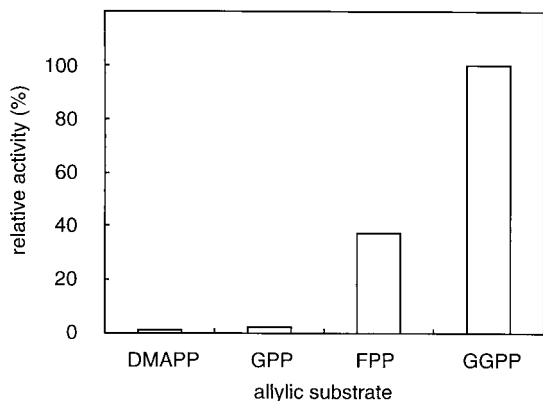
FIG. 2. Substrate specificity of *S. acidocaldarius* CPDS. The allylic substrate preference of *S. acidocaldarius* CPDS was determined by using DMAPP, GPP, FPP, or GGPP. One of the allylic substrates and IPP were added to the reaction mixture at a concentration of 5 μM and 0.5 μM, respectively. The other assay conditions were as described in the text. "Relative activity" means the activity normalized to the activity with GGPP as the allylic substrate, which is set at 100%.
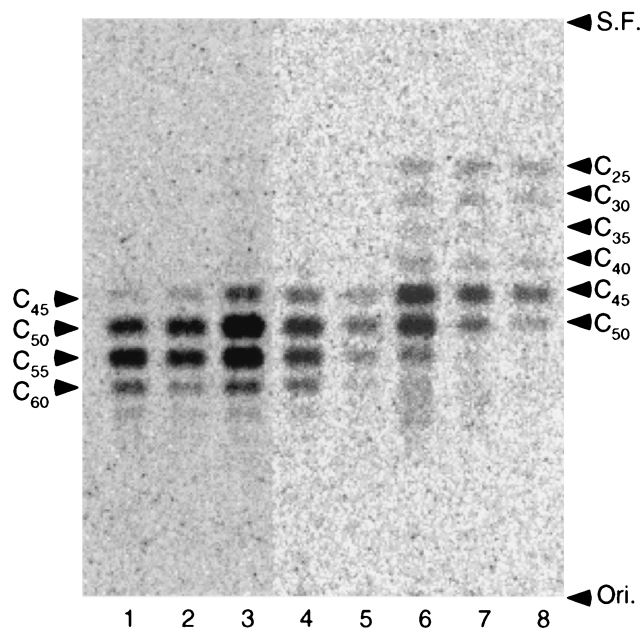

FIG. 3. Effect of substrate concentration on product distribution of *S. acidocaldarius* CPDS. Each reaction mixture contained various concentrations of [^{14}C]IPP and 5 μM GGPP. The concentration (and specific radioactivity) of [^{14}C]IPP in each reaction mixture is as follows: lane 1, 100 μM (1 Ci/mol); lane 2, 50 μM (1 Ci/mol); lane 3, 25 μM (5 Ci/mol); lane 4, 10 μM (5 Ci/mol); lane 5, 5 μM (5 Ci/mol); lane 6, 2.5 μM (55 Ci/mol); lane 7, 1 μM (55 Ci/mol); lane 8, 0.5 μM (55 Ci/mol). The conditions of enzyme reaction and product analysis are described in the text. The carbon numbers of the products are indicated on both sides. Ori., origin; S.F., solvent front.

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-H$_2$O (9:1). Authentic standard alcohols were visualized with iodine vapor, and the distribution of radioactivity was analyzed by a Molecular Imager (Bio-Rad). As shown in Fig. 3, the chain lengths of synthesized polyprenyl diphosphates depend on the concentration of IPP. Decreasing the concentration of IPP resulted in increasing amounts of intermediates with shorter chain lengths. This should not arise from the depletion of IPP, because the majority of the substrate remained unreacted after termination of the reaction. In general, long-chain polyprenyl diphosphate synthases require an excess amount of IPP compared to the allylic primer substrate to synthesize the conclusive product (9, 12). When an amount of IPP that was 20 times larger than that of GGPP was added to the reaction mixture, the archaeal enzyme mainly produced C$_{50}$ and C$_{55}$ polyprenyl diphosphates along with much smaller amounts of C$_{60}$, in a molar ratio, C$_{50}$/C$_{55}$/C$_{60}$, of 3.5:5:1.5. When FPP was used as an allylic substrate, the chain length of the conclusive product remained unchanged (data not shown). Because the names of prenyltransferases are traditionally determined in terms of their conclusive products, we regarded the enzyme as undecaprenyl diphosphate synthase. However, it must be noted that the divalent cation and the detergent in the reaction mixture might affect the conclusive product in its length as reported on dehydrodolichyl diphosphate synthase of rat liver (9).

In most bacteria and eucarya, which generally contain FPP synthases, FPP acts as the primer substrate for the biosynthesis of glycosyl carrier lipids (6). Because FPP is also the substrate of many enzymes, e.g., medium- and long-chain *trans*-prenyl diphosphate synthases which supply the precursors of the side chains of respiratory quinones, it is regarded as the central intermediate of isoprenoid biosynthesis in these organisms. However, GGPP synthase, not FPP synthase, is the only short-chain (all-*E*) prenyl diphosphate synthase that supplies the

primer substrate to CPDS in most archaea. Chen et al. (2) and Ohnuma et al. (13) proposed that the archaeal prenyltransferase would be a bifunctional GGPP or FPP synthase, based on the consideration that archaea also utilize FPP as the precursor of isoprenoid compounds. However, the substrate specificity of *S. acidocaldarius* CPDS strongly suggests that the allylic primer substrate for the biosynthesis of the glycosyl carrier lipid in the thermoacidophilic archaeon is GGPP, not FPP. To date it is not clear whether the enzymes that require FPP as a substrate exist in *S. acidocaldarius*, but it is conceivable that GGPP is the branch point of isoprenoid biosynthesis in this archaeon and that the metabolic specificity is generally in *Archaea* in contrast to the other domains of life.

REFERENCES

1. **Apfel, C. M., B. Takacs, M. Fountoulakis, M. Stieger, and W. Keck.** 1999. Use of genomics to identify bacterial undecaprenyl pyrophosphate synthetase: cloning, expression, and characterization of the essential *uppS* gene. J. Bacteriol. **181**:483–492.
2. **Chen, A., and C. D. Poulter.** 1993. Purification and characterization of farnesyl diphosphate/geranylgeranyl diphosphate synthase. A thermostable bifunctional enzyme from *Methanobacterium thermoautotrophicum*. J. Biol. Chem. **268**:11002–11007.
3. **Dennis, E. A.** 1973. Kinetic dependence of phospholipase A 2 activity on the detergent Triton X-100. J. Lipid Res. **14**:152–159.
4. **Dennis, E. A.** 1973. Phospholipase A2 activity towards phosphatidylcholine

in mixed micelles: surface dilution kinetics and the effect of thermotropic phase transitions. Arch. Biochem. Biophys. **158:**485–493.

5. **Fujii, H., T. Koyama, and K. Ogura.** 1982. Efficient enzymatic hydrolysis of polyprenyl pyrophosphates. Biochim. Biophys. Acta **712:**716–718.

6. **Hemming, F. W.** 1983. Biosynthesis of dolichols and related compounds, p. 305–354. *In* J. W. Porter and S. L. Spurgeon (ed.), Biosynthesis of isoprenoid compounds, vol. 2. John Wiley & Sons, New York, N.Y.

7. **Koyama, T., N. Shimizu, and K. Ogura.** 1998. Structure and function of polyisoprenoid chain elongation enzymes. *In* A. Steinbuchel (ed.), Biochemistry principles and mechanisms of biosynthesis and biodegradation of polymers. Proceedings of an international symposium. Wiley-VCH, Münster, Germany.

8. **Lechner, J., F. Wieland, and M. Sumper.** 1985. Biosynthesis of sulfated saccharides *N*-glycosidically linked to the protein via glucose. Purification and identification of sulfated dolichyl monophosphoryl tetrasaccharides from halobacteria. J. Biol. Chem. **260:**860–866.

9. **Matsuoka, S., H. Sagami, A. Kurisaki, and K. Ogura.** 1991. Variable product specificity of microsomal dehydrodolichyl diphosphate synthase from rat liver. J. Biol. Chem. **266:**3464–3468.

10. **Mescher, M. F., U. Hansen, and J. L. Strominger.** 1976. Formation of lipid-linked sugar compounds in *Halobacterium salinarium*. Presumed intermediates in glycoprotein synthesis. J. Biol. Chem. **251:**7289–7294.

11. **Mescher, M. F., and J. L. Strominger.** 1978. Glycosylation of the surface glycoprotein of *Halobacterium salinarium* via a cyclic pathway of lipid-linked intermediates. FEBS Lett. **89:**37–41.

12. **Ohnuma, S., T. Koyama, and K. Ogura.** 1992. Chain length distribution of the products formed in solanesyl diphosphate synthase reaction. J. Biochem. **112:**743–749.

13. **Ohnuma, S.-I., M. Suzuki, and T. Nishino.** 1994. Archaebacterial ether-linked lipid biosynthetic gene. Expression cloning, sequencing, and characterization of geranylgeranyl-diphosphate synthase. J. Biol. Chem. **269:**14792–14797.

14. **Sato, M., K. Sato, S. Nishikawa, A. Hirata, J. Kato, and A. Nakano.** 1999. The yeast *RER2* gene, identified by endoplasmic reticulum protein localization mutations, encodes *cis*-prenyltransferase, a key enzyme in dolichol synthesis. Mol. Cell. Biol. **19:**471–483.

15. **Shimizu, N., T. Koyama, and K. Ogura.** 1998. Molecular cloning, expression, and purification of undecaprenyl diphosphate synthase. No sequence similarity between *E*- and *Z*-prenyl diphosphate synthases. J. Biol. Chem. **273:**19476–19481.

16. **Zhu, B. C., R. R. Drake, H. Schweingruber, and R. A. Laine.** 1995. Inhibition of glycosylation by amphomycin and sugar nucleotide analogs PP36 and PP55 indicates that *Haloferax volcanii* β-glucosylates both glycoproteins and glycolipids through lipid-linked sugar intermediates: evidence for three novel glycoproteins and a novel sulfated dihexosyl-archaeol glycolipid. Arch. Biochem. Biophys. **319:**355–364.