Identification and Characterization of Heptaprenylglyceryl Phosphate Processing Enzymes in Bacillus subtilis

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In Archaea, ether lipids play an essential role as the main building blocks of the cellular membrane. Recently, ether lipids have also been discovered in the domain of Bacteria, and the key enzymes that catalyze their synthesis, glycerol 1-phosphate dehydrogenase and heptaprenylglyceryl phosphate synthase, have been described. In Bacillales, heptaprenylglyceryl phosphate does not become linked to a second polyprenyl moiety like ether lipids in Archaea but is dephosphorylated and acetylated. Here, we report on the enzymes that catalyze these reactions. We enriched the phosphatase activity from a B. subtilis cell extract and suppose that dephosphorylation is catalyzed by the phosphatase PhoB or by any other phosphatase in an unspecified manner. By screening a B. subtilis knock-out library for deficiency in acetylation, the yvoF gene product was identified to be the acetyltransferase. The acetyl-CoA-dependent enzyme YvoF is a close relative of maltose O-acetyltransferase (MAT). Its catalytic properties were analyzed and compared with MAT. YvoF and MAT partially overlap in substrate and product range in vitro, but MAT is not able to complement the yvoF knock-out in vivo.

The universal tree of life divides all organisms into the three phylogenetic domains of Eukaryota, Bacteria, and Archaea (1). A main difference between these domains is the chemical composition of the lipids forming their cellular membranes. Although bacterial and eukaryotic membrane lipids are ester lipids with a backbone of glycerol 3-phosphate, the archaean membranes consist of a backbone of glycerol 1-phosphate (G1P) to which isoprenoids, most frequently geranylgeranyl residues, are linked by an ether bond (2, 3). Until a few years ago, the occurrence of G1P-based ether lipids was considered to be unique for the domain of Archaea, and the emergence of the enzyme catalyzing the formation of this ether bond, geranylgeranyl glyceryl phosphate synthase (GGGPS), has been regarded to be a key event in the evolutionary separation between Archaea and Bacteria (4–9). Recently it was shown, however, that a G1P-producing enzyme (10) and polyrenyl glyceryl phosphate synthases exist in Bacteria, too. Although the synthases occurring in some Gram-negative species like Bacteroidetes have GGGPS activity like their archaean orthologues (11), the PcrB enzyme from Gram-positive species like Bacillus subtilis produces ether lipids with seven (heptaprenyl-) instead of four (geranylgeranyl-) isoprenoid units (12). The biochemical properties of these bacterial enzymes have been studied in detail in recent years (11–16), but the biological function of ether lipids in bacteria still remains an enigma.

Obviously, the product of the PcrB reaction in Bacillales, heptaprenylglyceryl phosphate (HepGP), does not become linked to a second polyprenyl moiety in subsequent reaction steps like in Archaea but is dephosphorylated and subsequently acetylated at the two glycerol hydroxyl groups in vivo (12). Such modifications of ether lipids have not been described so far. We have set out to elucidate the identity of the enzymes that catalyze those reactions (Fig. 1). We applied traditional biochemical methods to enrich the phosphatase activity and suppose from our results that HepGP can be dephosphorylated in an unspecified manner by different phosphatases. The screening of a B. subtilis knock-out library for acetylation-deficient strains revealed that YvoF is the acetyltransferase that acts on HepG. A biochemical characterization of YvoF shows that this acetyltransferase is acetyl-CoA-dependent and shows high homology to maltose O-acetyltransferase (MAT). The two homologues partially overlap in their substrate and product range in vitro, but MAT cannot complement the yvoF knock-out in vivo.

Results and Discussion

In a previous study, we have developed a method to visualize the formation of polyprenyl ether derivatives in vivo, based on feeding B. subtilis cells with radiolabeled [14C]G1P (12). The B. subtilis cells thereby provide the polyprenyl pyrophosphate substrate, particularly geranylgeranyl pyrophosphate (GGPP) and heptaprenyl pyrophosphate (HepPP) in about equal amounts. Because a large collection of individual B. subtilis knock-out strains is available from the National BioResource Project (NBRP) in Japan (17), it was apparent to use our methodology and screen this library to identify the phosphatase and acetyltransferase that act in the bacterial ether lipid synthesis pathway (Fig. 1). At the time of this study, single deletion mutants of 2514 out of 4422 (57% coverage of all genes) were available. We searched for all B. subtilis genes that have been annotated as a proved or putative phosphatase/pyrophosphatase (66 genes) and ordered all available knock-out strains at NBRP (26...
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strains. The strains were grown in the presence of \(^{14}\)C-labeled G1P, and lipids were extracted and analyzed by thin layer chromatography. No strain showed an altered lipid composition that would indicate phosphatase deficiency. The reason could be that a knock-out of the distinct phosphatase acting on HepGP was not available or, alternatively, that many phosphatases can complement each other, as discussed later.

As an alternative approach, we purified the HepGP phosphatase activity from \(B.\ subtilis\) wild type cell extracts using various conventional biochemical techniques, namely a combination of ammonium sulfate precipitation, followed by cation exchange chromatography and size exclusion chromatography. After each purification step, fractions were screened for GGGP dephosphorylation activity, and the most active fractions were used for the next enrichment step. We identified the enriched proteins by HPLC-coupled electrospray ionization-mass spectrometry. Among 11 proteins, one phosphatase was found, the alkaline phosphatase PhoB. A knock-out of \(phoB\) was not available from NBRP. We were able to express \(phoB\) heterologously in \(E.\ coli\) cells and tested the dephosphorylation of \(E.\ coli\) cells grown in rich medium, and therefore, we assume that PhoB is among the most abundant phosphatases even under those conditions. Like most other alkaline phosphatases, PhoB can hydrolyze a large number of phosphorylated components (19). The same is the case for calf intestinal phosphatase, which has already been used by others to dephosphorylate ether lipids (20), and therefore it has served as a positive control here. Consequently, it is very likely that HepGP gets dephosphorylated in an unspecified way, either by PhoB or by any other alkaline phosphatase existing in the cell. For that reason, we refrained from investigating the dephosphorylation reaction in further detail.

Identification of the Heptaprenylglycerol Processing Acetyltransferase YvoF—As for phosphatase activity, all \(B.\ subtilis\) genes with an annotation as a (putative) acetyltransferase, deacetylase, acetylase, or phosphotransacetylase were identified, resulting in 63 candidate genes. For 41 of them, knock-out strains were available from NBRP. All those strains were tested in the \(in\ vivo\) radiolabeling assay for their ability to acetylate HepG. Two of the knock-out strains showed an altered thin layer chromatography profile compared with the wild type, with missing spots for single acetylated Ac-HepG and double acetylated \(Ac_2\)-HepG (Fig. 3). In both strains (NBRP-codes MGNA-A332 and MGNA-A391), the same uncharacterized gene, \(yvoD\), is inactivated, which codes for a putative O-acetyltransferase. A \(ΔpcrB\) knock-out strain served as a background reference. It does not produce HepGP, and therefore no acetylated HepG as well (12). A knock-out strain of the \(maa\) gene encoding maltose \(O\)-acyltransferase (MAT) served as a positive control for the \(B.\ subtilis\) strain used at NBRP. As discussed later, MAT is the closest homologue to YvoF in \(B.\ subtilis\).

In the \(B.\ subtilis\) genome, the \(yvoD\) gene is located in one operon together with the genes of a serine/threonine protein kinase/phosphorylase (\(hprK\)), prelipoprotein diacylglycerol transferase (\(lgt\)), a putative integral inner membrane protein (\(yvoD\)), and a pyrophosphatase/glycerol-3-phosphatase (ppaX)
ground spots at low but comparable intensities, because [14C]G1P can go into /H9004 strains, including the background reference 
usuously between them, as discussed in the text (cf. Fig. 1). Please note that all 
strains, including the background reference ΔpcrB, produce some back-
ground spots at low but comparable intensities, because [14C]G1P can go into 
glycolysis and by this way into many cellular compounds (10). One of those 
faint spots superimposes with the Ac2-HepG spot (12).

(21). To enlighten a possible involvement of the products of 
these genes in the processing of heptaprenylglycerol phosphate, 
the corresponding knock-out strains were ordered from NBRP 
and subjected to in vivo labeling experiments as described 
before. No altered thin layer chromatography profile could be 
observed (Fig. 4). Therefore, an essential involvement of these 
gene products in the processing of HepGP is rather unlikely.

Acetyltransferase Activity of YvoF—To identify the localiza-
tion of the YvoF protein in the cell, fractionation experiments 
were performed (Fig. 5). A B. subtilis crude extract was sepa-
rated by centrifugation into a pellet fraction containing cell 
debris and insoluble proteins and a supernatant fraction con-
taining soluble proteins, including membranes. The super-
nant was further fractionated by ultracentrifugation, resulting 
in a supernatant fraction containing soluble proteins only, and 
a pellet fraction containing membranes and membrane pro-
teins only. All fractions were tested for acetyltransferase activity 
by thin layer chromatography. Most of the activity was pres-
ent in the pellet fraction of the crude extract. Only little activity 
was present in the supernatant, which could be concentrated by 
ultracentrifugation, resulting in a significant activity in the 
membrane fraction. No activity was left in the fraction of solu-
ble proteins. This indicates that YvoF is in some way associated 
to the membrane and is not present in the cytosol. Along these 
lines, we obtained only poor yields in our initial experiments to 
express yvoF heterologously in E. coli. Experiments to obtain a 
bigger amount of soluble protein suggested that YvoF is not an 
integral membrane protein, because high salt concentrations 
were sufficient to increase purification yield (Fig. 6). It has been

shown that solubilization by elevated salt concentrations is only possible for peripherally attached membrane proteins or pro-
teins with a lipid anchor but not for transmembrane proteins 
(22). Sequence analysis and comparisons with the related MAT 
protein, from which several structures have been solved (e.g. 
Protein Data Bank codes 3hj, B. anthracis; 1ocx, E. coli (23)),

FIGURE 3. Identification of acetyltransferase-deficient strains. Different 
B. subtilis strains were grown overnight in the presence of radiolabeled G1P. 
Lipids were extracted, separated on Silica 60 plates in ethyl acetate/hexane 
1:1 (v/v) and autoradiographed. Lane 1, B. subtilis WT (positive control); lane 2, 
B. subtilis ΔpcrB (background reference, produces no HepGP and thus no 
HepG); lane 3, B. subtilis ΔyvoF (NBRP code MGGNA-A332); lane 4, B. subtilis 
ΔyvoF (MGGNA-A391); lane 5, B. subtilis Δmao (MGGNA-B865). The origin of chro-
matography as well as spots of HepG, single acetylated Ac-HepG, and double 
acetylated Ac2-HepG are marked with arrows. Two separate spots occur for 
Ac-HepG, because there are two alternative positions for the first acetylation 
on the glycerol backbone. The acetyl group can most likely swap spontane-
ously between them, as discussed in the text (cf. Fig. 1).

FIGURE 4. Testing of yvoF operon-associated genes for deficiencies in 
HepGP processing. Different B. subtilis strains were grown overnight in the 
presence of radiolabeled G1P. Lipids were extracted, separated on Silica 60 
plates in ethyl acetate/hexane 1:1 (v/v), and autoradiographed. Lane 1, B. subtilis 
WT (positive control); lane 2, B. subtilis ΔpcrB (background reference); lane 3, B. subtilis 
ΔyvoF (NBRP code MGGNA-A332); lane 4, B. subtilis 
ΔyvoF (MGGNA-A391); lane 5, B. subtilis Δmao (MGGNA-B865). The samples 
were run on two separate TLC plates. The origin of chromatography as well as 
spots of HepG, single acetylated Ac-HepG, and double acetylated Ac2-HepG 
are marked with arrows.

FIGURE 5. Cellular localization of YvoF. Supernatant (SN) and pellet (P) frac-
tions of a B. subtilis (B. sub.) wild type crude extract were tested for acetyltrans-
ferase activity. The supernatant was further fractionated by ultracentrifuga-
tion (UC). The extracts were incubated with the substrate [14C]GGG for 2 h at 
40 °C. The generated products were extracted, separated by thin layer chro-
matography, and visualized by autoradiography. The origin of chromatogra-
phy (marked by residual GGGP from GGG production) and the product spots 
are marked by arrows. Lane 1, pellet crude extract; lane 2, supernatant crude 
extract; the supernatant was re-centrifuged: lane 3, supernatant after ultra-
centrifugation; lane 4, pellet after ultracentrifugation.

B. sub. wt

P

SN

SN

P

UC

UC

Ac2-GGG

Ac-GGG

GGG

GGGP

(1) (2) (3) (4)
also reveal no indications for YvoF being an integral membrane protein. How YvoF is associated to the membrane remains unknown to date.

We expressed yvoF heterologously in *E. coli* and tested the protein for acetyltransferase activity by incubation of protein samples with the *in vitro* produced substrate [14C]GGG and analysis via thin layer chromatography (Fig. 7). First, we tested crude extracts of *E. coli* expression cultures. No acetyltransferase activity could be observed in the *E. coli* crude extract without overexpressed YvoF (background control). This excludes a disturbing acetyltransferase cross-contamination of purified YvoF from heterologous expression. *E. coli* crude extracts with overexpressed YvoF show significant acetyltransferase activity. However, acetyltransferase activity is lost when testing a purified YvoF sample. This indicates that the second substrate providing the acetyl moiety is present in the *E. coli* cell extract but not in the purified protein solution. When adding 250 µM acetyl-CoA to the assay with purified YvoF, acetyltransferase activity is regained. This demonstrates that acetyl-CoA is the cosubstrate of the YvoF reaction.

Interestingly, the thin layer chromatography profile differs between lane 2 and lane 4 (Fig. 7). In Fig. 7, lane 2, only the monoacetylated Ac-GGG can be found, whereas in lane 4 both products are visible, the mono- as well as the diacetylated GGG. Because we assumed that the generation of mono- and diacetylated product might be dependent on acetyl-CoA concentration, an activity assay with varying acetyl-CoA concentrations was performed (Fig. 8A). With increasing acetyl-CoA concentrations, the diacetylated Ac2-GGG band gets more prominent, but interestingly, the mono-acetylated Ac-GGG band diminishes again after reaching a maximum at lower concentrations of about 5 µM. To gain more insight into the kinetics of mono- and diacylation, the accumulation of the products over time was followed at a constant acetyl-CoA concentration of 250 µM (Fig. 8B). Under those conditions, the formation of the diacylated product is favored at the beginning. During the first 10 min after starting the reaction, only a tiny but constant fraction of Ac-GGG can be detected, although the amount of Ac2-GGG increases. Later than 10 min after starting the reaction, the concentration of monoacetylated product is increasing, although the amount of the diacylated product almost remains constant. We conclude from the two latter experiments that when acetyl-CoA is redundantly available at high concentration (i.e. at the beginning of the reaction shown in Fig. 8B), the second acetylation takes place before the monoacetylated product can diffuse away from the active site of the enzyme. With decreasing concentrations of acetyl-CoA, more monoacetylated product is released before the second acetylation can take place.

As mentioned in the legend to Fig. 3, there are two alternative hydroxyl groups on the glycerol backbone for the first acetylation to take place, giving two spots on the chromatograms in varying intensities. The acetyl group most likely swaps spontaneously between the two hydroxyl groups by acyl migration. This isomerization makes a putative regiospecificity of YvoF irrelevant under physiological conditions and a detailed kinetic study very difficult. Acyl migration has first been described on acylated esters of glycerol (24) and is a well known hindering factor in the isolation, characterization, and synthesis of lipids (25–27).

We used both a DTNB-coupled assay (Fig. 9) and a discontinuous radiometric assay to determine the catalytic parameters of YvoF. The results of both assays are in good agreement (Table 1). The high *Kₘ* value for acetyl-CoA supports that when this substrate is present at low non-saturating concentrations (<<*Kₘ*), Ac-GGG may be released before a second acetylation of the same acceptor molecule can take place (Fig. 8).

**Evolutionary and Functional Relationship between YvoF and Maltose Acetyltransferase (MAT)**—The closest homologue to YvoF is the MAT enzyme, the *maa* gene product (28, 29), which is in turn a close relative of the galactoside O-acetyltransferase, the *lacA* gene product (30). MAT and galactoside O-acetyltransferase acetylate a large variety of hexoses with different efficiencies, with a focus of MAT on glucosides and galactoside O-acetyltransferase on galactosides (23). The structures of MAT from *E. coli* (code 3hij, see Ref. 23) and *Bacillus anthracis* (code 1oxc) have been solved, but the biological functions of MAT and galactoside O-acetyltransferase have not yet been
verified experimentally. It is supposed that they act as detoxifying enzymes at excessive intracellular sugar concentrations, because the acetylated sugars can easily diffuse out of the cell, but are not actively transported back (28, 31). Both enzymes belong to the family of hexapeptide repeat proteins (32). This family includes many acetyltransferases, and they all share a characteristic structural architecture, a large left-handed parallel β-helix that is built from repetitive hexapeptide motifs. The β-helix domain mediates the formation of a trimer, and three molecules of the substrate acetyl-CoA can be bound at the contact interfaces between the protomers. The sequence identity between YvoF and MAT is around 60% within a C-terminal sequence of ~50 amino acids (total sequence length of YvoF, 172) that forms the central part of the β-helix domain, including the (putative) acetyl-CoA-binding site. This is why we suppose that YvoF has the same basic structural features like MAT. However, the N-terminal section of YvoF differs significantly in sequence from known acetyltransferases and might provide the specificity for polyprenylglyceryl substrates, perhaps by controlling the cellular localization of the enzyme.

MAT cannot complement the YvoF function in vivo. The ΔyvoF strains have intact maa genes, but nevertheless they are deficient in HepG acetylation (Fig. 3). However, this might be due to a different cellular localization of YvoF and MAT than due to different substrate ranges of the two enzymes. We provide evidence that YvoF is membrane-associated (Fig. 5) like its homologous MAT protein, and qualitatively tested the overlap of substrate acceptance of YvoF and MAT in vitro, using GGG and maltose.

FIGURE 8. Dependence of product formation by purified YvoF on substrate concentration and time. A, YvoF was incubated with [14C]GGG and increasing concentrations of acetyl-CoA for 2 h at 40 °C. The generated products were extracted, separated by thin layer chromatography, and visualized by autoradiography. B, YvoF was incubated with [14C]GGG and 250 μM acetyl-CoA for different times at 40 °C. The generated products were extracted, separated by thin layer chromatography, and visualized by autoradiography. The origin of chromatography (marked by residual GGGP from GGG production) and the product spots are marked by arrows. The samples were run on two separate TLC plates each.

FIGURE 9. Steady state kinetic measurements of acetyltransferase activity of YvoF. A, 350 nM YvoF was incubated with 0–258 μM of the substrate [14C]GGG and 0–870 μM of acetyl-CoA. Reactions were performed in 50 mM Tris, pH 7.5, 10 mM MgCl2, 0.2% Tween 80 plus 2 mM DTNB at 40 °C and started by adding the enzyme. The increase of absorbance at 412 nm was followed, and reaction velocities were calculated from the protein concentration and the initial slopes. The experiment was done in triplicate; the error bars show standard deviations. Kinetic constants were obtained by fitting the Michaelis-Menten equation to the data, using SigmaPlot 12.0 (Table 1).

TABLE 1

Catalytic parameters of YvoF

The parameters were determined independently with a DTNB-coupled assay in triplicate (given with standard deviations) and a discontinuous radiometric assay without replicates (values in parentheses). Saturation curves of the DTNB assay are shown in Fig. 9.

| Substrate | GGG | Acetyl-CoA |
|-----------|-----|------------|
| K_m (μM)  |      |            |
| k_cat (μM⁻¹ s⁻¹) |    |            |
| k_cat/K_m (μM⁻¹ s⁻¹) |    |            |

| Substrate | GGG | Acetyl-CoA |
|-----------|-----|------------|
| K_m (μM)  |      |            |
| k_cat (μM⁻¹ s⁻¹) |    |            |
| k_cat/K_m (μM⁻¹ s⁻¹) |    |            |

* Values in parentheses are standard deviations.

** Values in parentheses are calculated from k_cat/K_m values with the assumption K_m = 8 μM.

** Because the acetyl-CoA-dependent reaction was assayed under non-saturating concentrations for GGG, k_cat/K_m (AcCoA) could only be estimated from the calculated k_cat and the experimentally determined K_m (AcCoA).
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**FIGURE 10. Overlap of substrate acceptance of YvoF and MAT.** A, GGG acetyltransferase activity of purified YvoF and MAT. Same amounts of protein (5 μM) were incubated with the substrate [14C]GGG for different times at 40 °C. The generated products were extracted, separated by thin layer chromatography, and visualized by autoradiography. The origin of chromatography (marked by residual GGPG from GGPG production) and the product spots are marked by arrows. As negative control (Neg.), the substrate [14C]GGG was incubated solely with acetyl-CoA without enzyme. B, maltose acetyltransferase activity of YvoF and MAT. Same amounts of protein (4 μM) were incubated with two different concentrations of the substrate [14C]maltose (1.25 μM, lanes 4 and 6; and 2.5 μM, lanes 5 and 7) overnight at 40 °C. The generated products were extracted, separated by thin layer chromatography, and visualized by autoradiography. M, [14C]maltose without added enzyme and acetyl-CoA; M(I), [14C]maltose in 50 mM potassium phosphate, no enzyme; M(II), [14C]maltose in 50 mM potassium phosphate, 250 μM acetyl-CoA, no enzyme; M(III), [14C]maltose that has been completely acetylated by incubation with acetic anhydride. The origin of chromatography is marked by an arrow.

as acetyl acceptors (Fig. 10). It is obvious that each enzyme accepts both substrates but with different efficiencies and product specificities. The polyprenylglyceryl acetyltransferase activity of MAT is somewhat lower than that of YvoF under the applied specific conditions (Fig. 10A). In contrast, the maltose acetyltransferase activity of YvoF is about the same as that of MAT, but the product spots obviously differ (Fig. 10B). Without reference substances or detailed analysis, it is impossible to identify them specifically, but we assume that they represent maltose derivatives that are (multiply) acetylated at different hydroxyl groups. MAT is reported to acetylate maltose exclusively at the C6 position of the nonreducing glucose moiety (23). Although these experiments only provide qualitative results, they allow two conclusions. First, YvoF and MAT have only partially overlapping activities and therefore certainly results, they allow two conclusions. First, YvoF and MAT have only partially overlapping activities and therefore certainly serve different cellular functions. Second, because MAT has significant polyprenylglyceryl acetyltransferase activity in vitro, but cannot complement the yvoF knock-out in vivo, it is very likely that YvoF acts in a membrane-associated manner, as indicated by the previous experiments.

**Occurrence of YvoF-like Enzymes**—Finally, we analyzed the species distribution of YvoF enzymes. To this end, we searched the NCBI RefSeq database using BLAST and B. subtilis YvoF as query and visualized the species distribution. Only hits better than an E-value threshold that we set by the best hit with a “maltose acetyltransferase” annotation were included (754 hits). Soon below the threshold, a rapid drop of E-values occurred, and the hits did not cover the whole sequence length of YvoF anymore. This proves that the selected sequences represent almost all YvoF orthologues that are known today. Most hits (95%) were among Bacillales (Bacillaceae, Listeriaceae, and Staphylococcaceae) and Clostridiales. This means that the species distribution of YvoF exactly coincides with that of PcrB, the enzyme that produces HepGP (11), and supports that both enzymes in fact belong to a common biologically relevant pathway. Interestingly, there were a significant number of additional hits among the archaeal Halobacteria, the best ones with 44% sequence identity over the complete sequence length to B. subtilis YvoF, and it will be a challenging task to elucidate their function.

**Conclusion**—In this study, we have identified the catalytic activities that dephosphorylate and acetylate the product of the PcrB reaction, HepGP. Although we have identified the phosphatase PhoB after enriching the HepGP phosphatase activity from a B. subtilis cell extract, we assume that any other phosphatase might catalyze as well dephosphorylation in an unspecified manner. Acetylation, however, is certainly catalyzed by a specific enzyme, YvoF. Although YvoF is closely related to MAT and the two enzymes overlap in their specificities in vitro, MAT cannot complement the yvoF knock-out in vivo. It remains to be clarified how this in vivo specialization is achieved, and one answer might be that the cellular localization of the two enzymes is different, as the YvoF substrate HepG is associated with the cellular membrane. The biological function of acetylated ether lipids in B. subtilis is still unknown. However, the identification of two enzymes involved in their biosynthesis pathway brings us closer to answering this intriguing question.

**Experimental Procedures**

**Cloning**—The yvoF, maa, and phoB genes were amplified by PCR from B. subtilis subsp. subtilis str. 168 genomic DNA. The primers used for cloning were as follows: YvoFfw, 5’-CTCATGAGAAAAACAGATCGTCATCC-3’, and YvoFrev, 5’-TGCGCTGAGTTCAAGGGACTTTTCTGATC-3’; MATfw, 5’-CTCATTATGTCTGAGCAAGAAAAAGAAAAATGGC-3’, and MATrev, 5’-TGCGCTGAGCAATTTGCTCTAAAAATCTTGCGGGGG-3’; PhoBfw, 5’-CTCCATATGAGGTGAAATACTGCAGAGAATCGAATCTG-3’; PhoBrev, 5’-TGCAGCTGCTGTGAAATGCAGG-3’. The PCR fragments of yvoF and maa were cloned via the Ndel/Xhol restriction sites into a derivative of the expression vector pET28a (pET28atrXN), which provides a C-terminal hexahistidine (His<sub>6</sub>) tag for purification plus an N-terminally fused thioredoxin (TrxA) for improved solubility of the recombinant protein (11, 33). The PCR fragment of phoB was cloned via the
Production of Recombinant Proteins—Heterologous expression was performed in the *E. coli* strain BL21-CodonPlus(DE3)-RIPL (Agilent Technologies). For genes cloned into pET28a or NXN, the transformed cells were grown at 37 °C and shaken (150 rpm) in 1-liter volumes of lysogeny broth (LB) containing kanamycin (50 μg/ml) and chloramphenicol (30 μg/ml). When $A_{600}$ reached 0.6–0.8, expression was induced with isopropyl β-D-1-thiogalactopyranoside (1 mM), and growth was continued overnight. For genes cloned into pET21a, the transformed cells were grown as described above in LB containing ampicillin (150 μg/ml) and chloramphenicol (30 μg/ml). When $A_{600}$ reached 0.6–0.8, expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside, and growth was continued for four h.

*muc* and *phoB* Expression, *Expression of yvoF for DTNB-coupled Assay*—After harvesting by centrifugation, cells were resuspended in 50 mM potassium phosphate, pH 7.5, 300 mM KCl, 10 mM imidazole and disrupted by sonication. Cells for *yvoF* expression were resuspended in 50 mM potassium phosphate, pH 7.5, 300 mM KCl, 10 mM imidazole, 10 mM MgCl$_2$, 9 mM CHAPS and disrupted by incubation on ice for 1 h with repeated vortexing. The proteins were purified from the clarified cell extract by metal chelate affinity chromatography. An ÄKTApurifier system with a HisTrap FF crude column (5 ml, GE Healthcare) was used, and a linear gradient of imidazole was applied to elute the protein. Interfering imidazole and salt were removed from the purified proteins by dialysis against 50 mM potassium phosphate, pH 7.5. Protein concentrations were determined either by absorbance spectroscopy ($\varepsilon_{280} = 35,473 \text{ M}^{-1} \text{ cm}^{-1}$ for Trx-YvoF, $\varepsilon_{280} = 27,118 \text{ M}^{-1} \text{ cm}^{-1}$ for Trx-MAT, and $\varepsilon_{280} = 40340 \text{ M}^{-1} \text{ cm}^{-1}$ for PhoB, calculated from the amino acid sequence by ProtParam) or by a Bradford assay (protein assay kit II; Bio-Rad). Protein yields were around 9 mg/liter of culture (MAT), 2 mg/liter (PhoB), and 0.4 mg/liter (YvoF). YvoF in 50 mM potassium phosphate, pH 7.5, was quite unstable and needed to be used immediately. The other proteins could be dropped into liquid nitrogen and stored at −80 °C.

*Expression of yvoF for Other Assays*—YvoF could be more stabilized by adding DTT and NaCl to the buffer. DTT and other reducing agents are incompatible with the DTNB-coupled assay, but YvoF purified as follows was used for all other assays. After harvesting by centrifugation, cells were resuspended in 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole, 10 mM MgCl$_2$, 9 mM CHAPS and disrupted by incubation on ice for 1 h with repeated vortexing. The protein was purified using an ÄKTApurifier system with a HisTrap FF crude column (5 ml, GE Healthcare), and a linear gradient of imidazole (10–500 mM) in 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM MgCl$_2$, was applied to elute the protein. Interfering imidazole and salt were removed from the purified protein by dialysis against 50 mM Tris, pH 8.0, 300 mM NaCl, 10 mM MgCl$_2$, 1 mM DTT at 4 °C. Protein yield was about 0.4 mg/liter culture. Purified protein was dropped into liquid nitrogen and stored at −80 °C.

Because *yvoF* expression yields were poor in standard lysis buffer, we optimized lysis conditions as follows: 5-ml aliquots of a single 50-ml *yvoF* expression culture, 3 h after induction, were harvested and resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole, 10 mM MgCl$_2$) containing either 9 mM CHAPS, 1 mM NaCl, or 0.4 mM Triton X-100. After 1 h of repeated vortexing, one-half of each sample was sonicated, and the other half was directly used for further analysis. The samples were centrifuged, and the supernatants were purified via SpinTrap columns (GE Healthcare), and equal volumes of the eluate were analyzed by SDS-PAGE and blotting on a PVDF membrane. Detection was done with an anti-His$_6$ tag antibody (Roche Diagnostics, product no. 11965085001).

*Enrichment of Phosphatase Activity from *B. subtilis* Cell Extracts*—For purification of phosphatase activity from *B. subtilis* wild type, cells were harvested by centrifugation, resuspended in 1 × PBS, disrupted by lysozyme (300 μg/ml) for 1 h at 40 °C, and sonicated. Ammonium sulfate solution was added slowly at 4 °C to an end concentration of 60%, and the solution was stirred for 30 min on ice. Precipitated proteins were removed by centrifugation (14,000 × g, 15 min at 4 °C), and the remaining soluble proteins containing the phosphatase activity were dialyzed against 50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM MgCl$_2$, and 1 mM DTT. Subsequently, cation exchange chromatography was performed on an ÄKTApurifier system with a Resource S column (1 ml, GE Healthcare). A linear gradient of NaCl (0–1 M) in 50 mM Tris, pH 8.0, 10 mM MgCl$_2$, and 1 mM DT was applied to elute the protein. Interfering salt was removed from the purified proteins by dialysis against 50 mM Tris, pH 8.0, 300 mM NaCl, 10 mM MgCl$_2$, 1 mM DT at 4 °C. After pooling the active fractions, size exclusion chromatography was carried out as last purification step. An ÄKTApurifier system with an S75 column (25 ml, GE Healthcare) was used. Phosphatase activity was assayed after each purification step as described below. Proteins in the active fraction after size exclusion chromatography were identified by electrospray ionization-mass spectrometry (ESI-MS) after a tryptic digest.

*Membrane Fractionation*—The localization of YvoF in *B. subtilis* wild type was determined by ultracentrifugation. A *B. subtilis* crude extract was clarified by centrifugation for 20 min at 4 °C and 4000 × g. Subsequent ultracentrifugation of the cleared extract was done for 1 h at 4 °C and 100,000 × g for membrane fractionation. Supernatant and pellet fractions of each centrifugation step were used as protein samples for activity assays.

*In Vivo Radiolabeling Experiments*—[14C]G1P was synthesized as described by Guidan et al. (12). To test *B. subtilis* 168 wild type and the knock-out strains from NBRP for a loss of function, the cells were grown overnight in 5 ml of lysogeny broth (LB) containing 1 μCi of [14C]G1P (1.34 μCi). To stabilize the knock-out strains, erythromycin (0.8 μg ml$^{-1}$) was added for their cultivation. Cells were harvested by centrifugation and disrupted by lysozyme (300 μg ml$^{-1}$ in 1 × PBS). Lipids were extracted according to the method of Bligh and Dyer (34) as modified by Kates (35), analyzed by thin layer chromatography on Silica 60 plates developed in ethyl acetate/hexane 1:1 (v/v), and visualized with a phosphorimagery system (PerkinElmer Life Sciences).
**HepGP Processing Enzymes in Bacillus subtilis**

**GGGP/GGG-dependent In Vitro Activity Assays**—The use of the natural substrate HepGP or HepG was not feasible in the in vitro assays of PhoB, YvoF, and MAT due to its hydrophobicity and the associated insolubility under physiological conditions. Instead, the shorter substrates GGGP or GGG were used, which are less hydrophobic and more soluble in aqueous solutions. Because of the fact that PcrB also accepts GGPP as substrate, although with less efficacy than HepPP (11, 12), we assumed that YvoF, MAT, as well as PhoB do not exhibit a strict selectivity with respect to the number of polypropenyl moieties of the substrate, too.

To test the activity of PhoB, YvoF or MAT under different conditions, [14C]GGGP and [14C]GGG, respectively, were synthesized, and radiolabeling assays were performed. Briefly, [14C]G1P was produced from [U-14C]glucose as described earlier (12). GGPP (150 μM; Sigma) was incubated with [14C]G1P (1 μCi, 22.3 μM) and GGPS from Archaeoglobus fulgidus (AfGGGPS; 5 μM; purified as described earlier (12)) in 10 mM MgCl₂, 0.02% Tween 80, 5 mM 2-mercaptoethanol, 50 mM Tris, pH 7.5, in a volume of 300 μl for 2 h at 40 °C to produce radiolabeled [14C]GGGGP. If [14C]GGG was needed, it was produced through dephosphorylation by calf intestinal alkaline phosphatase (New England Biolabs) for 1 h at 37 °C. The product was extracted according to the method of Bligh and Dyer (34) as modified by Kates (35). The solvent was evaporated to dryness in a rotary evaporator, and the remaining [14C]GGGP or [14C]GGG was dissolved in 40 μl of chloroform to a concentration of 167.5 μM under the assumption of 100% turnover.

In case of phosphatase, the *B. subtilis* wild type crude extract or samples after chromatographic separation were incubated with [14C]GGG (37.5 nCi, 2.5 μM) in a volume of 100 μl of 50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT for 2 h at 40 °C (equivalent to pH 8.0 at room temperature). In case of YvoF and MAT, conditions were used as follows. For initial activity assays (Fig. 7), [14C]GGG (37.5 nCi, 2.5 μM) was incubated with crude extract or 1 μM purified enzyme in a volume of 100 μl of 50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT for 2 h at 40 °C. For acetyl-CoA-dependent assays (Fig. 8A), [14C]GGG (37.5 nCi, 2.5 μM) was incubated with 1 μM purified enzyme and 0–250 μM acetyl-CoA (Sigma) in a volume of 100 μl of 50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT for 2 h at 40 °C. For time-dependent assays (Fig. 8B), [14C]GGG (37.5 nCi, 2.5 μM) was incubated with 1 μM purified enzyme and 250 μM acetyl-CoA (Sigma) in a volume of 100 μl of 50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT for 0–120 min at 40 °C. For comparison of YvoF and MAT (Fig. 10A), [14C]GGG (37.5 nCi, 2.5 μM) was incubated with 5 μM purified enzyme and 250 μM acetyl-CoA in a volume of 100 μl of 50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT for 0–3 h at 40 °C (Fig. 10B). [14C]maltose (1.25 or 2.5 μM) was incubated with 4 μM purified enzyme and 250 μM acetyl-CoA in a volume of 100 μl of 50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT overnight at 40 °C.

The products were extracted according to the method of Bligh and Dyer (34) as modified by Kates (35), analyzed by thin layer chromatography on Silica 60 plates developed in ethyl acetate/hexane 1:1 (v/v), and visualized with a phosphorimagery system (PerkinElmer Life Sciences).

**Discontinuous Radiometric Kinetic Assay of YvoF**—For acetyl-CoA-dependent kinetics, GGPP (30.8 μM; Sigma) was incubated with [14C]G1P (1.2 μCi, 16.08 μM) and AfGGGPS (1 μM) in 50 mM Tris, pH 7.5, 10 mM MgCl₂, 0.02% Tween 80, 5 mM 2-mercaptoethanol in a volume of 500 μl for 3 h at 40 °C to produce [14C]GGGP. [14C]GGG was produced by dephosphorylation with calf intestinal alkaline phosphatase (New England Biolabs) for 1 h at 40 °C. To initiate the acetylation reaction, YvoF (20 nm) in 50 mM Tris, pH 8.0, 300 mM NaCl, 10 mM MgCl₂, 1 mM DTT containing 0–500 μM of acetyl-CoA in a volume of 300 μl was added. After 2, 4, 6, and 9 min (80 and 120 μM acetyl-CoA) or 1, 3, 4, and 6 min (200–500 μM acetyl-CoA), 200 μl of the sample were removed, and the reaction was stopped immediately by extraction as described above. For GGPP-dependent kinetics, 0–410 μM GGPP (Sigma) were incubated with half-stoichiometric amounts of [14C]G1P and AfGGGPS (1 μM) in 50 mM Tris, pH 7.5, 10 mM MgCl₂, 0.02% Tween 80, 5 mM 2-mercaptoethanol in a volume of 900 μl for 3 h at 40 °C to produce [14C]GGGP. Following dephosphorylation by calf intestinal alkaline phosphatase (New England Biolabs) for 1 h at 40 °C, [14C]GGG was produced. The GGG concentration (0–8.6 μM in the final setup) was calculated in retrospect to account for losses during synthesis (see below). To initiate the reaction, YvoF (5 nm) in 50 mM Tris, pH 8.0, 300 mM NaCl, 10 mM MgCl₂, 1 mM DTT containing acetyl-CoA (1 mM) in a volume of 100 μl was added. After 1, 2, 3, and 5 min, 200 μl of the sample were removed, and the reaction was stopped immediately by extraction. Extraction and analysis of the products by thin layer chromatography was performed as described above. Intensities of the spots were quantified using the software OptiQuant 3.0 (PerkinElmer Life Sciences), and reference spots with known amounts of radioactivity served for calibration. The product concentrations could be calculated from the reaction stoichiometry, and the known specific activity of the initial substrate [U-14C]glucose. The starting GGG concentrations were calculated in retrospect from the total radioactivity per lane. Finally, reaction velocities were calculated from the protein and product concentrations and incubation times. Kinetic constants were obtained by fitting the Michaelis-Menten equation to the data, using SigmaPlot 12.0.

**DTNB-coupled Kinetic Assay of YvoF**—To determine the concentration of the substrate GGG, it was traced with 14C and synthesized similar to that described above. Briefly, [14C]G1P was produced from [U-14C]glucose as described earlier (12). GGPP (0.6 mM; Sigma) was incubated with [14C]G1P (1.4 μCi, 8.4 μM), cold G1P (1.2 mM, Sigma), and GGPS from Chitinophaga pinensis (CpGGGPS; 20 μM; purified as described earlier (11)) in 1100 μl of 50 mM Tris, pH 7.5, 10 mM MgCl₂, 0.2% Tween 80 overnight at 40 °C to produce [14C]GGGP. [14C]GGG was produced through dephosphorylation by adding calf intestinal alkaline phosphatase (New England Biolabs) and incubated for 2 h at 40 °C. To inactivate and remove the proteins, the solution was heated to 95 °C for 5 min and centrifuged for 5 min at 14,000 × g and 4 °C. Five GGPP preparations of this scale were pooled and stored at 4 °C until kinetic analysis. To determine the GGG concentration, GGG was analyzed by thin layer chromatography as described above. The intensity of the GGG spot was quantified using the software OptiQuant 3.0.
(PerkinElmer Life Sciences), and reference spots with known amounts of radioactivity served for calibration. The GGG concentration could be calculated from the known specific activity of the initial substrate [U-¹⁴C]glucose.

DTNB was obtained from Sigma and used without further purification. The DTNB stock solution (10 mM in 50 mM Tris 8.0) was prepared immediately before use and kept on ice in aluminum foil before pipetting into the assay medium. The acetylation reaction was followed by measuring the TNB²⁻ formation at 412 nm (ε₄₁₂ = 14.150 M⁻¹ cm⁻¹) using a V650 spectrophotometer (Jasco; d = 1 cm). Acetyl-CoA (Sigma) was dissolved in water to a concentration of 10 mM and stored at −80 °C. For acetyl-CoA-dependent kinetics, GGG (290 μM), acetyl-CoA (0 – 870 μM), and DTNB (2 mM) were mixed with YvoF (350 nM) in a total volume of 220 μl of 50 mM Tris, pH 7.5, 10 mM MgCl₂, 0.2% Tween 80 and incubated at 40 °C. The reaction was started by adding the enzyme. For GGG-dependent kinetics, GGG (0 – 258 μM), acetyl-CoA (909 μM), and DTNB (2 mM) were mixed with YvoF (350 nm) in a total volume of 220 μl of 50 mM Tris, pH 7.5, 10 mM MgCl₂, 0.2% Tween 80 and incubated at 40 °C. Reaction velocities were calculated from the protein concentration and the initial slopes. Kinetic constants were obtained by fitting the Michaelis-Menten equation to the data, using SigmaPlot 12.0.

Maltose-dependent in Vitro Activity Assay—To test the activity of MAT or YvoF on the substrate maltose, [¹⁴C]maltose was used in radiolabeling assays. Briefly, [U-¹⁴C]maltose (0.15 μCi, 1.25 μM, or 0.3 μCi, 2.5 μM; Hartmann Analytic, Braunschweig, Germany) was incubated with MAT or YvoF (4 μM) in 50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT containing 0.25 mM acetyl-CoA in a volume of 200 μl overnight at 40 °C to produce radiolabeled acetyl-maltose. To exclude that the observed acetyltransferase activity is a background artifact, [¹⁴C]maltose was incubated in 50 mM potassium phosphate, pH 7.5 (0.15 μCi, 1.25 μM), with or without acetyl-CoA as described above. The resulting products were dried in a rotary evaporator, dissolved in 30 μl of water, analyzed by thin layer chromatography on Silica 60 plates developed in acetone/chloroform/methanol/water 15:2:2:1 (v/v/v/v), and visualized with a phosphorimager system (PerkinElmer Life Sciences). As standards, [¹⁴C]maltose in water (2.5 nCi, 8.3 μM) and acetylated [¹⁴C]maltose (1.5 nCi, 1.25 μM) were analyzed. Maltose was acetylated in vitro by incubation in 50 μl of acetic anhydride overnight at room temperature.

Author Contributions—M. L. did all the experimental work, established or refined the experimental setups, and drafted the manuscript. D. P. supervised a part of the experimental work and revised the manuscript. R. S. assisted with advice and revised the manuscript. P. B. conceived the study, coordinated experiments, and wrote the manuscript. All authors read and approved the final manuscript.

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