

A thermostable dolichol phosphoryl mannose synthase responsible for glycoconjugate synthesis of the hyperthermophilic archaeon *Pyrococcus horikoshii*

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Abstract Dolichol phosphoryl mannose synthase (DPM synthase) is an essential enzyme in the synthesis of N- and O-linked glycoproteins and the glycosylphosphatidylinositol anchor. An open reading frame, PH0051, from the hyperthermophilic archaeon *Pyrococcus horikoshii* encodes a DPM synthase ortholog, PH0051p. A full-length version of PH0051p was produced using an *E. coli* in vitro translation system and its thermostable activity was confirmed with a DPM synthesis assay, although the in vitro productivity was not sufficient for further characterization. Then, a yeast expression vector coding for the N-terminal catalytic domain of PH0051p was constructed. The N-terminal domain, named DPM(1-237), was successfully expressed, and turned out to be a membrane-bound form in *Saccharomyces cerevisiae* cells, even without its hydrophobic C-terminal domain. The membrane-bound DPM(1-237) was solubilized with a detergent and purified to homogeneity. The purified DPM(1-237) showed thermostability at up to 75°C and an optimum temperature of 60°C. The truncated mutant DPM(1-237) required Mg²⁺ and Mn²⁺ ions as cofactors the same as eukaryotic DPM synthases. By site-directed mutagenesis, Asp⁸⁹ and Asp⁹¹ located at the most conserved motif, DXD, were confirmed as the catalytic residues, the latter probably bound to a cofactor, Mg²⁺. DPM(1-237) was able to utilize both

acceptor lipids, dolichol phosphate and the prokaryotic carrier lipid C₅₅-undecaprenyl phosphate, with K_m values of 1.17 and 0.59 μM, respectively. The DPM synthase PH0051p seems to be a key component of the pathway supplying various lipid-linked phosphate sugars, since *P. horikoshii* could synthesize glycoproteins as well as the membrane-associated PH0051p in vivo.

Keywords Dolichol phosphoryl mannose synthase · Membrane protein · Hyperthermophilic archaea · Pyrococcus · Glycoprotein synthesis · Glycoconjugate · Glycosylphosphatidylinositol anchor · Thermostable flippase

Introduction

The glycosylation of proteins plays an important role in the maintenance of conformation and stability, surface recognition, and protection against proteolytic enzymes (Upreti et al. 2003). Dolichol phosphate mannose synthase (DPM synthase, E.C.2.3.1.83) catalyzes the transfer of a mannosyl residue from GDP-mannose to dolichol monophosphate to synthesize dolichol phosphoryl mannose (Dol-P-Man) and is an essential enzyme in the synthesis of glycoproteins. A mannosyl residue of Dol-P-Man is transferred to GlcNAc₂Man₅-PP-dolichol by several glycosyltransferases located on the endoplasmic reticulum to assemble Glc₃GlcNAc₂Man₉-PP-dolichol, a precursor of N-linked oligosaccharides (Burda and Aebi 1999). A mannosyl residue of Dol-P-Man is also used in the synthesis of O-linked glycoproteins and the glycosylphosphatidylinositol (GPI) anchor (Menon et al. 1990; Orlean 1990).

DPM synthases have been purified from eukaryotes, and the genes cloned in some cases. DPM synthases can be

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classified into two types; the first type, including DPM synthases from *S. cerevisiae* (Orlean et al. 1988), *Trypanosoma brucei* (Mazhari-Tabrizi et al. 1996), *Leishmania mexicana* (Ilgoutz et al. 1999), and *Thermoplasma acidophilum* (Zhu and Laine 1996) is a single subunit possessing a short hydrophobic polypeptide at its C-terminus. The hydrophobic region was proposed to be the dolichol recognition sequence, but later reports clarified that the truncation of this region had little effect on the enzyme activity (Zimmerman and Robbins 1993). The second type, including DPM synthases from Humans (Maeda et al. 2000), *Trichoderma reesei* (Kruszewska et al. 2000), and *Schizosaccharomyces pombe* (Colussi et al. 1997), consists of three subunits, with the catalytic subunit lacking the hydrophobic sequence at the C-terminus. The catalytic subunit of the second-type DPM synthase associates with the endoplasmic reticulum through the interaction of the membrane-integrated subunit (Ashida et al. 2005).

Dol-P-Man synthesis occurs on the cytosolic side of the endoplasmic reticulum (ER), while the transfer of a mannosyl residue from Dol-P-Man to Glc₃GlcNAc₂Man₉-PP-dolichol occurs on the luminal side (Prado-Figueroa et al. 1994; Perez and Hirschberg 1986; Helenius and Aebi 2002). Therefore, the transfer of Dol-P-Man from the cytosolic space to the luminal side through the ER membrane is necessary (Burda and Aebi 1999). Additional factors, such as flippases, are required to transfer Dol-P-Man from the cytoplasmic to luminal side. Recently, Zhou and Troy 2005 determined the complex structure of polyisoprenyl recognition polypeptide with dolichol phosphate by NMR analysis, and suggested the participation of the C-terminal hydrophobic domain for the *S. cerevisiae* DPM synthase in the translocation of Dol-P-Man from the cytosolic space to the luminal side. However, the mechanism of translocation of Dol-P-Man through the ER membrane is still unknown.

Glycosylation is not limited to eukaryotes. Archaea also possess the ability to synthesize dolichol-linked oligosaccharides and have the enzymes required for the biosynthesis of glycoproteins (Eichler 2000; Moens and Vanderleyden 1997). *P. horikoshii* is a hyperthermophilic euryarchaeon that grows at temperature above 100°C, and its genome sequence has been available (González et al. 1998; Kawarabayasi et al. 1998). In the genome sequence of *P. horikoshii*, an open reading frame, named PH0051, showed high similarity to eukaryotic DPM synthase. Although the eukaryotic DPM synthase has been studied in detail, little is known about the archaeal DPM synthase. In this study, we elucidated the unique enzymatic properties of PH0051p in comparison with those of the eukaryotic DPM synthases.

Materials and methods

Strains and media

Escherichia coli XL2-blue (Stratagene, La Jolla, CA) was used as the host strain for cloning experiments and cultured in 2YT medium (Sambrook et al. 1989). *Saccharomyces cerevisiae* W303-1A (Mat a, *leu2-3.112*, *his3-11.15*, *ade2-1*, *ura3-1*, *trp1-1*, *can1-100*) was used as the host strain for the production of recombinant protein, and was grown in SD medium (0.67% yeast nitrogen base without amino acid, 2% glucose, 0.002% histidine, 0.002% adenine, 0.002% tryptophan, 0.002% leucine, 0.002% uracil, pH 7.0). *P. horikoshii* (JCM 9974) was obtained from the Japanese Collection of Microorganisms (JCM), Saitama, Japan. *P. horikoshii* cells were cultivated anaerobically in a chemically defined medium consisting of 13.5% NaCl, 4% Na₂SO₄, 0.7% KCl, 0.2% NaHCO₃, 0.1% KBr, 0.03% H₃BO₃, 10.8% MgCl₂·6H₂O, 1.5% CaCl₂, 0.025% SrCl₂, 0.0002% rezaurin, 1% Bacto yeast extract, and 5% Bactotrypton. Sterile sulfur (2 g/l) was added to the medium after autoclaving. The medium was gassed with CO₂ and 2 ml of sterile 15% Na₂S was added to remove oxygen. Cells were grown in 1-liter glass bottles containing 700 ml of the medium at 95°C for 3 days.

General DNA manipulation

Restriction endonucleases and KOD Plus DNA polymerase were purchased from Takara Shuzo (Shiga, Japan) and Toyobo (Osaka, Japan), respectively, and used as recommended by their manufacturers. Plasmids were isolated from *E. coli* with Qiaprep columns (Qiagen). Genomic DNA from *P. horikoshii* was isolated according to a method reported previously (Higashibata et al. 2003). DNA fragments were separated by agarose gel electrophoresis and individual fragments were isolated with the Qiaquick gel extraction kit (Qiagen). DNA sequencing was performed by the dideoxy chain termination method using ABI PRISM big-dye terminator cycle sequencing ready reaction kits on a ABI PRISM 310 DNA sequencer. The transformation of recombinant plasmids into *S. cerevisiae* was performed according to the LiOAc method (Kaiser et al. 1994), and transformants were selected on SD medium without uracil.

Construction of yeast expression vectors for PH0051p and truncation mutants

The expression vectors for PH0051p and its truncated form were constructed using the following procedure. The full-length PH0051 gene was amplified by PCR using 0051 F (5'-ATATTGGTGGTGAATTCATGAAAGTCTCAATAA

TAG-3') and 0051 R (5'-CTTCTGGAGGAGGGTTCGAC TGATTGAGCCCATG-3'). The 3'-deleted PH0051 gene encoding the N-terminal 1-237 region was amplified using 0051 F (5'-ATATTGGTGGTGAATTCATGAAAGTCCT CAATAATAG-3') and 0051 Δ C1 (5'-ATCCGACGTCGAC TCACTTTAAAATGCGATCTAC-3'). The 3'-deleted PH0051 gene encoding the N-terminal 1-219 region was amplified using 0051 F and 0051 Δ C2 (5'-TTTCATCA GCCTGTGACTCACCTTAGATA-3'). Amplified DNA fragments were digested with *EcoRI* and *SalI* and inserted into the *EcoRI/SalI* site of the vector YEP₃₅₂GAP.

Site-directed mutagenesis

The mutants of DPM(1-237) altered at Asp³⁸, Asp³⁹, Asp⁸⁹, and Asp⁹¹ were constructed using an overlap PCR method (Kadowaki et al. 1989). In the first PCR, 0051 F and 0051 Δ C1 were used as common primers. To convert Asp³⁸, Asp³⁹, Asp⁸⁹, and Asp⁹¹ to alanine, the following four sets of complementary primers were designed for the first PCR: 38A F 5'-AATTGTAGCTGATGATTCACCG GAT-3' and 38A R 5'-TCCGGTTGAATCATCAGCTA CAATT-3' for the D38A mutant, 39A F 5'-AATTGTA GATGCTGATTCACCGGAT-3' and 39A R 5'-ATCCGG TGAATCAGCAGCATCTACAATT-3' for the D39A mutant, 89A F 5'-GTATTCGTCGTTATGGCTGCCGA CCTTC-3' and 89A R 5'-GAAGGTCCGCAGCCATAA CGACGAATAC-3' for the D89A mutant, and 91A F 5'-TCGTTATGGATGCCGCTCTCAGCATCCC-3' and 91A R 5'-GGGATGCTGAAGAGCGGCATCCATAA CCGA-3' for the D91A mutant. The amplified fragments were collected, and a second PCR was performed using 0051 F and 0051 Δ C1. The amplified fragment was digested with *EcoRI* and *SalI*, and inserted into the *EcoRI/SalI* site of YEP₃₅₂GAP.

Production of PH0051p by the *in vitro* translation system

The full-length product of the PH0051 gene was little expressed in the *E. coli* host strains. Then, an *E. coli* *in vitro* translation system was chosen to overcome this problem. The expression unit was constructed as follows. Linear template DNA was constructed by a two-step overlap extension PCR method using an RTS *E. coli* linear template generation set, His-Tag (Roche). The forward primer 5'-CTTTAAGAAGGAGATATACCATGGTAGA TACAGAAAGTCTCTCTGCCCCCTCT-3' and reverse primer 5'-TGATGATGAGAACCCCCCGTACATGT CCCTGAG-3' were used for the amplification of the PH0051 gene in the first PCR. Then, the T7-promoter and T7-terminator were introduced upstream of and behind the

PH0051 gene by a second PCR using primers in the kit. The amplified DNA was inserted into pCR-TOPO (Invitrogen), and the resultant plasmid, designated pCR-PH0051, was used as a template. PH0051p with a histidine-tag at its C-terminus was produced using an RTS500 *E. coli* HY kit (Roche) according to the manual supplied. The reaction device was filled with 1 ml of reaction solution and 10 ml of feeding solution separated by a semipermeable membrane. When necessary, 0.5% (w/v) of Brij 35 (Sigma) was added in the reaction device. The reaction devices were incubated at 30°C for 24 h. The solution from the reaction compartment was removed and insoluble protein was removed by centrifugation at 5,000×g for 5 min. The supernatant was used for a Western blot analysis or enzyme activity assay.

DPM synthase assay

DPM synthase assay was carried out by the method of Orlean (Orlean et al. 1988) with a few modifications. Dolichol phosphate was firstly dried under N₂ gas, and dispersed in the 50 mM Tris-HCl (pH 7.5) containing 1% Triton X-100 by sonication. The standard reaction mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 μg of dolichol phosphate or undecaprenyl phosphate, and 8 ng of enzyme, in a final volume of 100 μl. The reaction mixture was pre-incubated at 60°C for 10 min, and the reaction was initiated by the addition of 0.1 μCi of GDP-¹⁴C-mannose (3.7 GBq/mmol; New England Nuclear). The reaction was stopped by the addition of 0.5 ml of chloroform-methanol (1:1, v/v) and 150 μl of H₂O. The mixtures were centrifuged at 12,000×g for 10 min and the lower organic phase was saved. The upper inorganic phase was re-extracted with 125 μl of chloroform, and the lower phase was combined with the first organic fraction. The combined mixtures were washed with 250 μl of chloroform-methanol-H₂O (3:48:47, v/v). After centrifugation at 12,000×g for 10 min, the radioactivity of the lower organic phase was measured by liquid scintillation counting.

Preparation of cell-free extract from recombinant *S. cerevisiae* cells

The yeast cells harboring recombinant plasmids were cultivated in 20 ml of SD medium at 30°C for 24 h with vigorous shaking. Harvested cells were suspended in 200 μl of 50 mM Tris-HCl (pH 7.5), and then 500 U of Zymolyase and 25 μg of DNase were added. After 10 min at 37°C, the tubes were centrifuged at 10,000×g for 10 min to remove cell debris. The supernatant was used for assaying DPM synthase activity.

Solubilization with Triton X-100 and purification of wild type (WT) and truncated mutants of PH0051p from *S. cerevisiae* cells

The yeast transformants were cultivated in SD medium without uracil at 30°C for 24 h with vigorous shaking. Cells were harvested from 8 l of culture by centrifugation, and suspended in 40 ml of maleic acid buffer (50 mM maleic acid, and 10% glycerol, pH 6.0). Cells were disrupted by using a French pressure cell press, and cell debris was removed by centrifugation at 15,000×g for 10 min. The supernatant was centrifuged at 100,000×g for 60 min to collect the membrane fraction. The precipitant was suspended in maleic acid buffer containing 2% TritonX-100, and incubated at 4°C for 60 min with gentle rotation. The solution was heated at 60°C for 10 min, and the denatured proteins were removed by centrifugation at 15,000×g for 10 min. The supernatant was applied to a Hitrap SP column (Amersham Biosciences) equilibrated with maleic acid buffer containing 0.1% TritonX-100, using the fast protein liquid chromatography system (Amersham Biosciences). After the column had been washed with maleic acid buffer, the proteins were eluted with a linear gradient of 0–400 mM NaCl. The active fractions were concentrated using a Centricon YM-10 (Millipore) and applied to a gel filtration column of Superose 12 HR 10/30 (Amersham Biosciences) with maleic acid buffer containing 0.02% Triton X-100 using the FPLC system. The fractions containing enzyme were collected, concentrated with a Centricon YM-10, and stored at 4°C.

Preparation of membrane-protein fraction and soluble-protein fraction from *P. horikoshii* cells, and detection of glycoproteins and PH0051p by Western blotting

Pyrococcus horikoshii cells were cultivated in the chemically defined medium at 90°C for 3 days. Harvested cells were suspended in 50 mM Tris–HCl buffer (pH 8.0) and disrupted by sonication on ice. Cell debris was removed by centrifugation at 15,000×g for 10 min. The resultant solution was further centrifuged at 100,000×g for 60 min. The supernatant was used as the soluble-protein fraction. The precipitant was suspended with 50 mM Tris–HCl buffer (pH 8.0) and used as the membrane-protein fraction.

The glycoproteins of *P. horikoshii* were detected by Western blotting using alkaline phosphatase-conjugated concanavaline A (Molecular Probe). The proteins were separated by SDS-PAGE, and transferred onto a nitrocellulose membrane. After a wash with TBST buffer, the blotted membrane was incubated with alkaline phosphatase-conjugated concanavaline A. The resultant membrane was washed again with TBST buffer, and the glycoprotein bands on the membrane were visualized by treatment with

Fig. 1 a The amino acid sequence alignment for DPM synthase homologues from *Pyrococcus horikoshii* (*P. horikoshii*, GenBank accession no. BA000001), *Pyrococcus furiosus* (*P. furiosus*, AE010132), *Trypanosoma brucei* (*T. brucei*, AJ866775), *Leishmania mexicana* (*L. mexicana*, AJ131960), *Saccharomyces cerevisiae* (*S. cerevisiae*, U25842), *Homo sapiens* (Human, D86198), *Caenorhabditis briggsae* (*C. briggsae*, AF007874), *Aeropyrum pernix* (*A. pernix*, BA000002), *Methanococcoides burtonii* (*M. burtonii*, NC007955), and *Sulfolobus solfataricus* (*S. solfataricus*, NC002754). Conserved residues are shaded. The catalytic residues Asp⁸⁹ and Asp⁹¹ in the most conserved DXD motif are indicated with asterisks. **b** Hydropathy plot of PH0051. The Kyte-Doolittle hydropathy plot was produced with the program DNASIS (Hitachi software). Hydrophobic regions at the C-terminus are noted by an arrow

the color-producing reagents 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium (Promega).

PH0051p was detected using an anti-DPM(1-237) antiserum raised against the purified DPM(1-237). The proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. After being washed with TBS buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl) at room temperature, the membrane was incubated with the anti-DPM(1-237) antiserum diluted with TBST buffer (20 mM Tris–HCl, pH 7.5, 500 mM NaCl and 0.05% Tween 20). The membrane was washed with TBST buffer and soaked into TBST buffer containing horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Promega). After incubation, the protein was visualized using an ECL plus Western blotting detection system (Amersham Biosciences).

Protein assay and SDS-PAGE

Protein concentrations were determined using the Bio-Rad protein assay system with bovine serum albumin as a standard (Bio-Rad). SDS-PAGE analysis of proteins was performed using a 10–15% gradient gel using the Phast-system (Amersham Pharmacia). The gel was stained with Coomassie brilliant blue R-250. The molecular weights of proteins were estimated by comparison with protein markers (Bio-Rad).

Results and discussion

PH0051p is a thermostable DPM synthase

An open reading frame (1062 bp), PH0051 encodes a protein consisting of 353 amino acids. The deduced molecular mass and *pI* values calculated from the amino acid sequence were 41012 Da and 9.58, respectively. The amino acid sequence alignment among DPM synthase homologues obtained from databases (<http://mbgd.genome.ad.jp/>) is shown in Fig. 1a. The N-terminal half of PH0051p showed high similarity to eukaryotic DPM synthases, and the DXD

A

<i>P. furiosus</i>	1	-----MKVSVIIPTYNERENLEELFSRIDNAIQG-----LNYEIVVDDDS
<i>P. horikoshii</i>	1	-----MKVSIIVPTYNERDNLEELFSRISSALKG-----YDYBIIIVDDDS
<i>T. brucei</i>	1	-----MAVKYSIIVPAYKBCGNLEELPTKQVFDALADDGFSKNEVEMVIIVDDNS
<i>L. mexicana</i>	1	-----MOYSIIVPAYKBCGNLEELPVRVFAAVREQGLPTQNVEMLIIVDDNS
<i>S. cerevisiae</i>	1	-----MSIEYSVIVPAYHEKLNKPKPTTRRLFAGMSP--EMAKKTELEIVDDNS
Human	1	MASLEVSRSPRRSRRELEVRSPRQNKYSVLLPTYNBERENLPLIVVLLVKS PSES--GINYEIIVHDDGS
<i>C. briggsae</i>	1	-----MIAVLSTPKYSIILPTYNBERENLPLICIWLTKEYLKG-----ISYBIIIVDDAS
<i>A. pernix</i>	1	-----MSKPPCGEPEYSVIVPTLNERDNIRPFIYVGLKKSLEGAG--LECFEIVIVDDSS
<i>M. burtonii</i>	1	-----MKRSIVPSFNERENIPVIVDKLQNVKLD-----IDYEIIVVDDNS
<i>S. solfataricus</i>	1	-MKRRESSPLISTILGIRYGRVCMGNSIVIVPTYNERDNIVKLVVEEINRIVYPN-----SRILIVDDNS

* *

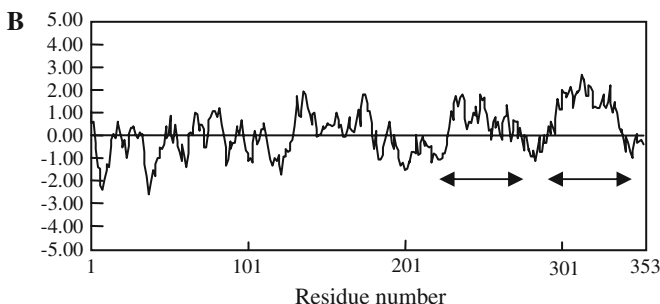
<i>P. furiosus</i>	42	PDRTWKKAQELS-SKYPVKVIRRTKEK--GLSSAVIRGFKEA---SGDVFVMDADLQHPPEVIVPKLIEA
<i>P. horikoshii</i>	42	PDKTWPKAMELS-KLYPVKVIKRVNEK--GLSSAVIRGFSEA---SGDVFVMDADLQHPPEVIVPSLLRE
<i>T. brucei</i>	49	RDGSEVVEKVRNEGYGVRIEVRTNDR--GLSSAVIHGISVS---KGSFIVMDADLQHPPEVIVPSLLRA
<i>L. mexicana</i>	47	CDGSEVVEVRFHKEGFNVSMDEVRTTER--GLSSAVIHGLRHT---SGYKLVMDADLQHPPEVIVPSLLFA
<i>S. cerevisiae</i>	47	QDGSVEVDALAHQGYNVRITVIRTNER--GLSSAVLKG FYEA---KQOYLVMADADLQHPPEVIVPSLLFES
Human	68	PDGTRDVAEQLKLIYGS DRILLRPREKKLGLGTAYTHGMKHA---TGNYIIMDADLSHHPKFFIPEFIRK
<i>C. briggsae</i>	49	PDGTQDVAKLEKEYGENKILIKPRAGKLGLTAYSHGLSFA---RGEFIIMDADLSHHPKFFIPEMIAL
<i>A. pernix</i>	53	TDGTIEEVEVRLSRSDPRVRLIVRGEK--GLSRVLEGLRRA---RGRVAVMDADLQHPPEVIVPSLLVEK
<i>M. burtonii</i>	42	PDKTDWDLVQMSHEDSRVKVIRIRGRN--GLSSAVIRGFLEA---TGEYLVMDADLQHPPEVIVPSLLTMLNE
<i>S. solfataricus</i>	64	PDGTAALIQELN--IHNLTVLIHNER--GLGSALRYGISKALIELESDFIVTMDADFSHNPKYLPEMMKI

<i>P. furiosus</i>	106	IKNGS-DIAIGSRVYKGGKVEN-WPFYRKLISKGAIMVGRIALPKIRDIDPVS GFFALRKEVVE---GV
<i>P. horikoshii</i>	106	IEKGN-DIAIASRVYKGGKVEN-WPFYRRLISRGAILIGRLALPKIAGIKDPVSGFFALKRSSVVE---GV
<i>T. brucei</i>	114	LEKPGVEFVCGPRYAGVEIDKDWPLHRRFISWGARLLARPPT---PLSDPMSGFFGLRVDVFG--RGRE
<i>L. mexicana</i>	112	LSRDGVEFVCGPRYAGVEIDKNWPAHRRFISWGARLLSRPPT---TLDSPMSGFFGIRDCVFK--RHAG
<i>S. cerevisiae</i>	112	LHDH--AFTLGRYAPVSGIDKDWPMYRRVISTARMARPPT---IASDPMSPFGLQKYLECNRHP
Human	135	QKEGNFDIVSGTRYKNGCGVYG--WDLKRAKISRGANPLTQILLR--PGASDLTCSERLYRKEVEKLKIE
<i>C. briggsae</i>	116	QQYKLDIVTGRYKNGCGVSG--WDLKRAKISRGANPLAQFLN--PGVSDLTCSERLYRKEVEKLATLIA
<i>A. pernix</i>	118	VAGGEADLAVASRYAPGGRVEG--WSLVRRISRGAVLAWLIPESRRTRDPVSGFFAVNLERVR---PA
<i>M. burtonii</i>	107	IEENDDLVIASRYTETKDVEG--WSRTRLFISDVATKLAQLVIK--YKVTDPMSGFFVLLKKSIVQEN-VE
<i>S. solfataricus</i>	130	ALNENCDFVIGSRVYVIGGIGTEN--WLSLRRIISKGANYLFKLVSHS---PIMDNTSNRYIYSRRAALLALE

<i>P. furiosus</i>	171	ELNPIGFKILMBILIKGKYS----KVVEVPPTFGIARGESKLGKGTIFEYLRHIIYRLMKWEGEIDRIV
<i>P. horikoshii</i>	171	ELNPIGFKILMBILIKGKYS----RVTEVPPTFSTKFGESKLGKGTIMVNYLRHVYRLMKWEGEVDRII
<i>T. brucei</i>	179	VVNPICGKIALLFLVFKCAVR---KYEVGQNFPAARTVGESKLTGKVIINYLEHEKLLYFYVYGTALTIV
<i>L. mexicana</i>	177	EVNSIGYKIGLELFFVKCRVQ---CFEVEGQNFATRTYGESKLTGKVIILHYLEHEYALYLFKLRGLFYV
<i>S. cerevisiae</i>	176	DINSQGGKHALLELLAKLPLPRDPRVAIEGVPFTFGVTEGESKLSGKVIILQYLLQKELVYVFKFGANNLI
Human	201	KCVSKGYVFMEMIVRAKQLN---YTIQEVPTSFVDVYVYGESKLGQNEIVSFLKGLLTLFATT-----
<i>C. briggsae</i>	182	ESVSKGYVFMEMIVRAKQSG---YTIQEVPTSFVDVYVYGESKLGQNEIVVAKGILYLFVIVW-----
<i>A. pernix</i>	184	IVSGRGEFKILLEILYTNPYAR---HVDVPTFRGSRSGESKLGPTIILDVYLLQVLLQSSIPRFIAVGA
<i>M. burtonii</i>	173	KFYGKGEKILLDIMSVKNDLR---HVEIPVFTFNRLHGESKLGNDVIFQYVEFLELRFQKXVETEYI
<i>S. solfataricus</i>	196	CDTNGYEFQICSIFFKIIRNN--LKIKREYPIIEVDKRTGKSKLNLKLEILNWFYLIIRLSLSP-----

<i>P. furiosus</i>	236	KFSIVGLSGILVNEGFLWLFVNLG-IPKEIAVIPAVELSILNFFWNDIWTFKDIRRGSIFSRLKPFHIA
<i>P. horikoshii</i>	236	KFSIVGLSGVGVNEGFLWLFVNFPHISKELGVIPSTELSILNFFWNDLWTFKDIKKSIVIERLAKFHV A
<i>T. brucei</i>	244	LLVLLPLIFYCFYILL-----
<i>L. mexicana</i>	242	FVVAVMFAVYLLAFLYHSLF-----
<i>S. cerevisiae</i>	246	LFITFWSLFFYVYQLYHLVF-----
Human		-----
<i>C. briggsae</i>		-----
<i>A. pernix</i>	249	LGSLVNLAVYTLAFSATGVHPLSSILGFPEAGLIHNAILHDRITFRNRSMWRS----HSFLRRLAKYHAT
<i>M. burtonii</i>	238	KYMIVGSFGALLHFSILTLYFKNIGYYSISLAIILVAAILFNYYNNIWTFKDYRLTDISLITGLYLYKN
<i>S. solfataricus</i>		-----

<i>P. furiosus</i>	305	ALSGAVVNFIVYWILLFLG-IHYLIANLVGIVLSFGVRYVINRHVTWAT---
<i>P. horikoshii</i>	306	AFVGAVAQFLVYWILLFLG-IHYLLANLFGIGASFVRYIFNRRHITWAQ---
<i>T. brucei</i>		-----
<i>L. mexicana</i>		-----
<i>S. cerevisiae</i>		-----
Human		-----
<i>C. briggsae</i>		-----
<i>A. pernix</i>	314	SAAGIAGVAASTILYSLAGLHPLLSQALGIGAGFIVNFTLASRYIWR---
<i>M. burtonii</i>	308	LLCFV GALANYSVSMYLDHMSWIIASMVGA FVGANNNYLTNSIYTWKTKHP
<i>S. solfataricus</i>		-----



motif, putative catalytic residues among β -glycosyltransferases (Saxena et al. 1995), was conserved in PH0051p (residue number ⁸⁹DA⁹¹D). PH0051p and archaeal DPM

synthase homologues except for the *Sulfolobus solfataricus* enzyme possess non-conserved long stretches of amino acid sequences at the C-terminus. The hydropathy plot of

PH0051p is shown in Fig. 1b. The plot indicated the presence of hydrophobic regions at the C-terminus of PH0051p, noted by arrows in Fig. 1b. The sequence $^{313}\text{QFLVY-WILLFL}^{324}\text{G}$ of PH0051p corresponding to the C-terminal hydrophobic sequence of *S. cerevisiae* seemed to be related to the putative dolichol recognition sequence (FI/VXF/YXXIPXF/Y) (Mazhari-Tabrizi et al. 1996).

Using the in vitro translation system, PH0051p was produced to confirm its DPM synthase activity. The positive signal of a 40 kDa band was detected in the reaction mixture containing the PH0051 gene, although the control reaction without the PH0051 gene gave no signal in the immunoblotting pattern. The molecular mass, 40 kDa was well consistent with that calculated from the amino acid sequence of PH0051p, indicating that the product was the full-length PH0051p.

Then, DPM synthase activity was measured using the in vitro translation mixture. The enzyme activity was detected in the reaction mixture with the histidine-tagged PH0051p, but no enzyme activity was detected in the control reaction without PH0051p. The Western blotting pattern and the enzyme activity indicated that PH0051p was a thermostable DPM synthase. Interestingly, both the amount and the level of activity of PH0051p were remarkably increased by the addition of the surfactant Brij 35 to the in vitro translation reaction mixture. The effect might be due to the stabilization of PH0051p on covering the C-terminal hydrophobic region with the surfactant.

The core domain of PH0051p, DPM(1-237), is sufficient for thermostability and enzymatic activity

Although we succeeded in producing intact PH0051p as an active form using the in vitro translation system, the amount of PH0051p synthesized was not sufficient for the purification and investigation of its enzymatic properties. Therefore, we changed strategy and decided to produce PH0051p using a yeast expression system. The PH0051 gene was inserted into the vector Yep₃₅₂GAP, and the resultant expression plasmid, named Yep-DPM(1-353), was introduced into *S. cerevisiae*. To investigate the region essential for the enzyme activity, the PH0051 gene was truncated at the 3'-ends. The resultant plasmids were named Yep-DPM(1-237) and Yep-DPM(1-219) as shown in Fig. 2a.

At the first step in the purification process, the DPM synthase activities of the wild type (WT) and truncated PH0051p were roughly assayed with the cell-free extracts from the recombinant yeast cells. The results are shown in Fig. 2b. The endogenous yeast DPM synthase was inactivated at 60°C, and no enzyme activity was detected with the cell-free extract from the yeast transformant harboring the empty Yep₃₅₂GAP vector. On the contrary, enzyme activity

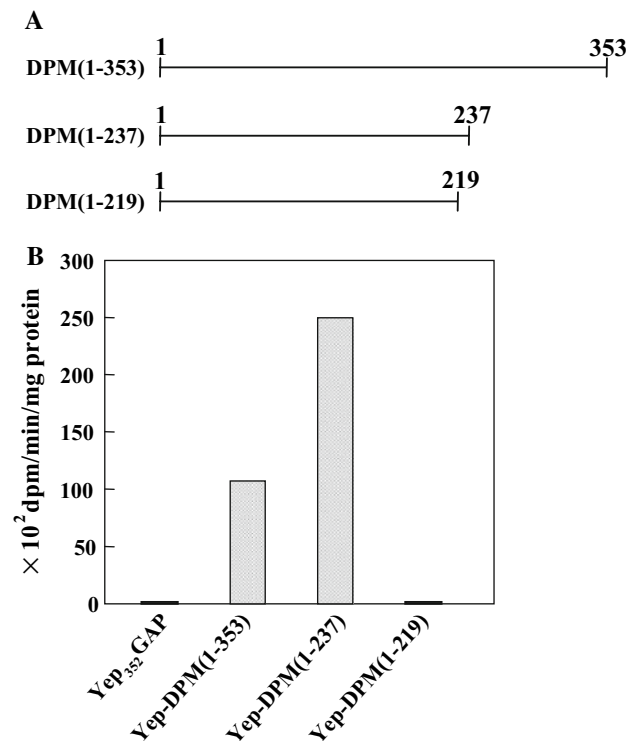


Fig. 2 Production of PH0051p and truncated proteins in *S. cerevisiae*. **a** Schematic representation of various PH0051p truncated proteins. Numbers indicate the site where PH0051p was shortened. **b** DPM synthase activity in the cell lysate from the *S. cerevisiae* transformant. Cell lysate was prepared by Zymolyase plus DNase treatment as described in “Materials and methods”. Ten microliters of cell lysate containing about 100 µg of protein was added to the reaction mixture, and the reaction was performed at 60°C for 10 min. The synthesized Dol-P-[¹⁴C]-Man was extracted with chloroform methanol, and radioactivity was measured by scintillation counting

was detected with extracts from the transformants harboring Yep-DPM(1-353) and Yep-DPM(1-237), respectively, indicating that the thermostable DPM synthases were produced in *S. cerevisiae*, whereas it turned out afterwards that the expression product from the transformant with Yep-DPM(1-353) was not intact, probably due to the C-terminal truncation of the product by the endogenous protease in the *S. cerevisiae* cells (data not shown). In addition, the result indicates that the hydrophobic domain at the C-terminus corresponding to between the 238th and 353rd residues was dispensable for the enzyme assay. When the enzyme activity was assayed with the lysate from the transformant harboring Yep-DPM(1-219), no activity was detected. The results indicated that the C-terminal 18 residues of DPM(1-237) were also indispensable for activity and/or thermostability.

Before purifying DPM(1-237), the localization of DPM(1-237) in *S. cerevisiae* was investigated. To our surprise, almost all of the enzyme activity was detected in the membrane fraction (data not shown). This finding

indicated that DPM(1-237) could associate with membranes without the hydrophobic domain at the C-terminus. Then, DPM(1-237) was purified from the membrane fraction obtained by ultracentrifugation with the cell lysate from the yeast transformant harboring Yep-DPM(1-237), followed by several steps of purification including solubilization, heat treatment, ion exchange, and gel filtration. DPM(1-237) was thermostable and purified to homogeneity, and one band corresponding to 27 kDa was detected by SDS-PAGE analysis (data not shown). The molecular mass, 27 kDa, is well consistent with that calculated with the amino acid sequence of DPM(1-237). The N-terminal sequence of the purified DPM(1-237) sample determined by the peptide sequencing, MKVSIIV, was identical to that coded for by the initial sequence of the PH0051 gene. Using the purified DPM(1-237), its biochemical properties were investigated. The optimum temperature was approximately 60°C as shown in Fig. 3a. Full enzyme activity remained even after incubation at 75°C for 30 min (Fig. 3b). The optimum pH was 8.5 (Fig. 3c).

Furthermore, the requirement of metal ions was investigated. The results suggested that DPM(1-237) required Mg^{2+} or Mn^{2+} as a cofactor in the same manner as the other DPM synthases (Mazhari-Tabrizi et al. 1996; Maeda et al. 2000; Babczinski et al. 1980). Taking the activity with 10 mM Mg^{2+} as 100%, the relative activity of each divalent and monovalent cation at the same concentration was as follows: none; 0%, Mn^{2+} ; 146%, Ca^{2+} ; 31%, Ni^{2+} ; 7%, Zn^{2+} ; 13%, Cu^{2+} ; 5%, Fe^{2+} ; 4%, Na^+ ; 3%, and K^+ ; 4%.

It has been reported that phospholipids activate eukaryotic DPM synthases and protect from thermal inactivation (Jensen and Schutzbach 1985; Villagómez-Castro et al. 2000). The effect of phospholipids on the enzyme activity was investigated by measuring the activity in the presence of 10 μ g of phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylethanolamine (PE), or phosphatidylcholine (PC), respectively. The enzyme activity in the presence of each phospholipid is shown in Table 1. When PG and PS were added to the reaction mixture, DPM(1-237) showed three times more activity than that without phospholipid. Non-bilayer phospholipids such as PE and PC were reported to activate eukaryotic DPM synthases, however, PE and PC had little effect on the activity of DPM(1-237) as shown in Table 1. Since the polar head groups of phospholipids, ethanolamine, L-serine, glycerol, and choline are found throughout the three domains (*Archaea*, *Bacteria*, and *Eucarya*) (Koga and Morii 2007), the head groups, glycerol and L-serine of phospholipids might protect PH0051p from thermal inactivation in vitro.

The enzyme activity against the dolichol phosphate or C_{55} -undecaprenyl phosphate is shown in Fig. 4. The substrate, C_{55} -undecaprenyl phosphate consisting of eleven isoprene units with an unsaturated α -isoprene unit as shown

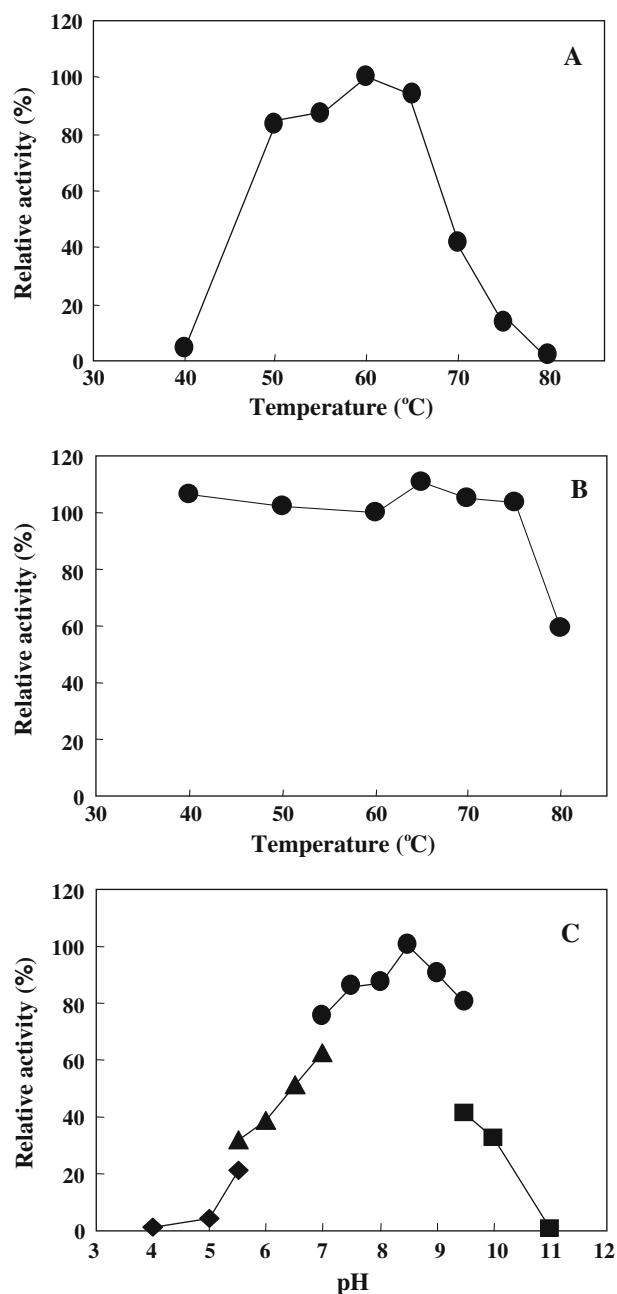


Fig. 3 The optimum temperature (a), thermostability (b), and optimum pH (c) of DPM(1-237). Eight nanograms of the purified enzyme was added to the reaction mixture containing 10 μ g of dolichol phosphate. **a** The optimum temperature was measured between 40 and 80°C. **b** The heat stability was investigated by measuring residual activity after incubating the enzyme for 30 min at various temperatures. **c** The optimum pH was measured at 60°C in 50 mM buffer. filled diamond sodium acetate; filled triangle maleic acid; filled circle Tris-HCl; filled square cyclohexylaminopropane-sulfonic acid

in Fig. 4, was reported to be essential for the biosynthesis of bacterial cell wall polymers such as peptidoglycan, lipopolysaccharides, and teichoic acids (Zhou and Troy 2005). The enzyme assay showed that DPM(1-237) is able

to utilize both dolichol phosphate and undecaprenyl phosphate as an acceptor lipid, as shown in Fig. 4. The K_m values for C₈₅-, C₁₁₀-, C₁₂₀-dolichol phosphate and C₅₅-undecaprenyl phosphate were determined to be 2.3, 0.59, 1.59, and 1.17 μM , respectively. The specificity of DPM(1-237) against these acceptor lipids with different numbers of isoprene units and with a double bond at the α -isoprene unit was low. The K_m value of DPM(1-237) for dolichol phosphate was close to that of *S. cerevisiae* DPM synthase (10.6 μM , Jensen and Schutzbach 1985). Furthermore, DPM synthase from thermophilic archaeon *Thermoplasma acidophilum* had been purified and characterized (Zhu and Laine 1996). It was reported that the K_m value of *T. acidophilum* DPM synthase for dolichol phosphate was

2.6 μM , and that the temperature and pH optima were 65°C and 6.0, respectively. Dolichol phosphate was preferable to undecaprenyl phosphate for *T. acidophilum* DPM synthase, although the K_m value for undecaprenyl phosphate had not been determined. *T. acidophilum* DPM synthase contained a trace of dolichyl-phosphate glucose (DPG) synthase activity (Zhu and Laine 1996), whereas no DPG synthase activity was detected for DPM(1-237) (data not shown). These facts indicate that the substrate specificity of PH0051p for acceptor lipid and sugar is distinct from that of *T. acidophilum* DPM synthase.

Asp⁸⁹ and Asp⁹¹ are catalytic residues of PH0051p

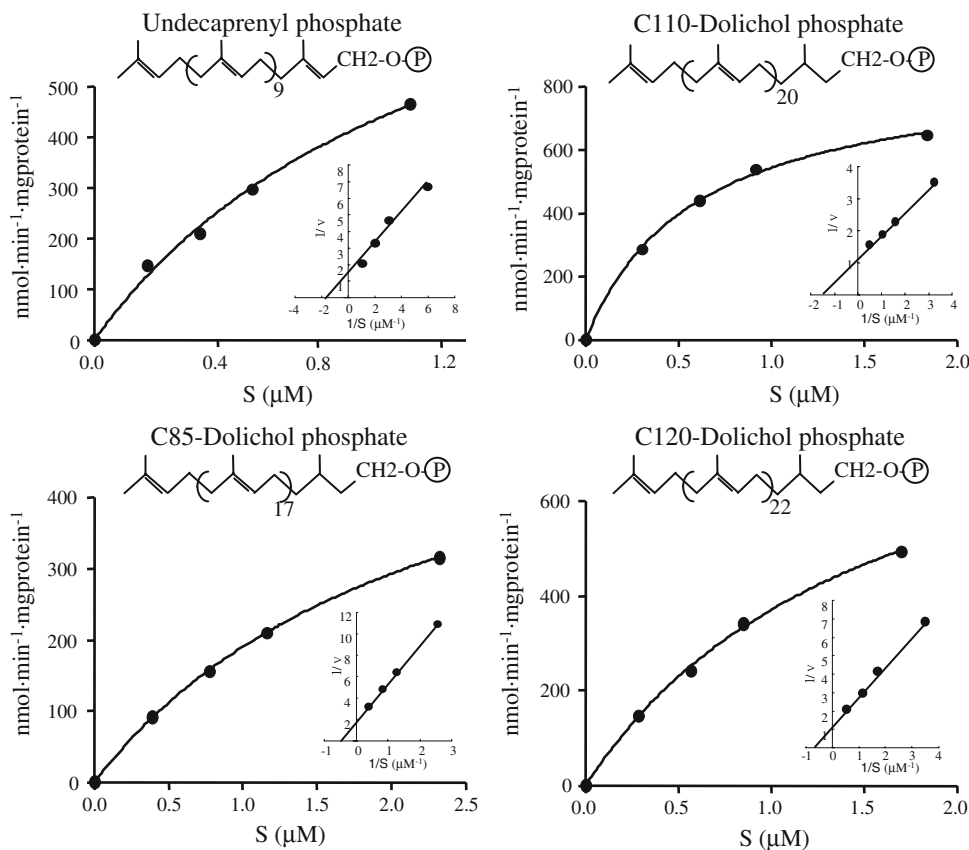
According to the sequence alignment shown in Fig. 1, two aspartate residues of the DXD motif and one aspartate residue corresponding to Asp⁴⁴ of *S. cerevisiae* DPM synthase were putatively identified as candidates for catalytic residues of PH0051p (Asp³⁸, Asp⁸⁹, and Asp⁹¹) (Maeda et al. 2000; Saxena et al. 1995). These aspartate residues of DPM(1-237) were replaced with alanine by site-directed mutagenesis to investigate their function in the catalytic reaction. Four alanine mutants, named D38A, D39A, D89A, and D91A, were produced in *S. cerevisiae* and purified from the solubilized membrane fraction in the

Table 1 Effect of phospholipid on DPM synthase activity

Phospholipid (10 μg)	Relative activity (%)
None	100
Phosphatidylcholine	122
Phosphatidylglycerol	356
Phosphatidylserine	342
Phosphatidylethanolamine	125
Phosphatidylinositol	70

Fig. 4 Kinetic characterization of purified DPM synthase.

Enzyme activities were measured for undecaprenyl phosphate (a), C110 dolichol phosphate (b), C85 dolichol phosphate (c), and C120 dolichol phosphate (d). Undecaprenyl phosphate and dolichol phosphate were dried under gas, and dispersed in the 50 mM Tris-HCl (pH 7.5) containing 1% Triton X-100 by sonication. Enzyme reactions were carried out at 60°C for 5 min. The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.06 μCi GDP-[¹⁴C]-mannose, 77 ng of enzyme and different concentrations of undecaprenyl phosphate or dolichol phosphate. The synthesized undecaprenyl-phosphoryl-[¹⁴C]-Man or Dol-P-[¹⁴C]-Man was extracted with chloroform-methanol, and radioactivity was measured by scintillation counting



same manner as for the wild type (WT) DPM(1-237). Results of the SDS-PAGE of the purified alanine mutants are shown in Fig. 5a. All mutants were stable after heating at 60°C for 10 min, and were purified to homogeneity. The enzyme activity of the purified mutants was measured using C₈₅₋₁₂₀ dolichol phosphate as a substrate at a temperature of 60°C (Fig. 5b). The assay revealed that D38A and D39A showed 5.8 and 2% of the activity of the wild type (WT) DPM(1-237), respectively. On the contrary, D89A and D91A showed 0.09 and 0.17% activity of the WT enzyme. These results indicate that Asp⁸⁹ and Asp⁹¹ are catalytic residues, and Asp³⁸ and Asp³⁹ play important roles in the enzymatic reaction.

Detection of glycoprotein and PH0051p in *P. horikoshii* cells

The glycoproteins from several kinds of archaea have been reported (Upreti et al. 2003; Abu-Qarn and Eichler 2006; Weinberg et al. 2005). Western blotting using alkaline phosphatase-conjugated concanavaline A specific for the α -D-mannosyl residue or α -D-glucosyl residue in N-linked glycoprotein was carried out to confirm whether *P. horikoshii* possesses the ability to synthesize glycoprotein or not (Fig. 6a). The membrane-protein fraction and soluble-protein fraction from *P. horikoshii* cells, cultivated in a chemically defined medium at 90°C for 3 days, were prepared by ultracentrifugation and subjected to Western blot analysis. As shown in Fig. 6a, no detectable protein band was found in the soluble-protein fraction, but a positive band at the position of 75 kDa was detected in the membrane-protein fraction. This result indicated that at least one glycoprotein was located at the cell membrane of *P. horikoshii* as for *P. furiosus* (Weinberg et al. 2005). Next, endogenous DPM synthase activity was checked using the membrane-protein fraction and soluble-protein fraction of *P. horikoshii* cells. The reaction was carried out at 60°C (Fig. 6b). When dolichol phosphate was added to the reaction mixture, a high level of enzyme activity was observed for the membrane-protein fraction. On the contrary, the activity was scarcely detected in the reaction mixture with the soluble fraction and dolichol phosphate. The results indicated that *P. horikoshii* cells possessed the membrane-associated thermostable DPM synthase, and that no activity was detected without addition of the external dolichol phosphate. This might be due to the insufficient amount of endogenous polyisoprenoid in the *P. horikoshii* cell membrane.

We also confirmed that PH0051p was present in the *P. horikoshii* cell membrane by Western blot analysis using anti-DPM(1-237) antiserum (Fig. 6c). One positive band of approximately 35 kDa was found in the membrane fraction, whereas no detectable signal was observed in the

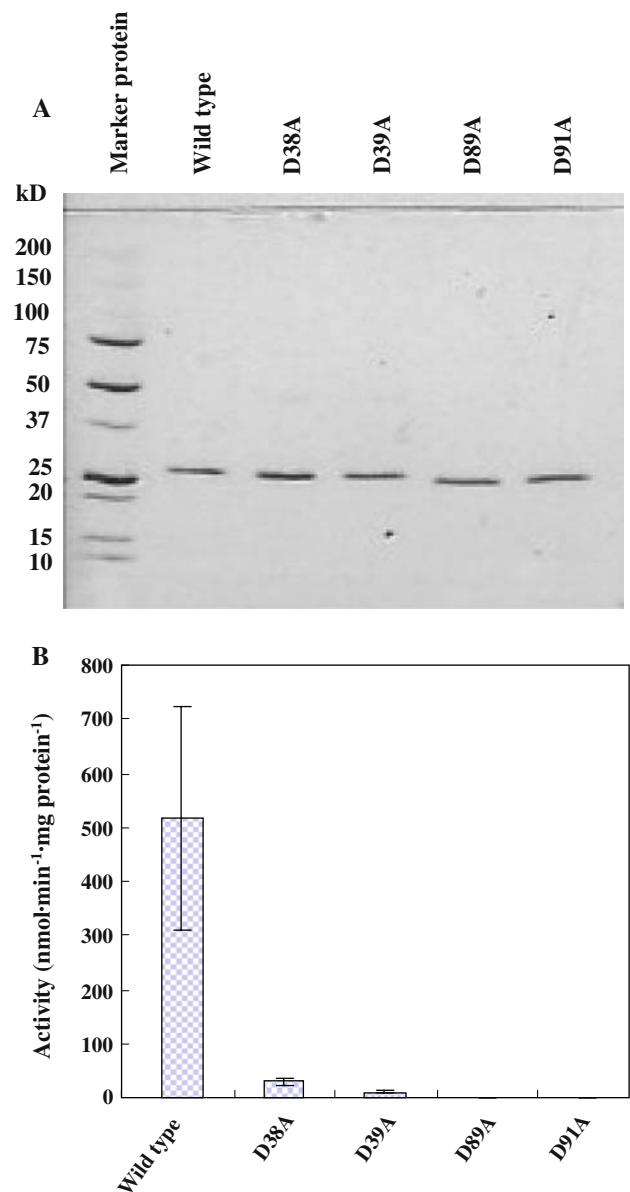


Fig. 5 DPM synthase activity of wild type and alanine mutants of DPM(1-237). **a** SDS-PAGE of the purified DPM(1-237) alanine mutants of DPM(1-237). The expression and purification of alanine mutants were carried out in the same manner as for DPM(1-237). **b** DPM synthase activity of wild type and alanine mutants of DPM(1-237). The purified DPM(1-237) (10 ng), D38A (48 ng), D39A (36 ng), D89A (24 μ g), and D91A (24 μ g) were used as the enzyme solution. The reactions were performed at 60°C. The synthesized Dol-P-[¹⁴C]-Man was extracted with chloroform methanol, and radioactivity was measured by scintillation counting

soluble-protein fraction. The production of PH0051p in the *P. horikoshii* cell membrane was confirmed, although the molecular mass, 35 kDa, was smaller than that calculated. When the purified DPM(1-237) was used as a control, a 25 kDa major band and 50 kDa minor band were detected as shown in the first lane from the right side in Fig. 6c, suggesting DPM(1-237) to be present as a dimer in the

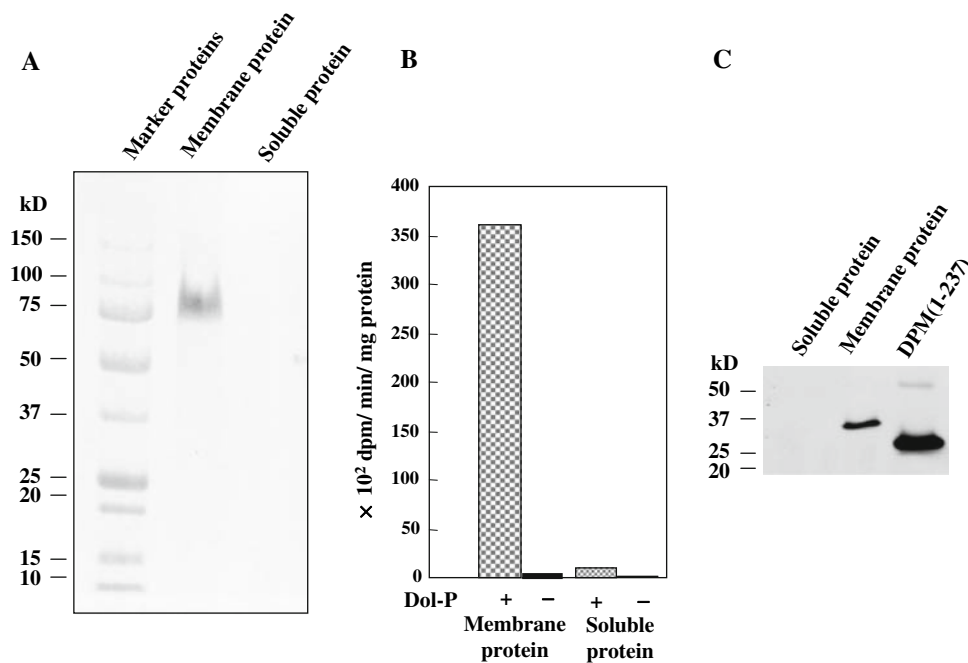


Fig. 6 Detection of glycoprotein and PH0051p in *P. horikoshii* cells. **a** Detection of glycoprotein. *P. horikoshii* cells were disrupted by sonication, and the membrane-protein fraction and soluble-protein fraction were separated by ultracentrifugation. Membrane-proteins or soluble-proteins were separated by SDS-PAGE, and subjected to Western blot analysis using alkaline phosphatase-conjugated

concanavaline A. **b** Endogenous DPM synthase activity in *P. horikoshii* cells. The enzyme activity in the membrane-protein fraction and soluble-protein fraction was assayed at 60°C for 10 min. **c** Detection of PH0051p in *P. horikoshii* cells. Membrane-proteins or soluble-proteins were separated by SDS-PAGE, and subjected to Western blot analysis using anti-DPM(1-237) antiserum

native form. These results indicated that *P. horikoshii* could synthesize glycoprotein using lipid-linked mannose as in the eukaryotic glycosylation system, and that the membrane-associated PH0051p might be a crucial enzyme to produce glycoproteins in *P. horikoshii* cells.

Implication for GtrA-like motif observed at C-terminus of PH0051p

PH0051p consists of two domains; the N-terminal half (a catalytic domain), and the C-terminal half (the hydrophobic domain presumably anchored to the cell membrane). The existence of a hydrophobic domain at the C-terminus suggests that PH0051p is the same DPM synthase as that of *S. cerevisiae*, abbreviated as Sc-DPM. However, the hydrophobic domain of PH0051p is much longer than that of Sc-DPM, and the long stretch of the hydrophobic domain was only found from the archaeal DPM synthases as shown in Fig. 1. The C-terminal domain, DPM(238–353), of PH0051p is not essential for the catalytic reaction as shown in Fig. 2. An analogous result was reported for Sc-DPM. The truncation of the C-terminal 25 residues of Sc-DPM had no effect on enzyme activity and affinity for the dolichol phosphate (Zimmerman and Robbins 1993). The result that the mutant DPM(1-237) truncated at the C-terminal hydrophobic domain could associate with the cell

membrane of *S. cerevisiae*, indicated the possibility that the hydrophobic domain possesses another function. By a computer-assisted motif search (<http://pfam.janelia.org/hmmsearch.shtml>), interestingly, the GtrA-like motif was observed at the C-terminus of PH0051p as shown in Fig. 7. GtrA was discovered from *Shigella flexneri* bacteriophage X as a small highly hydrophobic protein (13.2 kDa) with four putative transmembrane domains (Guan et al. 1999), and was proposed to be a membrane-integrated protein important to translocate undecaprenyl phosphoryl glucose from the cytoplasmic side to periplasmic side through the cell membrane (Korres et al. 2005). An NMR study of the polyisoprenyl recognition peptide suggested that a single polyisoprenole molecule was bound to multimeric recognition peptides which formed a membrane channel (Zhou and Troy 2005). Since DPM(1-237) might be a dimeric molecule in solution as shown in Fig. 6c, the dimeric DPM(238–253) might form a membrane channel to translocate dolichol phosphate mannose as for the polyisoprenyl recognition sequence. Further studies should clarify the real function of the C-terminal hydrophobic domain, DPM(238–353), of PH0051.

As PH0051p and Sc-DPM share the following enzymatic properties; amino acid sequence, catalytic residues, metal ion requirement, substrate specificity, and membrane-associated form, it is conceivable that *P. horikoshii* possesses a

Fig. 7 Comparison of amino acid sequence between GtrA from *Shigella flexneri* bacteriophage X (accession no. Af056939) and DPM(237-353) from PH0051p. Sequences were aligned using Clustal W (<http://clustalw.genome.jp/>). Identical residues are indicated with asterisks

GtrA PH0051 (237-353)	MLKLFVKYTSIGVLNLTLIHVVFVGVCIYAAHTSQALANFTGFVVAVSFSFFANARFTFKA -----KFSIVGLSGVGVNEGFLWLFVNFHHSKELGVIPSTELSLNNFLWDLWTFKD *:::*:..::.::::.*:*..:::.*:*:***
GtrA PH0051 (237-353)	S-----TTAMRYMLYVGFMG-ILSVIVGWAADKCSLPPIVTLITFSAISLVCGFVYSKFI IKKGSVIERLAKFHVAAVFGAVAQFLVYVILLFLGIHYLLANLFGIGASFVVRVYIFNRHI : : ..** : ..** * : : : : .** : : : : *
GtrA PH0051 (237-353)	VFRDAK TWAQ-- .: :

glycoprotein synthetic pathway like that of eukaryotes. Further structure/function studies about Pyrococcal hyperthermostable glycosyltransferases including DPM synthase might provide us novel biotechnological tools to synthesize artificial oligosaccharides useful for glycobiology.

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