Enzyme Discovery

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Functional Assignment of an Enzyme that Catalyzes the Synthesis of an Archaea-Type Ether Lipid in Bacteria**

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The universal tree of life divides all organisms into the three phylogenetic domains eukaryota, bacteria, and archaea.[1] A main difference between these domains is the chemical composition of the lipids forming cellular membranes.[2–5] Phospholipids from bacteria and eukaryota are composed of a *sn*-glycerol-3-phosphate (G3P) core to which fatty acids are bound via ester linkages, while phospholipids from archaea consist of a *sn*-glycerol-1-phosphate (G1P) with two isoprenoid chains attached by ether bonds (Figure 1).

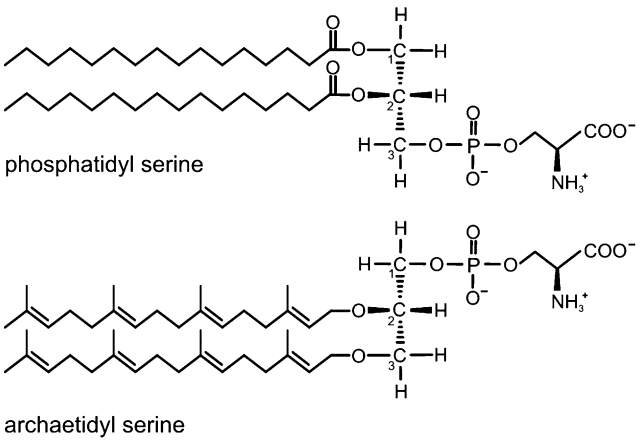

Figure 1. Depiction of phospholipids typical of bacteria and eukaryota (top) and archaea (bottom). In bacteria and eukaryota, G3P is bound to two fatty acids by ester linkages. In archaea, G1P is bound to two isoprenoid derivatives by ether linkages. The main polar head groups (here L-serine) are found in all three phylogenetic domains.

This difference suggests that the emergence of the archaea during evolution was linked to the advent of glycerol-1-phosphate dehydrogenase (G1PDH) and geranylgeranylglyceryl phosphate synthase (GGGPS).[6–9] These consecutively acting enzymes catalyze the first two steps leading to G1P-based ether lipids, which are the reduction of dihydroxyacetone phosphate (DHAP) to G1P and its condensation with the activated isoprenoid geranylgeranyl pyrophosphate (GGPP; 20 carbon atoms), giving rise to geranylgeranylglyceryl phosphate (GGGP). GGGP is then stepwise converted into G1P-based ether lipids, such as the one shown in Figure 1. As G1P and GGGP are considered to be typical of archaea, the discovery of proteins with significant sequence similarities to G1PDH and GGGPS within certain species of the bacteria was unexpected.[6,10] We are interested in identifying the function of these proteins, and recently showed that the AraM enzyme from the gram-positive bacterium *Bacillus subtilis*, which is homologous to the archaeal G1PDH, catalyzes the $NADH^+$-dependent reduction of DHAP to G1P.[11]

We have now deciphered the function of the bacterial PcrB family, whose members are homologues of the archaeal GGGPS. Our approach was based on the ability of the enzyme to convert radioactively labeled G1P with a second, hitherto unknown polyprenyl substrate being provided by *B. subtilis* cells. The characterization of the formed products demonstrates that PcrB catalyzes in vivo the condensation of G1P with heptaprenyl pyrophosphate (HepPP; 35 carbon atoms) to heptaprenylglyceryl phosphate (HepGP). HepGP, which is the first archaea-type G1P-based ether lipid being identified within the phylogenetic domain of the bacteria, was found to be subsequently dephosphorylated and acetylated. Moreover, we show that the different substrate specificities of the archaeal GGGPS and the bacterial PcrB, which bind polyprenyl moieties containing 20 and 35 carbon atoms, respectively, are caused by a single amino acid difference at the bottom of the active site.

The structural superposition of the archaeal GGGPS from *Archaeglobus fulgidus* (afGGGPS) with PcrB from *B. subtilis*, which share a sequence identity of 35%, revealed that the binding pocket for G1P is completely conserved and that GGPP can be modeled into the active sites of both enzymes (Figure 2). This finding motivated us to test whether PcrB is able to catalyze the GGGPS reaction in vitro. For this purpose, the genes coding for PcrB from *B. subtilis* (bsPcrB), *B. anthra*cis (baPcrB), *Geobacillus kaustophilus* (gkPcrB), *Listeria monocytogenes* (lmPcrB), and *Staphylo-*

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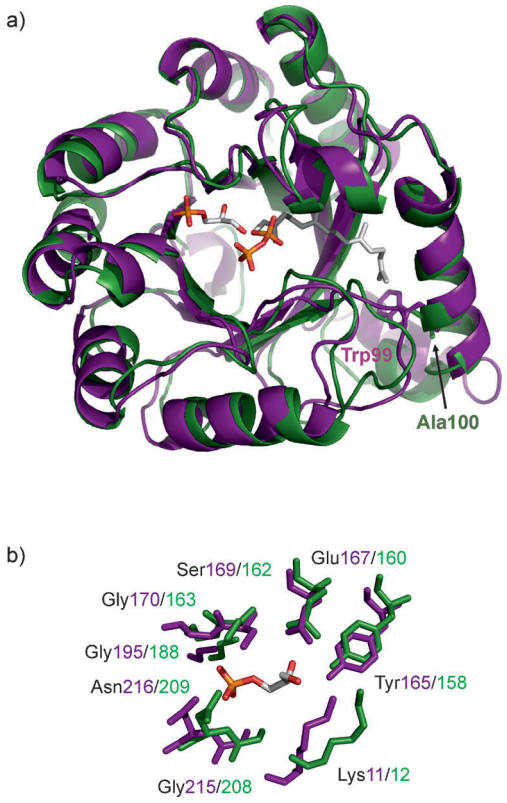


Figure 2. Structural superposition of afGGGPS and bsPcrB with modeled ligands. a) Ribbon diagrams of afGGGPS (PDB code: 2F6U[10]) and bsPcrB (PDB code 1VIZ[12]) are depicted in violet and green. G1P and GGPP are colored according to the CPK convention. The side chain of Trp99 at the tip of the substrate binding groove of afGGGPS limits the length of the polyprenylic side chain to 20 carbon atoms. It is replaced by Ala100 in bsPcrB. b) The G1P binding site is completely conserved between afGGGPS and bsPcrB. G1P-coordinating residues are colored according to (a) and labeled.

coccus aureus (saPcrB) were expressed in *E. coli*, and the recombinant proteins were purified to homogeneity by metal chelate chromatography. Purified PcrB was then incubated with GGPP and ^{14}C-radiolabelled G1P or G3P, which were produced enzymatically from glucose and ATP (Supporting Information, Scheme S1). The analysis of the reaction products by thin layer chromatography (TLC) and autoradiography showed that all tested bacterial PcrB enzymes catalyze the condensation of G1P with GGPP with the same stereospecificity as purified afGGGPS, which served as control (Supporting Information, Figure S1). This result confirms a recent report that PcrB accepts GGPP as substrate in vitro.[13] However, these data do not allow for conclusions about the polyprenyl pyrophosphate substrate used by PcrB in vivo. In the context of this question, it has been noted that afGGGPS contains a conserved tryptophan residue (Trp99) at the tip of its substrate binding pocket. Its bulky hydrophobic side chain has been assumed to limit the size of the polyprenyl moiety to 20 carbon atoms.[10] Remarkably, in the active site of PcrB proteins, the tryptophan is replaced by a small aliphatic residue (in most cases alanine), which indicates that the bacterial homologue of archaeal GGGPS might be able to accommodate longer polyprenyl side chains (Figure 2).

We developed a protocol for the identification of the unknown native polyprenyl substrate of PcrB, which is based on its reaction with ^{14}C-G1P and the subsequent isolation of the radiolabeled product. For this purpose, wild-type *B. subtilis* cells and *ΔpcrB* cells lacking the chromosomal *pcrB* gene[14] were incubated with ^{14}C-G1P and grown overnight. The cells were harvested by centrifugation, and their lipids were extracted and separated on silica TLC plates. The

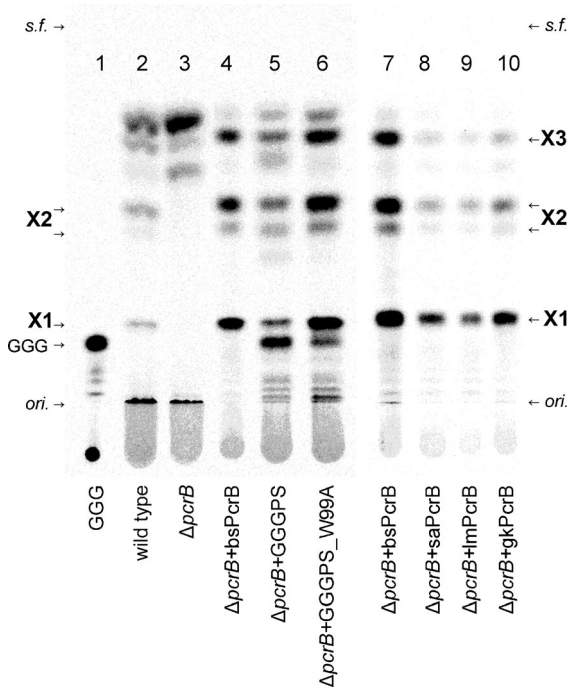


Figure 3. Thin layer chromatographic separation of ^{14}C-labeled glycerolipids being produced in *B. subtilis* by various PcrB proteins and afGGGPS. Cells were grown overnight in the presence of radiolabeled G1P. Lipids were extracted, dephosphorylated to reduce their polarity, separated on silica 60 plates in ethyl acetate/hexane 1:1 (v/v), and autoradiographed. Lane 1: GGG (dephosphorylated GGGP) = reference; lane 2: *B. subtilis* wild-type cells; lane 3: *ΔpcrB* cells; lane 4: *ΔpcrB* cells over-expressing the plasmid-encoded gene for bsPcrB; lane 5: *ΔpcrB* cells over-expressing the plasmid-encoded gene for afGGGPS; lane 6: *ΔpcrB* cells over-expressing the plasmid-encoded gene for the afGGGPS_W99A mutant protein; lanes 7–10: *ΔpcrB* cells over-expressing the plasmid-encoded genes for bsPcrB, saPcrB, lmPcrB, and gkPcrB. Samples 1–6 and 7–10 were run on separate TLC plates. Sample 7 is identical to sample 4 and serves as a reference for lines 8–10. The origin of the TLC spots (*ori.*), the solvent front (*s.f.*), and also spots X1, X2, and X3 are marked with arrows.

comparison of the autoradiograms showed that two faint spots, X1 and X2, generated by *B. subtilis* wild-type cells were missing in the *ΔpcrB* control cells (Figure 3).

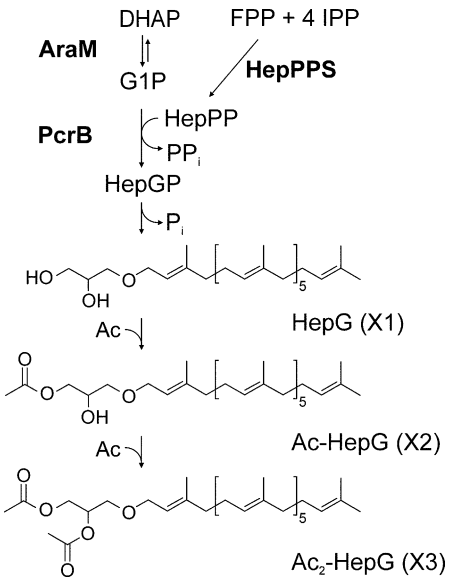
To confirm that PcrB is responsible for the formation of spots X1 and X2, we over-expressed the plasmid-encoded *pcrB* genes from *B. subtilis*, *G. kaustophilus*, *S. aureus*, and *L. monocytogenes* in *B. subtilis ΔpcrB* cells. Lipid extracts from these cells yielded stronger signals for X1 and X2 compared with *B. subtilis* wild-type cells; furthermore, this experiment showed that X2 splits in two spots, for reasons discussed below. Moreover, an additional spot, X3, appeared

which in wild-type cells was too weak to be discriminated from the background signal. The TLC spots X1, X2, and X3 migrate faster than the afGGGPS product GGGP and thus are more hydrophobic than the latter one, which served as control (Figure 3). This finding indicated that the native polyprenyl substrate of PcrB contains more carbon atoms than GGPP.

The *B. subtilis* PcrB products X1, X2, and X3 were isolated and characterized by MS and NMR. To obtain sufficient amounts of the pure substances, we used HPLC instead of TLC for lipid separation. The elution of ^{14}C-labeled X1, X2, and X3 was followed by online scintillation detection (Supporting Information, Figure S2). The experiment was then repeated with non-labeled G1P, and the HPLC fractions containing X1, X2, and X3 were collected on the basis of the retention times recorded with the labeled substances. The analysis of X1 by MS, MS-MS, high-resolution MS, and NMR (Supporting Information, Figure S3–S5) unambiguously identified this compound as heptaprenylglycerol (HepG). We concluded that the second substrate of PcrB besides G1P must be heptaprenyl pyrophosphate (HepPP), and that the reaction product HepGP was subsequently dephosphorylated in vivo. High-resolution MS analysis of X2 showed that its mass is higher than that of X1 by 42.011 Da, which corresponds to a single acetyl group. Depending on whether this group is attached to the C1 or the C2 atom of the glyceryl backbone of HepG, one of two possible isomers is formed, which explains the double spot obtained for X2 (Figure 3). Compared to X2, the mass of X3 is increased by another 42 Da, corresponding to HepG with two acetyl groups. We were able to reproduce the acetylation of X1 in vitro using acetic anhydride, converting it into X2 and X3.

When the plasmid-encoded gene for afGGGPS was over-expressed in the *ΔpcrB* strain, a slower migrating spot appeared which corresponds to GGGP (Figure 3). This result demonstrates that GGPP, although being available in *B. subtilis* cells, is discriminated by all of the tested PcrB enzymes against their preferred substrate HepPP. This result was rationalized by modeling studies, which showed that the long polyprenyl moiety of HepPP fits perfectly into the deep substrate binding pocket of PcrB, and that the end of the polyprenyl chain interacts with several conserved amino acids and a modified loop at the tip of the groove (Supporting Information, Figure S6). The afGGGPS over-expressing strain also generated small amounts of X1, X2, and X3 (Figure 3), indicating that the enzyme can accept polyprenyl substrates with more than 20 carbon atoms, albeit with low propensity. This result suggests that the side chain of Trp99, the assumed ruler to limit substrate size to 20 carbon atoms (Figure 2), may occasionally flip out of the binding groove and thus allow for the binding of longer polyprenyl derivatives. In line with these conclusions, the afGGGPS_W99A mutant generated similar quantities of X1, X2, and X3 as PcrB (Figure 3).

The isolation and identification of X1, X2, and X3, together with the previous finding that AraM is a G1PDH,[11] reveal a hitherto unknown pathway for the biosynthesis of archaea-type ether lipids in gram-positive bacteria. In this pathway, AraM reduces DHAP to G1P, which then reacts at the active site of PcrB with HepPP, giving rise to HepGP and pyrophosphate. Subsequently, HepGP is dephosphorylated and acetylated (Scheme 1).



Scheme 1. A novel biosynthetic pathway for the generation of archaea-type ether lipids in *B. subtilis* and other gram-positive bacteria. G1P is produced by AraM, and HepPP is provided by the HepPP synthase (HepPPS). PcrB catalyzes the reaction of G1P and HepPP to HepGP, which is subsequently dephosporylated and acetylated by hitherto unknown enzymes. The identity of the three shown ether lipids with the TLC spots X1, X2, X3 from Figure 3 is indicated. FPP = farnesyl pyrophosphate, IPP = isopentenyl pyrophosphate, PP_i = pyrophosphate.

The genomes of numerous bacillales code for PcrB proteins (Supporting Information, Figure S7), and our analysis of a representative subset revealed a consistent preference for HepPP as substrate over GGPP (Figure 3). We therefore postulate that all bacterial members of the PcrB family catalyze the same reaction as bsPcrB. In line with this conclusion, all species possessing a *pcrB* gene also contain a gene for HepPPS (Supporting Information, Table S1), which has previously been described as the source for HepPP in the production of menaquinone in gram-positive bacteria.[15] However, not all of those microorganisms have an AraM-like G1PDH, suggesting that G1P might be produced in those species by a non-homologous G1PDH or the stereospecific phosphorylation of glycerol by an unknown kinase.

The functional assignments of AraM and PcrB show that G1P-based polyprenyl ethers do also occur in the bacterial domain of life. However, as HepG and its acetylated derivatives represent only a minor fraction of the total lipid in *B. subtilis* (unpublished data), our findings do not contradict the idea that the occurrence of G1PDH and GGGPS was the key event leading to the emergence of the archaea.[6,8] The nearly exclusive occurrence of PcrB proteins in the bacillales is in favor of a single horizontal gene transfer event from an archaeal species, which was followed by the acquisition of a new substrate specificity (Supporting Information, Figure S7).

We have observed that cells of the *ΔpcrB* knockout strain show a cloggy growth. Moreover, we have noticed that the various HepG derivatives identified in this study are associated with the *B. subtilis* cell membrane, suggesting that they might influence its structural and functional properties. Further experiments are necessary to substantiate this hypothesis and to elucidate the physiological function of the first discovered ether lipids within the phylogenetic domain of the bacteria.

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