Submitted to ***Biochemical and Biophysical Research Communications***

Title

A heteromeric *cis*-prenyltransferase is responsible for the biosynthesis of glycosyl carrier lipids in *Methanosarcina mazei*

Authors

Koh-ichi Emi[a], Kitty Sompiyachoke[b], Miyako Okada[a], Hisashi Hemmi[a]

Affiliation

[a]Graduate School of Bioagricultural Sciences, and [b]School of Agriculture, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 460-8601, Japan

Corresponding author

Hisashi Hemmi

Phone: +81-52-789-4134

Fax: +81-52-789-4120

E-mail: hhemmi@agr.nagoya-u.ac.jp

Abbreviations

cPT, *cis*-prenyltransferase; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GPP, geranyl pyrophosphate; IPP, isopentenyl pyrophosphate

Highlights

- Three *cis*-prenyltransferase homologs from *M. mazei* were expressed in *E. coli*.
- Two of these homologs must co-exist to exhibit *cis*-prenyltransferase activity.
- The other homolog acts alone and produces shorter polyprenyl pyrophosphates.
- The heteromeric enzyme is responsible for glycosyl carrier lipid biosynthesis.

Abstract

Cis-prenyltransferases are enzymes responsible for the biosynthesis of glycosyl carrier lipids, natural rubber, and some secondary metabolites. Certain organisms, including some archaeal species, possess multiple genes encoding *cis*-prenyltransferase homologs, and the physiological roles of these seemingly-redundant genes are often obscure. *Cis*-prenyltransferases usually form homomeric complexes, but recent reports have demonstrated that certain eukaryotic enzymes are heteromeric protein complexes consisting of two homologous subunits. In this study, three *cis*-prenyltransferase homolog proteins, MM_0014, MM_0618, and MM_1083, from the methanogenic archaeon *Methanosarcina mazei* are overexpressed in *Escherichia coli* and partially purified for functional characterization. Coexistence of MM_0618 and MM_1083 exhibits prenyltransferase activity, while each of them alone has almost no activity. The chain-lengths of the products of this heteromeric enzyme are in good agreement with those of glycosyl carrier lipids extracted from *M. mazei*, which are likely di- and tetra-hydrogenated decaprenyl phosphates, suggesting that the MM_0618/MM_1083 heteromer is involved in glycosyl carrier lipid biosynthesis. MM_0014 acts as a typical homomeric *cis*-prenyltransferase and produces shorter products.

Keywords

cis-prenyltransferase; archaea; glycosyl carrier lipid; heteromer; isoprenoid

Introduction

Cis-prenyltransferases (cPTs) are enzymes that catalyze the iterative *cis*-condensation of isopentenyl pyrophosphate (IPP), generally using short-chain (all-*E*) prenyl pyrophosphates as the primer substrate for the chain elongation process to form (*E,Z*-mixed) polyprenyl pyrophosphate [1, 2]. Eukaryotic cPTs yield longer, typically $C_{70\text{-}120}$, products as precursors of dolichyl phosphates, which act as glycosyl carrier lipids used in *N*-glycosylation and possess a saturated isoprene unit at the α-terminus. Bacterial and archaeal cPTs usually produce shorter, typically $C_{40\text{-}60}$ polyprenyl pyrophosphates. In bacteria, the products act as precursors of glycosyl carrier lipids for peptidoglycan biosynthesis, typically undecaprenyl phosphate. In archaea, the cPT products are used for the biosynthesis of short-chain dolichyl phosphate, which is exploited for protein *N*-glycosylation and usually possesses saturated isoprene units at the ω-terminus in addition to that at the α-terminus [3]. Additionally, in the biosynthesis of secondary metabolites, some plant and bacterial cPT family enzymes yield much shorter products such as C_{10} neryl pyrophosphate [4], or catalyze unusual and intriguing condensation reactions between allylic prenyl pyrophosphates [5, 6, 7]. cPTs are also essential in the biosynthesis of long-chain, high molecular weight polyprenyl compounds, i.e. natural rubber in plants [8].

Biochemical properties of cPTs have been well-studied using bacterial enzymes, which commonly form homodimeric complexes that interact with the cell membrane but do not possess transmembrane regions. Detailed characterizations such as kinetic and mutagenic studies have been established, and several crystal structures of bacterial cPTs have been solved to date, enabling inferences of their catalytic, substrate-recognition, or product-determination mechanisms. In contrast, information about eukaryotic cPTs is relatively limited. Eukaryotic cPTs, such as those from human and *Arabidopsis thaliana*, were once considered to be homomeric enzymes, and indeed, their characterization has also been performed with recombinant enzymes [9, 10]. However, recent studies have revealed that they, as well as cPTs responsible for natural rubber biosynthesis, form heteromeric complexes with membrane-anchoring subunits. Human dehydrodolichyl pyrophosphate synthase is a heteromer composed of hCIT and NgBR [11]. NgBR exhibits low homology with hCIT but possesses transmembrane regions. Some hCIT orthologs such as Rer2p and Srt1p from *S. cerevisiae*, SlCPT3 from tomato, and AtCPT1 from *A. thaliana* were proven to form a complex with NgBR orthologs such as *S. cerevisiae* Nus1p, tomato SlCPTL2, and *A. thaliana* AtLEW1 respectively [11, 12, 13]. Rubber synthase from *Hevea brasiliensis* is also an enzyme complex containing the hCIT homolog subunit HRT1 and the NgBR homolog subunits

HRBP [8]. Given the strongly hydrophobic, bulky nature of the products from these eukaryotic cPTs, membrane anchoring via the NgBR-like subunit likely accelerates the release of the products into the membrane. However, such advantages of heteromer formation remain elusive because of the difficulty in handling membrane proteins and the purification and biochemical characterization of a heteromeric cPT was only recently reported for the first time using the human hCIT/NgBR complex transiently expressed in human culture cells [14].

The discovery of heteromeric cPTs provides clues to understanding why certain organisms, not only eukaryotes but also some bacteria and archaea, have multiple genes encoding cPT homologs. Formation of a heteromeric complex of two cPT homolog proteins may explain this apparent redundancy or otherwise, each cPT homolog may have a different physiological role. A methanogenic archaeon, *Methanosarcina acetivorans,* encodes three cPT homologs, all of which are unlike NgBR homologs in that they lack apparent transmembrane regions. This number is exceptionally high among archaeal species and therefore evokes our interest in the physiological roles of the homologs in *M. acetivorans*. Recently, Grabi´nska et al. reported that the introduction of two of the cPT homologs, i.e. MA3723 and MA4402, can complement the growth of cPT-deficient *Saccharomyces cerevisiae* [14]. They also showed that the

homologs potentially form a heteromeric cPT, which had never been identified in prokaryotes, by performing an *in vitro* assay using crude membrane protein from the yeast strains expressing the homologs and/or their inactive mutants. However, the product of this heteromeric cPT was not reported and the physiological role of the enzyme remains in question. We previously characterized the cPT homologs from *M. acetivorans* via recombinant expression in *E. coli*, which led us to propose that the remaining homolog MA1831 might be responsible for glycosyl carrier lipid biosynthesis [15]. In that study, however, we could not detect in vitro activity of MA3723 and MA4402 even when they were mixed, likely because of the formation of inclusion bodies during MA3723 expression.

In the present study, we characterized the cPT homologs found in *Methanosarcina mazei*, a close relative of *M. acetivorans*, to overcome the previous problem in protein expression. The three homologs from *M. mazei,* MM_0014, MM_0618, and MM_1083, correspond to the three cPT homologs from *M. acetovorans*, MA1831, MA3723, and MA4402, respectively. The cPT homologs from *M. mazei* could be independently expressed in *E. coli* as fusion proteins with an N-terminal affinity tag. The proteins were partially purified and used for enzyme assays and product analyses. The glycosyl carrier lipids from *M. mazei* were also extracted and

analyzed, allowing us to conclude that the heteromeric cPT composed of MM_0618 and MM_1083 is responsible for the biosynthesis of glycosyl carrier lipids in *M. mazei*.

Materials and Methods

Materials

Precoated reversed-phase thin-layer chromatography (TLC) plates, RP-18 F_{254S}, were purchased from Merck Millipore. [1-^{14}C]IPP was purchased from GE Healthcare. Non-labeled IPP, dimethylallyl pyrophosphate (DMAPP), and (all-*E*)-farnesyl pyrophosphate (FPP) were donated by Dr. Chikara Ohto, Toyota Motor Co. geranyl pyrophosphate (GPP) and (all-*E*)-geranylgeranyl pyrophosphate (GGPP) was purchased from Sigma-Aldrich. Polyprenol mixture was provided by Dr. Tokuzo Nishino, Tohoku University.

Phylogenic analysis

The multiple alignment of known cPTs and their homologs and the construction of the phylogenetic tree via the neighbor joining method were performed using the MEGA7 program, with settings as follows: Test of phylogeny, Bootstrap method (1,500 trials); Model/Method, Poisson model; Rates among Sites, Gamma Distributed (G); Gamma Parameter, 2; Gaps/Missing Data Treatment, Complete deletion.

Expression and purification of recombinant cPT homologs from *M. mazei*

The genes of the cPT homologs from *M. mazei*, *MM_0014*, *MM_0618* and *MM_1083*, were amplified from genomic DNA using KOD DNA polymerase (TOYOBO), and the primer pairs are listed below: for *MM_0014*, 5'-gctgcggtaccagatggatataccaaaatttaaaagactg-3' and 5'-cggatgaattcttacccacccagtgtaatatcc-3'; for *MM_0618*, 5'-actgtccatgggggggaaaagaaataattaacc-3' and 5'-gtatgggatccttaggagactccctgctttttaaag-3'; and, for *MM_1083*, 5'-actgtccatggacctgctttcctttgg-3' and 5'-gtatgggatccttatttcccgtaccttctctg-3'. The amplified DNA fragment of *MM_0014* was digested with *Kpn*I and *Eco*RI and then ligated with a *Kpn*I/*Eco*RI-digested pET48b(+) expression vector (Novagen) to construct pET48b-MM0014. The amplified DNA fragments of *MM_0618* and *MM_1083* were digested with *Nco*I and *Bam*HI and then ligated with a *Nco*I/*Bam*HI-digested pET32a(+) expression vector (Novagen) to construct pET32a-MM0618 and pET32a-MM1083, respectively. After construction of the plasmids, the sequences of the inserted genes were verified by DNA sequencing.

The plasmids pET48b-MM0014, pET32a-MM0618, and pET32a-MM1083 were independently introduced into *E. coli* C41(DE3). The transformants were cultured in 1.0 L LB medium supplemented with 50 mg/L kanamycin for C41(DE3)/pET48b-

MM0014 and 100 mg/L ampicillin for the other strains at 37°C until formation of the log phase, followed by overnight cultivation at 22°C after the addition of IPTG to a concentration of 0.5 mM. The cells harvested from the culture were disrupted by sonication using a UP200S ultrasonic homogenizer (Hielscher Ultrasonics) in a binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH7.4). The homogenate was centrifuged at 24,000 g for 30 min, and the supernatant was recovered as a crude extract. The crude extract was loaded onto a 1 mL HisTrap crude FF column (GE Healthcare). The column was washed with a 10 mL wash buffer (20 mM sodium phosphate, 0.5 M NaCl, 60 mM imidazole, pH7.4), and the recombinant protein was eluted from the column with a 5 mL elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH7.4). For purification of MM_0014, imidazole concentration in the wash buffer was reduced into 40 mM. The level of purification was confirmed via 10% SDS-PAGE.

Radiometric assay of cPTs and TLC analysis of their products

Reaction mixtures contained 0.10 nmol [1-^{14}C]IPP (55 Ci/mol), 0.10 nmol allylic prenyl pyrophosphate (DMAPP, GPP, FPP, or GGPP), 10 μmol 3-morpholinopropanesulfate-NaOH, pH 7.0, 200 nmol $MgCl_2$, and 2.4-23.3 pmol of the

purified *cis*-prenyltransferase homolog in 100 μL were incubated at 37 °C for 1 hour using an aluminum heat block, and the reactions terminated by cooling in an ice-cooled aluminum block. When DMAPP was used as the prenyl acceptor substrate, 4.0 nmol DMAPP, 0.10 nmol [^{14}C]FPP, which was enzymatically prepared as described elsewhere [15], and 0.10 nmol of the purified MM_0014 were utilized for reaction. The reaction mixture was mixed with 600 μL 1-butanol saturated with saline and 200 μL saturated NaCl, and the radiolabeled products were extracted in the 1-butanol layer. For measurement of *cis*-prenyltransferase activity, radioactivity in 300 μL of the 1-butanol layer after centrifugation was measured using an LSC-7200 liquid scintillation counter (Aloka). Specific activity of the cPTs was expressed in nmol IPP·min^{-1}·mg protein^{-1} that indicates the amount of IPP incorporated into the mixture of hydrophobic products. For radio-TLC analysis, the butanol extract containing radiolabeled products/substrates was treated overnight with 2 mg acid phosphatase from potato (Sigma Aldrich) in a 2:1 mixture of methanol and 0.5 M sodium acetate buffer (pH 4.6), following a method developed by Fujii et al. [16]. The resultant prenyl alcohols were extracted with *n*-pentane and analyzed with a RP18 reversed-phase TLC plate developed with acetone/H_2O (19:1). The distribution of radioactivity on the plate was visualized with a Typhoon FLA9000 multifunctional scanner (GE Healthcare).

Structural analysis of glycosyl carrier lipids from *M. mazei*

Total lipid was extracted with a method established by Bligh and Dyer [17] with modification described elsewhere [15]. LC-ESI-MS analysis was performed with an Esquire 3000 ion trap system (Bruker Daltonics) connected to an Agilent 1100 Series HPLC (Agilent Technologies). The compounds eluted from a InertSustain C18 packed column (2.1 ID×150 mm, GL Science) were detected via UV absorption at 210 nm and ESI-MS in the negative mode. The mobile phase used for the analysis was 2-propanol/methanol/10 mg·L^{-1} CH_3COONa (10:9:1) at a flow rate of 0.2 mL·min^{-1}. The MS parameters were as follows: sheath gas, N_2 of 30 psi; dry gas, N_2 of 7.0 L·min^{-1}, 320°C; scanning range, 100-1,200 *m/z*; scan speed, 13,000 *m/z*·sec^{-1}; ion charge control target, 20,000; maximum accumulation time, 100 ms; averages, 10; and, rolling averaging, 1.

Results

Cloning, recombinant expression, and purification of *M. mazei* cPTs

The phylogenetic tree of several identified cPTs and archaeal cPT homologs in Figure 1 clearly shows the close relation between each corresponding cPT homolog pair from *M. mazei* and *M. acetivorans*, which have amino acid sequence identities of 83-87%, much higher than the 29-36% identities among *M. mazei* cPT homologs themselves. The tree can be divided into two branches: one contains NgBR and archaeal cPT homologs, including MM_1083; and the other, much larger, contains hCIT, archaeal and bacterial cPTs with known activities, and other archaeal cPT homologs, including MM_0014 and MM_0618. In the latter branch, MM_0014 exists in close proximity to homomeric cPTs from *Aeropyrym pernix* and bacteria, while MM_0618 resides at a position relatively close to hCIT. The presence of both hCIT- and NgBR-like homologs within a range of archaea implies the existence of heteromeric cPTs in the archaeal species as discussed later.

Each of the cPT homolog genes from *M. mazei* were heterologously expressed in *E. coli* and all highly expressed as fusions with an N-terminal thioredoxin-polyhistidine-tag, which could be purified using Ni-affinity chromatography. Although the recombinant protein solutions contain impurities, especially that of MM_0618

which likely contains partially degraded MM_0618, SDS-PAGE analysis showed that a major band was observed at the expected molecular mass of each fusion protein (Figure 2A).

Enzymatic assay and product analysis of *M. mazei* cPTs

To confirm if MM_0618 and MM_1083 form a heteromeric cPT, the enzyme activity of each protein or a 1:1 mixture of both of the partially-purified proteins was assessed using ^{14}C-labeled IPP and FPP as substrates (Figure 2B). The mixture of MM_0618 and MM_1083 indeed showed cPT activity, while MM_0618 or MM_1083 by themselves were almost inactive. The emergence of enzyme activity is by no means due to contamination of the endogenous of *E. coli* prenyltransferases, i.e. farnesyl pyrophosphate synthase, octaprenyl pyrophosphate synthase, and undecaprenyl pyrophosphate synthase, because each of these *E. coli* enzymes exhibit activity by themselves. This result supports the data from Grabi´nska et al. that the corresponding homologs from *M. acetivorans* form a heteromeric cPT [14], but conflicts with the data reported by Park et al. showing that co-translation, not just coexistence, of hCIT and NgBR subunits (or their corresponding orthologs) is required for cPT activity [11]. The heteromeric cPT from *M. mazei* efficiently uses both FPP and GGPP as the allylic

substrate but does not form any detectable products with shorter allylic substrates, which is characteristic of general cPTs [1] (Figure 2C). As the products of archaeal heteromeric cPTs have never been reported, we investigated the structures of the products of the *M. mazei* enzyme containing MM_0618 and MM_1083. The reversed-phase TLC analysis of the products from the reaction with ^{14}C-labeled IPP and FPP or GGPP demonstrated that C_{45} and C_{50} polyprenyl pyrophosphates were the main products (Figure 2D).

In our previous study, we had concluded that MA1831, which is highly homologous to MM_0014, is involved in glycosyl carrier lipid biosynthesis because the homomeric cPT from *M. acerivorans* could produce C_{55} polyprenyl pyrophosphate when a tenfold excess of IPP over allylic substrates was supplied in the absence of detergent [15]. In this study, we assessed the activity of MM_0014 under the same conditions used for MM_0618/MM_1083, with [1-^{14}C]IPP and an allylic substrate at a 1:1 ratio, and determined its products. MM_0014 showed comparable activity with various allylic substrates including DMAPP and GPP as the prenyl donor (Figure 2C), and the main products from the reactions were C_{25}-C_{40} polyprenyl pyrophosphates (Figure 2D), clearly shorter than those of MM_0618/MM_1083. In addition, like MA1831, MM_0014 accepted DMAPP as the prenyl acceptor substrate instead of IPP

and formed a product when ^{14}C-labeled FPP was used as the donor substrate (Figure 2E). The detailed structure of the product is yet to be determined, but from its R_f value on TLC, the product is likely the same as that of the "head-to-middle" condensation reaction catalyzed by MA1831, i.e., geranyllavandulyl pyrophosphate [15].

Analysis of glycosyl carrier lipid in *M. mazei*

The enzyme assays and product analyses demonstrated that the MM_0618/MM_1083 heteromer produces longer polyprenyl pyrophosphates than those synthesized by MM_0014 and are thus likely utilized for glycosyl carrier lipid biosynthesis in *M. mazei*. We therefore determined the structure of the glycosyl carrier lipid from *M. mazei*, which was expected to be dolichyl phosphate (α-saturated polyprenyl phosphate) but not yet reported. Whole lipid fraction, supposedly containing non-glycosylated dolichyl phosphate, was extracted from *M. mazei* cells. After acetone precipitation and acid phosphatase treatment, the non-polar lipid fraction was extracted with pentane. LC-ESI/MS analysis of the extracted lipids found ion peaks of lipids eluted immediately after authentic polyprenols (Figure 3). Their m/z values showed that the major peaks were likely derived from partially saturated C_{50} polyprenols. Mono-saturated C_{50} polyprenol, or dihydrodecaprenol, with m/z 724 showed a four-times

larger ion peak than that of di-saturated or tetrahydrodecaprenol, with m/z 726. A very scarce ion peak with m/z 792 was observed, suggesting that a slight amount of mono-saturated C_{55} polyprenol, or dihydroundecaprenol, was present, while ions corresponding with polyprenols shorter than C_{50} were not detected. The positions of saturated double bonds in the detected polyprenols could not be determined by MS/MS analyses (data not shown), and thus it is unclear if they are (α-saturated) dolichols or (α-unsaturated) polyprenols. We can only conclude that the glycosyl carrier lipids of *M. mazei* are likely dihydrodecaprenyl phosphate and tetrahydrodecaprenyl phosphate, which are shorter than those of *M. acetivorans* composed mainly of undecaprenyl phosphate and dihydroundecaprenyl phosphate [15].

Discussion

The main conclusion of this study is that it is the heteromeric cPT composed of MM_0618 and MM_1083, not the homomeric cPT MM_0014, that is responsible for the production of glycosyl carrier lipids, which are likely dihydrodecaprenl phosphate and tetrahydrodecaprenyl phosphate. Under this conclusion, the physiological role of MM_0014 remains unclear. Although the unusual substrate preference of the MM_0014, in that it can accept DMAPP as the prenyl acceptor substrate, makes its function obscure, the relatively short (C_{25}-C_{40}) products from the reaction with IPP and (all-*E*) prenyl pyrophosphates are likely of physiological function and allows for some suppositions. Because the products are too short to be used for the biosynthesis of the glycosyl carrier lipids but too long for C_{20} isoprenoid membrane lipids, MM_0014 is possibly involved in the biosynthesis of other isoprenoid compounds that have been discovered in *M. mazei,* including methanophenazine [18] and 2,6,10,15,19-pentamethylicosenes [19], both of which are C_{25} isoprenoids.

The distribution of archaeal heteromeric cPTs, which differ from eukaryotic counterparts in the absence of a transmembrane region in the NgBR-like subunit, is likely limited to a subsection of the phylum Euryarchaeota. The phylogenetic tree in Figure 1 suggests that, like *M. mazei* and *M. acetivorans*, methanogens such as *M.*

paludicola, and *M. thermautotrophicus*, a thermophile *A. fulgidus*, and a halophile *H. volcanii* seem to possess a NgBR-like subunit, that could potentially compose a heteromeric cPT with a highly conserved hCIT-like subunit. Each of them encodes two cPT homolog genes, whereas *M. mazei* and *M. acetivorans* uniquely encode three homolog genes as described above. In contrast, *M. jannaschii*, *M. burtonii*, and *P. horikoshii*, all of which belong to Euryarchaeota, likely do not have a NgBR-like subunit and possess only one cPT homolog gene, like archaea of the phylum Crenarchaeota such as *A. pernix* and *S. solfataricus*. The putative heteromeric cPTs from archaea attract our interest because they possibly provide clues to understand the reaction mechanism of eukaryotic heteromeric cPTs. The stoichiometry, strength, and mechanistic importance of the heteromer formation, as well as the structures of heteromeric cPTs, will be important targets of the studies on heteromeric cPTs, and such studies may be facilitated through the use of archaeal enzymes.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Numbers 17H05437, 18K19170, and 19H04651, and by grants-in-aid from the Institute for Fermentation, Osaka, Noda Institute for Scientific Research, and Nagase Scientific Technology Foundation, for H.H.

We thank Susumu Asakawa, Graduate School of Bioagricultural Sciences, Nagoya University, for his help with the cultivation of *M. mazei*. We thank Tohru Yoshimura and Tomokazu Ito, Graduate School of Bioagricultural Sciences, Nagoya University, for helpful discussions.

References

[1] S. Takahashi, and T. Koyama, Structure and function of *cis*-prenyl chain elongating enzymes. Chem Rec 6 (2006) 194-205. 10.1002/Tcr.20083.

[2] K.A. Grabinska, E.J. Park, and W.C. Sessa, *cis*-Prenyltransferase: New Insights into Protein Glycosylation, Rubber Synthesis, and Human Diseases. J Biol Chem 291 (2016) 18582-90. 10.1074/jbc.R116.739490.

[3] J. Eichler, and Z. Guan, Lipid sugar carriers at the extremes: The phosphodolichols Archaea use in *N*-glycosylation. Biochim Biophys Acta Mol Cell Biol Lipids 1862 (2017) 589-599. 10.1016/j.bbalip.2017.03.005.

[4] A.L. Schilmiller, I. Schauvinhold, M. Larson, R. Xu, A.L. Charbonneau, A. Schmidt, C. Wilkerson, R.L. Last, and E. Pichersky, Monoterpenes in the glandular trichomes of tomato are synthesized from a neryl diphosphate precursor rather than geranyl diphosphate. Proc Natl Acad Sci U S A 106 (2009) 10865-10870. 10.1073/Pnas.0904113106.

[5] Z.A. Demissie, L.A.E. Erland, M.R. Rheault, and S.S. Mahmoud, The Biosynthetic Origin of Irregular Monoterpenes in *Lavandula* ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF A NOVEL *cis*-PRENYL DIPHOSPHATE SYNTHASE GENE, LAVANDULYL DIPHOSPHATE SYNTHASE. J Biol Chem 288 (2013) 6333-

6341. 10.1074/jbc.M112.431171.

[6] T. Ozaki, P. Zhao, T. Shinada, M. Nishiyama, and T. Kuzuyama, Cyclolavandulyl Skeleton Biosynthesis via Both Condensation and Cyclization Catalyzed by an Unprecedented Member of the *cis*-Isoprenyl Diphosphate Synthase Superfamily. J Am Chem Soc 136 (2014) 4837-4840. 10.1021/ja500270m.

[7] R. Teufel, L. Kaysser, M.T. Villaume, S. Diethelm, M.K. Carbullido, P.S. Baran, and B.S. Moore, One-pot enzymatic synthesis of merochlorin A and B. Angew Chem Int Ed Engl 53 (2014) 11019-22. 10.1002/anie.201405694.

[8] S. Yamashita, H. Yamaguchi, T. Waki, Y. Aoki, M. Mizuno, F. Yanbe, T. Ishii, A. Funaki, Y. Tozawa, Y. Miyagi-Inoue, K. Fushihara, T. Nakayama, and S. Takahashi, Identification and reconstitution of the rubber biosynthetic machinery on rubber particles from *Hevea brasiliensis*. eLife 5 (2016). 10.7554/eLife.19022.

[9] M. Giladi, I. Edri, M. Goldenberg, H. Newman, R. Strulovich, D. Khananshvili, Y. Haitin, and A. Loewenstein, Purification and characterization of human dehydrodolychil diphosphate synthase (DHDDS) overexpressed in *E. coli*. Protein Expr Purif 132 (2017) 138-142. 10.1016/j.pep.2017.02.001

[10] S.K. Oh, K.H. Han, S.B. Ryu, and H. Kang, Molecular cloning, expression, and functional analysis of a cis-prenyltransferase from *Arabidopsis thaliana*.

Implications in rubber biosynthesis. J Biol Chem 275 (2000) 18482-8. 10.1074/jbc.M002000200

[11] E.J. Park, K.A. Grabinska, Z.Q. Guan, V. Stranecky, H. Hartmannova, K. Hodanova, V. Baresova, J. Sovova, L. Jozsef, N. Ondruskova, H. Hansikova, T. Honzik, J. Zeman, H. Hulkova, R. Wen, S. Kmoch, and W.C. Sessa, Mutation of Nogo-B Receptor, a Subunit of *cis*-Prenyltransferase, Causes a Congenital Disorder of Glycosylation. Cell Metabol 20 (2014) 448-457. 10.1016/j.cmet.2014.06.016.

[12] M.I. Brasher, L. Surmacz, B. Leong, J. Pitcher, E. Swiezewska, E. Pichersky, and T.A. Akhtar, A two-component enzyme complex is required for dolichol biosynthesis in tomato. Plant J 82 (2015) 903-914. 10.1111/tpj.12859.

[13] M. Kwon, E.J. Kwon, and D.K. Ro, *cis*-Prenyltransferase and Polymer Analysis from a Natural Rubber Perspective. Methods Enzymol 576 (2016) 121-45. 10.1016/bs.mie.2016.02.026.

[14] K.A. Grabinska, B.H. Edani, E.J. Park, J.R. Kraehling, and W.C. Sessa, A conserved C-terminal RXG motif in the NgBR subunit of *cis*-prenyltransferase is critical for prenyltransferase activity. J Biol Chem 292 (2017) 17351-17361. 10.1074/jbc.M117.806034.

[15] T. Ogawa, K. Emi, K. Koga, T. Yoshimura, and H. Hemmi, A *cis*-prenyltransferase from

Methanosarcina acetivorans catalyzes both head-to-tail and nonhead-to-tail prenyl condensation. FEBS J 283 (2016) 2369-2383. 10.1111/febs.13749.

[16] H. Fujii, T. Koyama, and K. Ogura, Efficient enzymatic hydrolysis of polyprenyl pyrophosphates. Biochim Biophys Acta 712 (1982) 716-8. 10.1016/0005-2760(82)90304-6

[17] E.G. Bligh, and W.J. Dyer, A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. Can J Biochem Physiol 37 (1959) 911-917. 10.1139/o59-099

[18] H.J. Abken, M. Tietze, J. Brodersen, S. Baumer, U. Beifuss, and U. Deppenmeier, Isolation and characterization of methanophenazine and function of phenazines in membrane-bound electron transport of *Methanosarcina mazei* Go1. J Bacteriol 180 (1998) 2027-32.

[19] S. Schouten, M.J.E.C. VanderMaarel, R. Huber, and J.S.S. Damste, 2,6,10,15,19-Pentamethylicosenes in *Methanolobus bombayensis*, a marine methanogenic archaeon, and in *Methanosarcina mazei*. Org Geochem 26 (1997) 409-414. 10.1016/S0146-6380(97)00011-9.

[20] T. Mori, T. Ogawa, T. Yoshimura, and H. Hemmi, Substrate specificity of undecaprenyl diphosphate synthase from the hyperthermophilic archaeon *Aeropyrum pernix*. Biochem Biophys Res Commun 436 (2013) 230-234. 10.1016/j.bbrc.2013.05.081.

[21] H. Hemmi, S. Yamashita, T. Shimoyama, T. Nakayama, and T. Nishino, Cloning, expression, and characterization of *cis*-polyprenyl diphosphate synthase from the thermoacidophilic archaeon *Sulfolobus acidocaldarius*. J Bacteriol 183 (2001) 401-4. 10.1128/JB.183.1.401-404.2001

[22] C.M. Apfel, B. Takacs, M. Fountoulakis, M. Stieger, and W. Keck, Use of genomics to identify bacterial undecaprenyl pyrophosphate synthetase: cloning, expression, and characterization of the essential *uppS* gene. J Bacteriol 181 (1999) 483-92.

[23] M.C. Schulbach, P.J. Brennan, and D.C. Crick, Identification of a short (C_{15}) chain *Z*-isoprenyl diphosphate synthase and a homologous long (C_{50}) chain isoprenyl diphosphate synthase in *Mycobacterium tuberculosis*. J Biol Chem 275 (2000) 22876-81. 10.1074/jbc.M003194200.

[24] S. Endo, Y.W. Zhang, S. Takahashi, and T. Koyama, Identification of human dehydrodolichyl diphosphate synthase gene. Biochim Biophys Acta 1625 (2003) 291-5. 10.1016/s0167-4781(02)00628-0.

[25] K.D. Harrison, E.J. Park, N. Gao, A. Kuo, J.S. Rush, C.J. Waechter, M.A. Lehrman, and W.C. Sessa, Nogo-B receptor is necessary for cellular dolichol biosynthesis and protein *N*-glycosylation. EMBO J 30 (2011) 2490-500. 10.1038/emboj.2011.147.

Figure legends

Figure 1. Phylogenetic tree of cPT homologs

Proteins shown in black are cPT homologs from archaea of the phylum Euryarchaeota: MM_0014, MM_0618 and MM_1083, from *M. mazei*; MA1831, MA3723, and MA4402, from *M. acetivorans*; Mbur_0041, from *Methanococcoides burtonii*; Mthe_0589 and Mthe_1499, from *Methanosaeta thermophila*; MCP1817 and MCP2939, from *Methanocella paludicola*; MJ1372, from *Methanocaldococcus jannaschii*; MTH232, from *Methanothermobacter thermautotrophicus*; HVO2315 and HVO2318, from *Haloferax volcanii*; AF0707 and AF1219, from *Archaeoglobus fulgidus*; and, PH1590, from *Pyrococcus horikoshii*. Proteins from *Methanosarcina* spp. are shown by bold letters. Others are cPTs whose activities have already been identified: ApUPPS, undecaprenyl pyrophosphate synthase from *A. pernix* [20]; SaUPPS, from *S. acidocaldarius* [21]; EcUPPS, from *E. coli* [22]; MtFPPS, *cis*-farnesyl pyrophosphate synthase from *Mycobacterium tuberculosis* [23]; hCIT, dehydrodolichyl pyrophosphate synthase from human [24]; and, NgBR, a heteromer subunit of human dehydrodolichyl pyrophosphate synthase [25]. These enzymes are denoted by the taxa of their origins: the phylum Crenarchaeota in red, bacteria in blue, and eukaryotes in green. Bootstrap

values over 60% were indicated at each node.

Figure 2. Radiometric assay of *M. mazei* cPTs and TLC analysis of their products

(A) SDS-PAGE analysis of the recombinant *M. mazei* proteins partially purified from *E. coli* cells. Protein bands indicated with open arrow heads are in good agreement with the estimated molecular masses of recombinant proteins with tag sequences: tagged MM_0014 (41,501 Da); tagged MM_0618 (54,174 Da); and tagged MM_1083 (42,843 Da). (B) cPT activities of MM_0618, MM_1083, and the mixture of MM_0618 and MM_1083 (MM_0618/1083). (C) Allylic substrate preferences of MM_0014 and MM_0618/1083. [^{14}C]IPP and the allylic substrate indicated were used at a 1:1 ratio for the reaction. Each reaction was performed in triplicate. (D) Reversed-phase TLC analysis of the products of MM_0014 and MM_0618/1083 from the reactions with [^{14}C]IPP and the indicated allylic substrate. (E) Reversed-phase TLC analysis of the MM_0014 product from the reaction with [^{14}C]FPP and DMAPP. s.f., solvent front; ori., origin

Figure 3. LC-ESI-MS analysis of glycosyl carrier lipids from *M. mazei*

Upper panel: UV chromatogram of authentic polyprenols containing decaprenol (C_{50},

open circle), undecaprenol ($C_{55,}$ open triangle), and dodecaprenol (C_{60}, open square). Second upper panel: UV chromatogram of phosphatase-treated total lipid extracted from *M. mazei*. Two peaks eluted immediately after that of authentic decaprenol are indicated by gray and black circles, respectively, and a peak eluted immediately after that of authentic undecaprenol is shown by a gray triangle. Middle, second lower, and lower panels: Extracted ion chromatograms with indicated *m/z* ranges. The *m/z* values of 724, 726, and 792 correspond to $[M+Na]^+$ of dihydrodecaprenol, tetrahydrodecaprenol, and dihydroundecaprenol, respectively. These ions co-eluted with the UV peaks in the second panel with the gray circle, black circle, and gray triangle, respectively.

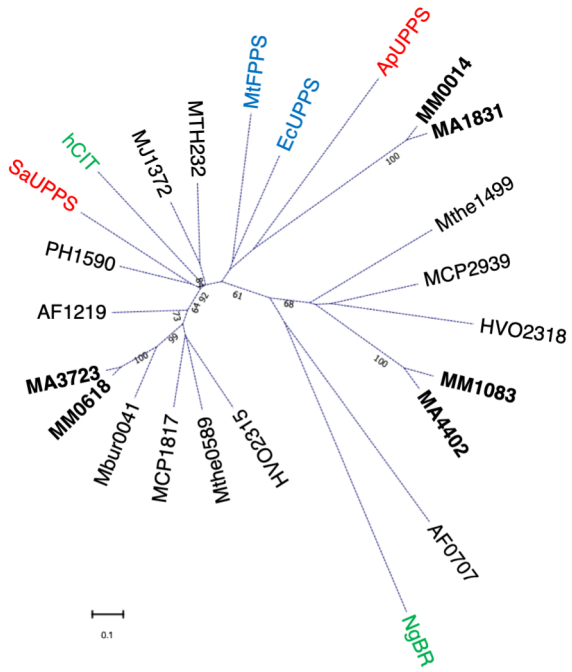
ApUPPS
MtFPPS
EcUPPS
MM0014
MA1831
MTH232
MJ1372
hCIT
SaUPPS
Mthe1499
PH1590
MCP2939
AF1219
HVO2318
MA3723
MM0618
MM1083
MA4402
Mbur0041
MCP1817
Mthe0569
HVO2315
AF0707
NgBR
100
100
100
100
0.1

Figure 1

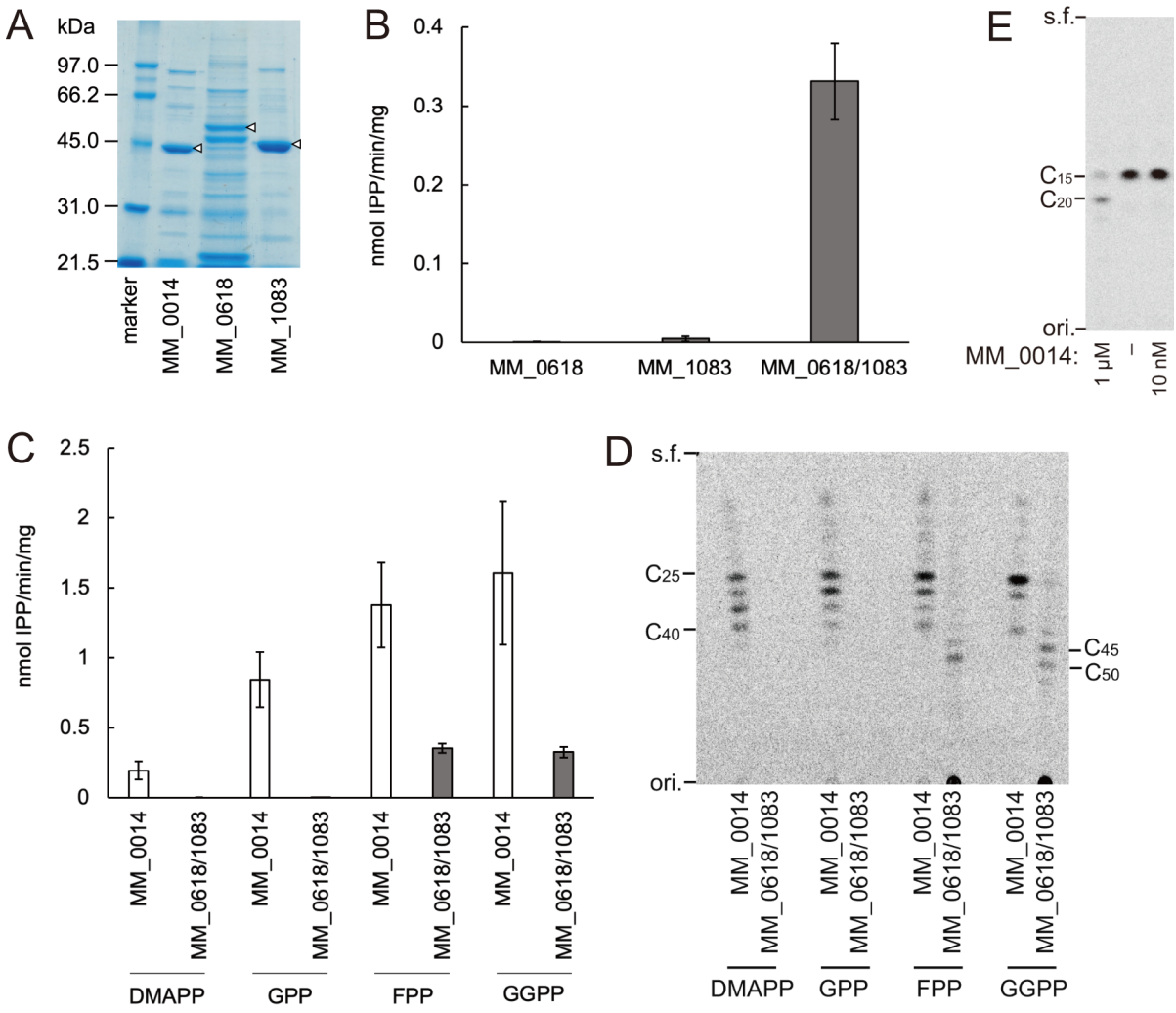
A
kDa
97.0
66.2
45.0
31.0
21.5
marker
MM_0014
MM_0618
MM_1083
B
nmol IPP/min/mg
0.4
0.3
0.2
0.1
0
MM_0618
MM_1083
MM_0618/1083
E
s.f.
C15
C20
ori.
MM_0014:
1 µM
–
10 nM
C
nmol IPP/min/mg
2.5
2
1.5
1
0.5
0
MM_0014
MM_0618/1083
DMAPP
GPP
FPP
GGPP
D
s.f.
C25
C40
C45
C50
ori.
MM_0014
MM_0618/1083
DMAPP
GPP
FPP
GGPP

Figure 2

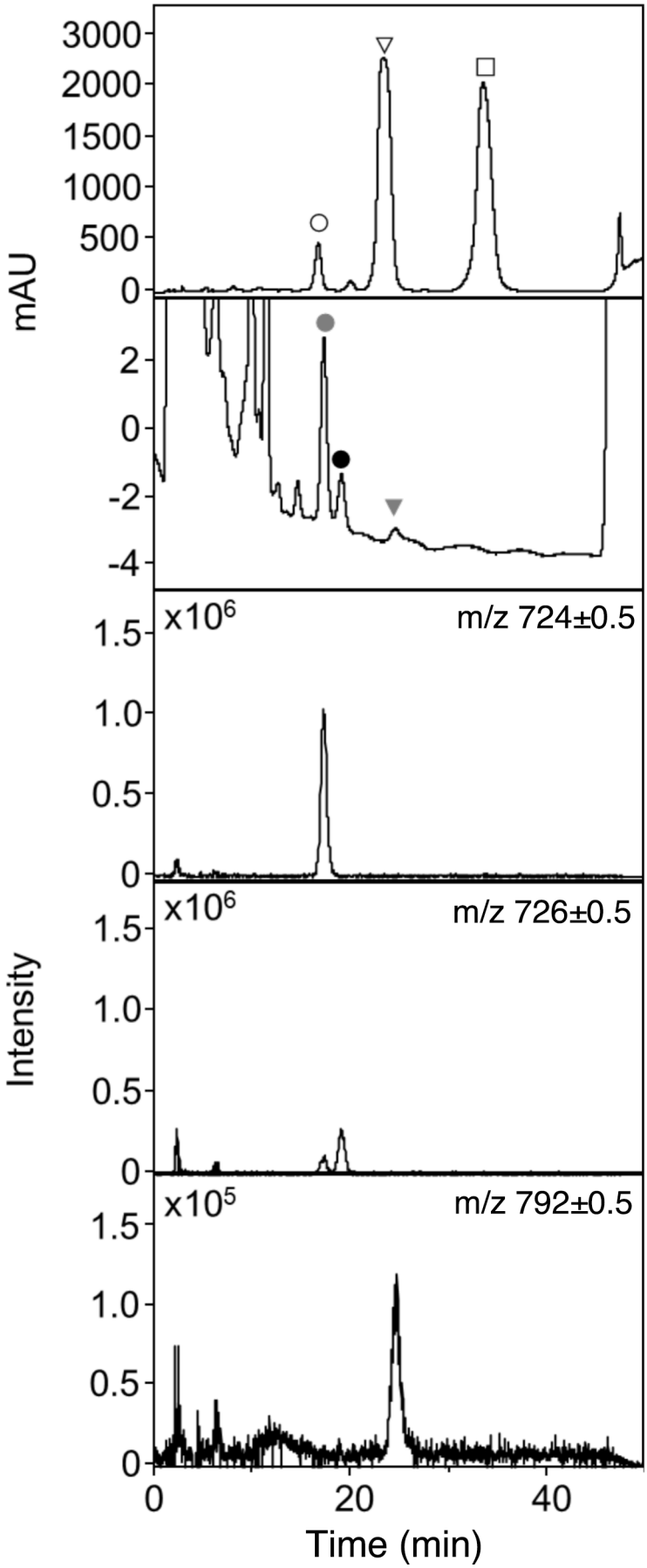
mAU
3000
2000
1500
1000
500
0
2
0
-2
-4
x10^6
m/z 724±0.5
1.5
1.0
0.5
0
x10^6
m/z 726±0.5
x10^5
m/z 792±0.5
Intensity
0
20
40
Time (min)

Figure 3