Bacillus subtilis YngB contributes to wall teichoic acid glucosylation and glycolipid formation during anaerobic growth

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UTP-glucose-1-phosphate uridylyltransferases are enzymes that produce UDP-glucose from UTP and glucose-1-phosphate. In Bacillus subtilis 168, UDP-glucose is required for the decoration of wall teichoic acid (WTA) with glucose residues and the formation of glucolipids. The B. subtilis UGPase GtaB is essential for UDP-glucose production under standard aerobic growth conditions, and gtaB mutants display severe growth and morphological defects. However, bioinformatics predictions indicate that two other UTP-glucose-1-phosphate uridylyltransferases are present in B. subtilis. Here, we investigated the function of one of them named YngB. The crystal structure of YngB revealed that the protein has the typical fold and all necessary active site features of a functional UGPase. Furthermore, UGPase activity could be demonstrated in vitro using UTP and glucose-1-phosphate as substrates. Expression of YngB from a synthetic promoter in a B. subtilis gtaB mutant resulted in the reintroduction of glucose residues on WTA and production of glucolipids, demonstrating that the enzyme can function as UGPase in vivo. When WT and mutant B. subtilis strains were grown under anaerobic conditions, YngB-dependent glucolipid production and glucose decorations on WTA could be detected, revealing that YngB is expressed from its native promoter under anaerobic condition. Based on these findings, along with the structure of the operon containing yngB and the transcription factor thought to be required for its expression, we propose that besides WTA, potentially other cell wall components might be decorated with glucose residues during oxygen-limited growth condition.

The cell envelope of bacteria is composed of several sugar-containing polymers, including peptidoglycan, capsular polysaccharides, and lipopolysaccharide, in gram-negative bacteria and secondary cell-wall polymers such as teichoic acids in gram-positive bacteria (1–5). Secondary cell-wall polymers in gram-positive bacteria can either be complex and made up of different repeating sugar units or more simple glycerol- or ribitol-phosphate polymers that are further decorated with sugar residues (6–8). Under standard aerobic growth conditions, the model gram-positive organism Bacillus subtilis strain 168 produces two different types of teichoic acid. Lipoteichoic acid (LTA) is a polyglycerol phosphate polymer that is linked by the glycolipid anchor diglucosyl-diacylglycerol (GlcC2-DAG) to the outside of the bacterial membrane and further decorated with D-alanine and GlcNAc residues, and wall teichoic acid (WTA) is a polyglycerol phosphate polymer covalently linked to peptidoglycan and decorated with D-alanine and glucose residues (9, 10). Both polymers are made up of glycerol phosphate repeating units but are produced by separate pathways. Whereas LTA is polymerized on the outside of the cell, WTA is polymerized within the cell (7, 8). Under phosphate-limiting growth conditions, at least part of the WTA is replaced with teichuronic acid, a nonphosphate-containing anionic cell-wall polymer (11, 12).

For the synthesis and decoration of bacterial cell-wall polymers, several important enzymes producing nucleotide-activated sugars are required (13, 14). For the decoration of LTA with GlcNAc residues, UDP-GlcNAc is utilized, which is also an essential precursor for peptidoglycan and WTA synthesis (7, 15). UDP-GlcNAc is produced from glucosamine-1-phosphate by the bifunctional enzyme GlmU, which has acyltransferase and uridylyltransferase activity (16). The nucleotide-activated sugar is subsequently used by a multienzyme glycosylation machinery for the modification of LTA with GlcNAc residues on the outside of the cell. For this process, it is thought that the membrane-linked glycosyltransferase CsbB transfers the GlcNAc residue from UDP-GlcNAc onto the lipid carrier undecaprenyl phosphate (C55-P) to generate the lipid-linked sugar intermediate...
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C₅₅-P-GlcNAc (17–19). Next, this intermediate is transferred across the membrane with the aid of the small membrane protein and proposed flippase enzyme GtcA, which belongs to the GtrA protein family (20, 21). The GlcNAc residues are finally added to the LTA polymer by the multimembrane–spanning GT-C-type glycosyltransferase YfH (19).

The glycosylation process of WTA in B. subtilis is much simpler. The glucose residues are attached to the polymer within the cytoplasm of the cell by the glycosyltransferase TagE using UDP-glucose as the substrate (22, 23). The nucleotide-activated sugar precursor UDP-glucose is produced from UTP and glucose-1-phosphate by UTP-glucose-1-phosphate uridylyltransferase (UGPase) enzymes (14). UGPases are widespread in bacteria and are often named GalU. For several gram-positive and gram-negative bacterial pathogens, GalU has been shown to be required for full virulence because of its involvement in biofilm formation and capsule and/or lipopolysaccharide biosynthesis (24–27). A well-characterized GalU equivalent in B. subtilis is GtaB, and the first gtaB mutants were isolated as part of studies investigating phage-resistant B. subtilis strains (10). These studies also revealed that the phage resistance is due to the lack of glucose decorations on WTA (28, 29). In B. subtilis 168, UDP-glucose is required not only for the decoration of WTA with glucose residues but also for the production of glycolipids. An abundant glycolipid found in the membrane is Glc₂-DAG, which serves as the lipid anchor for LTA and is produced by the transfer of two glucose molecules from UDP-glucose onto the membrane lipid DAG by the glycosyltransferase UgtP (or sometimes also named YpfP) (30–33). Several independently obtained B. subtilis gtaB mutants have now been characterized. These mutants are resistant to certain types of phages, display morphological and growth defects, lack glucose decorations on WTA, and are unable to produce glycolipids under standard laboratory growth conditions (31, 34). Furthermore, no UGPase activity could be detected in lysates prepared from gtaB mutant strains, indicating that GtaB is the sole functional UGPase in B. subtilis under these conditions (28). However, apart from GtaB, two other predicted UGPases, YtdA and YngB, are encoded in the B. subtilis 168 genome; as part of this study, we investigated the function of YngB (35, 36).

The yngB gene is thought to be part of the yngABC operon (35), but an additional internal promoter appears to be present in yngB driving yngC expression. Furthermore, expression of yngC has been reported to be under control of a sigma M-dependent promoter (37). YngA belongs to the GtrA protein family and could therefore, similar as proposed for the GtcA enzyme involved in the LTA glycosylation process, function as flippase enzyme and be required for the transfer of a sugar-linked C₅₅-P lipid intermediate across the membrane. YngC is a membrane protein belonging to the DdeA family of proteins. The function of the protein is not clear, but proteins belonging to this family have been shown to be important for multiple processes in other bacteria, including cell division, membrane composition, and antibiotic resistance (38–40). The transcription of the yngABC operon is activated by the transcription factor YclJ, which forms a two-component system (TCS) with the histidine sensor kinase YclK (35, 41, 42). The biological role of the YclJ-YclK TCS remains unknown, but it has been shown that the yclJK regulon is upregulated during low oxygen conditions (43, 44). Hence, the proposed UGPase YngB could potentially function under oxygen-limiting conditions. However, the expression might be more complex as the expression of the yclJK genes in turn is controlled by the ResDE TCS, a signal transduction system known to play a key role in the expression of both aerobic and anaerobic respiration-related genes in B. subtilis (45, 46).

In this study, we aimed to determine if YngB is a functional UGPase enzyme and to provide insight into its biological function. Based on its structure and in vitro enzymatic activity, we showed that YngB is a functional UGPase. YngB can also function in vivo as UGPase, leading to the decoration of WTA with glucose residues and glycolipid production in the absence of GtaB, when expressed from a synthetic promoter. YngB-dependent glycosylation of WTA and glycolipid production was also observed when B. subtilis was grown under anaerobic fermentative growth condition. This revealed that besides GtaB, YngB is expressed from its native promoter under anaerobic growth conditions and functions as UGPase. Based on these findings and previous reports on the transcription control of the yngABC and other operons in B. subtilis, the potential decoration of other cell wall structures with glucose residues during an oxygen-limited growth condition will be discussed.

Results

The crystal structure of the B. subtilis YngB protein reveals a putative UDP-glucose binding site

Three paralogous UGPases, GtaB (BSU_35670), YtdA (BSU_30850), and YngB (BSU_18180) are encoded in the genome of B. subtilis strain 168 (Fig. S1). GtaB has been characterized as a UGPase, and in its absence, B. subtilis lacks glucose decorations on WTA and is unable to produce glycolipids during vegetative growth (28, 31). Here we set out to determine whether YngB is a bona fide UGPase and to investigate its biological function. An amino acid sequence alignment of the B. subtilis proteins GtaB, YngB, and YtdA with the UGPase enzymes A4JT02 from Burkholderia vietnamiensis (PDB code: 5i1f), GalU_Hp from Helicobacter pylori (PDB code: 3juk), and GalU_Cg from Corynebacterium glutamicum (PDB code: 2pa4), for which structures with bound UDP-glucose are available (47, 48), revealed that most of the UDP-glucose binding residues (Fig. S1, colored in yellow) and the metal-chelating residue (Fig. S1, colored in cyan) are conserved in the 3 B. subtilis proteins (14, 47, 48). To determine the crystal structure of YngB, selenomethionine-substituted protein was produced in Escherichia coli and purified as a C-terminal His-tag fusion protein. The protein crystallized as a dimer in the asymmetric unit and the structure was solved at 2.80 Å by experimental phasing (Fig. 1A and Table 1). YngB displayed a Rossman fold with alternating α-helices and β-strands, which is commonly found in nucleotide-binding proteins (49). More specifically, the YngB monomer contains a central β-sheet surrounded by α-helices (47, 50).

Two examples of RNA-editing enzymes are shown in the Supporting Information. The first example is the RNA editing of the HIF-1α gene in response to hypoxia, which results in a change from a C to a T at position 4. The second example is the RNA editing of the CREB3L2 gene, which results in a change from a G to an A at position 71. These changes in the RNA sequence result in changes in the translation of the corresponding protein, with potential effects on the function of the protein.
Similar to the dimer interactions described for homologous UGPases, hydrogen bonds are formed between Tyr102 residues on the two β-strands located at the interface of the monomers, producing an extended central β-sheets spanning across the two subunits (50, 51). The overall structure of YngB is very similar to homologous UGPases including A4TT02, GalUHp and GalUCg as shown by superimposition (Fig. S2). The outermost C-terminal α-helix is one of the most variable regions and absent either in GalUHp or in a different conformation in GalUCg (Fig. S2). To locate the substrate-binding site, YngB and UDP-glucose–bound H. pylori GalUHp structures (PDB code: 3juk) (47) were overlayed. The structures superimposed with an RMSD of 1.61 Å and the putative UDP-glucose binding pocket could easily be identified in YngB (Fig. 1, B and C). Based on this alignment, it can be predicted that residues Gly110, Gln105, Ala13, Gly14, Glu29, and Lys28 in YngB interact with the uridine moiety; Asp133 chelating the magnesium ion; Asp134, Val 204, Gly172, and Glu191 interacting with the glucose moiety; and Lys 192 interacting with the diphosphate moiety.

### YngB shows UGPase activity in vitro

To determine if YngB has UGPase activity *in vitro*, enzyme assays were preformed using a method previously described for assessing the UGPase activity of the GalU enzyme from *Erwinia amylovora* (52). For this assay, purified proteins are incubated with α-glucose-1-phosphate (Glc-1-P), and UTP and active UGPases will convert these substrates into UDP-glucose and pyrophosphate. The generated pyrophosphate is then hydrolyzed by a pyrophosphatase to two molecules of inorganic phosphate, which is quantified calorimetrically. To assess the enzymatic activity of YngB, assays were performed with recombinant YngB protein using recombinant GtaB protein as control, and increasing concentrations of Glc-1-P with recombinant YngB protein using recombinant GtaB to assess the enzymatic activity of YngB, assays were performed using a method previously described. 

| Statistics in brackets refer to the highest resolution shell. |  |
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**Figure 1. YngB crystal structure and proposed UDP-glucose binding site residues.** A, crystal structure of YngB shown in cartoon representation. The protein crystalized as a dimer in the asymmetric unit, and individual monomers are shown in orange and cyan, respectively. B, overlay of the B. subtilis YngB (cyan) and the UDP-glucose–bound *Helicobacter pylori* GalUHp (3juk) (gray) structures. The structures are shown in ribbon representation, with UDP-glucose-binding residues shown in stick representation. C, enlarged view of the substrate binding site from the alignment shown in panel B. The magenta sphere represents a magnesium ion; ionic interactions with the magnesium ion and hydrogen bonds are shown as dashed lines. Proposed UDP-glucose binding residues in YngB are Gly110, Gln105, Ala13, Gly14, Glu29, and Lys28 interacting with the uridine moiety; Asp133 chelating the magnesium ion; Asp134, Val 204, Gly172, and Glu191 interacting with the glucose moiety; and Lys 192 interacting with the diphosphate moiety.
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Figure 2. Enzyme activity of GtaB and YngB. A–B, Michaelis–Menten kinetics using purified GtaB or YngB proteins and A, increasing concentrations of the substrate glucose-1-phosphate (Glc-1-P) or B, increasing concentrations of the substrate UTP. The number of UDP-glucose formed per molecule of GtaB or YngB per second is plotted versus substrate concentration. The measured apparent \( K_m \) values for glucose-1-phosphate were 45.6 ± 3.24 \( \mu \)M (GtaB) and 42.1 ± 20.2 \( \mu \)M (YngB) and for UTP, 49.5 ± 10.2 \( \mu \)M (GtaB) and 62.9 ± 13.8 \( \mu \)M (YngB), respectively. The experiment was performed three times with technical replicates. A representative graph from one experiment and plotting the mean and SD from the technical replicates is shown. The SD values for some data points were too small to be displayed on the graph. Michaelis–Menten curves were produced with Prism, and \( K_m \) values given are the mean ± SD from the three independent experiments.

In B. subtilis strain 168, glucose is transferred onto WTA by the glycosyltransferase TagE using UDP-glucose as substrate (23). Under standard aerobic growth conditions, GtaB appears to be the only enzyme that produces UDP-glucose, as glucose is absent from WTA in gtaB mutant strains (28, 34, 53). This is somewhat at odds with our data showing that B. subtilis YngB protein is a functional UGPase enzyme. Possible explanations could be that YngB uses a different sugar or nucleotide as substrate in vivo or that it is not expressed under standard aerobic growth conditions. To address these issues, the yngB gene or, as a control, the gtaB gene, was placed under control of the synthetic IPTG-inducible Phyper-spank promoter (short P_hyper promoter) and introduced into the chromosome of the \( \Delta \)gtaB single-mutant and the \( \Delta \)gtaB\( \Delta \)yngB double-mutant strain. The presence of glucose on WTA in the different B. subtilis strains was initially assessed by fluorescence microscopy after staining the bacteria with fluorescently labeled concanavalin A, a lectin that specifically binds to terminal glucose residues (34). The different B. subtilis strains were grown aerobiologically in a medium supplemented with IPTG, and culture samples were taken at the mid-log growth phase for microscopy analysis. A fluorescence signal was observed for the WT and \( \Delta \)yngB single-mutant strain but as expected was absent from the \( \Delta \)gtaB single- and the \( \Delta \)gtaB\( \Delta \)yngB double-mutant strains (Fig. 3). Consistent with previous observations, \( \Delta \)gtaB mutant bacteria displayed morphological defects and the cells were curved and showed some bulges, which was also seen for \( \Delta \)gtaB\( \Delta \)yngB double-mutant cells (Fig. 3).

Expression of either yngB or gtaB from the inducible promoter (P_hyper) in the \( \Delta \)gtaB single- or \( \Delta \)gtaB\( \Delta \)yngB double-mutant strains fully or at least partially complemented both phenotypes; the cells showed again binding to the fluorescent lectin, and bacteria expressing GtaB had a normal rod-shaped morphology and the bacteria expressing YngB showed an improved cell morphology and the cell were less curved (Fig. 3). These results indicate that YngB is not expressed from its native promoter under standard aerobic growth conditions; however, when expressed from an inducible promoter, YngB can function as UGPase in vivo. To further confirm that YngB expression leads to the decoration of WTA with glucose residues, WTA was isolated from WT, the \( \Delta \)gtaB mutant, and strains \( \Delta \)gtaB P_hyper-gtaB and \( \Delta \)gtaB P_hyper-yngB and analyzed by NMR and LC-MS. The \( ^{1} \)H NMR spectrum obtained for WTA isolated from the WT strain revealed a peak at 5.2 ppm likely derived from the hydrogen atom at the anomeric carbon of the glucose residue on WTA (23) (Fig. 4A). This peak was absent in the sample derived from the \( \Delta \)gtaB mutant strain but could again be detected in samples isolated from strains \( \Delta \)gtaB P_hyper-gtaB and \( \Delta \)gtaB P_hyper-yngB (Fig. 4A), indicating that WTA is indeed decorated with glucose residues upon expression of YngB from an inducible promoter. For the LC-MS analysis, the purified WTA was hydrolyzed with hydrogen fluoride and the depolymerized species were characterized. A species with a mass of 253.09 (m/z) corresponding to a glycerol-glucose repeating unit was detected for samples derived from the WT as well as strains \( \Delta \)gtaB P_hyper-gtaB and \( \Delta \)gtaB P_hyper-yngB (Fig. 4B) but was absent from the \( \Delta \)gtaB sample (Fig. 4B). These data highlight that YngB can function as UGPase in vivo and produce UDP-glucose, which can subsequently be used for the decoration of WTA with glucose moieties.

Expression of YngB from an inducible promoter leads to the formation of glycolipids

UDP-glucose is also used for the production of glycolipids in B. subtilis and the glycosyltransferase Ugtp transfers one or more glucose moieties onto the membrane lipid DAG (30, 32). It has been reported that glycolipids are absent in B. subtilis gtaB mutant strains (31, 54); however, our data suggest that expression of YngB in gtaB mutant strains should restore...
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In previous work, it has been shown that expression of genes in the yngABC operon is upregulated by the transcription activator YclJ (35). Expression of yclJ itself is under control of the transcription factor ResD, which is produced under oxygen-limitation conditions (42–44). It is therefore possible that YngB is expressed from its native promoter under anaerobic growth conditions through a pathway involving ResD and YclJ. To determine if YngB is expressed from its native promoter under anaerobic growth condition and contributes to the decoration of WTA with glucose molecules and glycolipid production, the WT *B. subtilis* strain 168 and the isogenic ΔgtaB and ΔyngB single- and ΔgtaBΔyngB double-mutant strains were grown in an anaerobic chamber under fermentative growth condition. The presence of glucose on WTA and the production of glycolipids were assessed by fluorescence microscopy and TLC analysis as described above. Clear fluorescence signals were observed for the WT and ΔgtaB mutant and ΔyngB mutant strains under anaerobic fermentative growth conditions, indicating that WTA is also decorated with glucose residues during anaerobic growth (Fig. 6A). Only cells of the ΔgtaBΔyngB double mutant were no longer stained (Fig. 6A). These data suggest that under these growth conditions, YngB is produced from its native promoter and functions as a second UGPase enzyme next to GtaB. Consistent with the fluorescence microscopy data, glycolipids could be detected by thin layer chromatography in WT, ΔgtaB and ΔyngB single mutant strains, but not in the ΔgtaBΔyngB double mutant after growth under anaerobic, fermentative growth condition (Fig. 6B). Some differences in the glycolipid profiles were observed for lipid samples isolated...
from the WT after growth under aerobic or anaerobic conditions (Fig. 6B). A number of the slower migrating glycolipids were absent in samples derived from the anaerobically grown cultures; however, bands likely corresponding to Glc2-DAG and Glc3-DAG were present in both samples (Fig. 6B). Furthermore, a reduced glycolipid signal was observed for the ΔgtaB mutant compared with the WT and ΔyngB mutant strain (Fig. 6B). This is consistent with the microscopy results and the observation that only some but not all of the ΔgtaB mutant cells seemed to contain glucose decorations on their WTA. Taken together, the data show that GtaB is the main UGPase enzyme producing UDP-glucose under both aerobic and anaerobic fermentative growth conditions. Furthermore, our data show that YngB is a functional UGPase that augments
UDP-glucose production under oxygen-limiting conditions. Under these conditions, it functions together with GtaB to produce UDP-glucose, for the production of glycolipids and the decoration of WTA with glucose residues and as discussed below potentially also other cell wall structures.

Discussion

In bacteria, nucleotide-activated sugars are key sugar donors for glycosylation processes (52, 55). In the gram-positive, spore-forming bacterium *B. subtilis*, one of the best characterized nucleotide-activated sugar-synthesizing enzymes is GtaB. Up to now, all UDP-glucose produced in *B. subtilis* has been attributed to the activity of GtaB despite the presence of two orthologous proteins, YngB and YtdA. Here we show that, based on its crystal structure and *in vitro* biochemical activity, the *B. subtilis* YngB protein is a functional UGPase (Figs. 1 and 2). The necessary glucose-1-phosphate and UTP substrate-binding residues could be identified in the YngB structure (Fig. 1), and it is likely that YngB synthesizes UDP-glucose via the same catalytic mechanism as proposed for other members of this family (47, 48, 52).

The main reason why all UDP-glucose production in *B. subtilis* has been attributed to GtaB is likely due to the specific growth conditions or developmental stages in which YngB and YtdA are produced. We present here experimental evidence that YngB is produced and can synthesize UDP-glucose when bacteria are grown under anaerobic conditions (Fig. 7). However, even under these conditions, GtaB appears to remain the main UGPase, as YngB activity could only be revealed in a gtaB mutant strain (Fig. 7).

Table 2

| Possible fatty acid chain length | Chemical formula | Predicted molecular mass | WT | ΔgtaB Phyper-gtaB | ΔgtaB Phyper-yngB |
|---------------------------------|------------------|--------------------------|----|------------------|------------------|
| Top band: Glc2-DAG              |                  |                          |    |                  |                  |
| (30:0)                          | C_{45}H_{84}Na_{1}O_{15} | 887.57                  | 887.51 | 887.94           | 887.90           |
| (31:0)                          | C_{46}H_{86}Na_{1}O_{15} | 901.59                  | 901.95 | 901.97           | 901.81           |
| (32:0)                          | C_{47}H_{88}Na_{1}O_{15} | 915.60                  | 915.95 | 915.87           | 915.91           |
| Mid band: Glc3-DAG              |                  |                          |    |                  |                  |
| (30:0)                          | C_{51}H_{94}Na_{1}O_{20} | 1049.62                 | 1049.85 | 1049.90          | 1049.77          |
| (31:0)                          | C_{52}H_{96}Na_{1}O_{20} | 1063.64                 | 1063.85 | 1063.90          | 1063.93          |
| (32:0)                          | C_{53}H_{98}Na_{1}O_{20} | 1077.65                 | 1077.85 | 1077.38          | 1077.90          |

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spores; however, no clear phenotype could be identified for a \textit{ytdA} mutant strain (36). Therefore, similar as done here, to reveal a function of YtdA as UDPglucose-phosphorylase (UGPase), its activity and contribution to the production of the polysaccharide layer on spores might need to be assessed in a \textit{gtaB} mutant strain.

Under aerobic growth condition, an aberrant morphology was observed for the \textit{ΔgtaB} mutant; in contrast to rod-shaped WT bacteria, the mutant cells were curved (Fig. 3), consistent with previous observations (31, 53, 57). It is thought that the aberrant morphology is due to the lack of glycolipids, rather than the absence of glucose residues on WTA. This is based on the observation that an \textit{ugtP} (\textit{ypfP}) mutant unable to produce glycolipids but still containing glucose residues on WTA has an aberrant morphology, whereas a \textit{tagE} mutant lacking glucose decorations on WTA but producing glycolipids does not display morphological defects (31, 53, 57). Consistent with these findings, the \textit{ΔgtaBΔyngB} double-mutant strain, which is also unable to produce glycolipids, showed similar morphological defects under aerobic growth conditions (Figs. 3 and 5). Interestingly, no aberrant morphology was observed for \textit{ΔgtaB} or \textit{ΔgtaBΔyngB} mutant bacteria when grown under anaerobic growth conditions and both strains produced short, rod-shaped cells (Fig. 7). Under these growth conditions, the \textit{gtaB} mutant is able to produce glycolipids, be it at reduced levels, whereas the \textit{ΔgtaBΔyngB} double mutant is unable to synthesize glycolipids (Fig. 7). These data not only show that YngB contributes to UDP-glucose production under anaerobic growth conditions, but they also indicate that the production of glycolipids is not essential for cells to maintain their normal rod shape under anaerobic growth conditions.

The finding that YngB contributes to the production of UDP-glucose under anaerobic growth conditions is consistent with reports on its expression control. Transcription of the \textit{yngABC} operon has been reported to be activated by YclJ, a transcription factor that is part of the YclJK TCS, whose expression itself is upregulated during oxygen limitation by the ResDE TCS (35, 42, 44). Previous studies have revealed the genes and operons regulated by YclJ, which include, besides the \textit{yngABC} operon, the two genes of the \textit{ykcBC} operon (35, 42) (Fig. 8A). Although we show here that the UDP-glucose produced by YngB can be utilized for the glucosylation of WTA and glycolipid production under anaerobic growth, given the predicted function of the proteins encoded by the \textit{yngABC} and \textit{ykcBC} operons, we speculate that the UDP-glucose produced by YngB could potentially be utilized for the glucosylation of other extracellular cell-wall components (Fig. 8). As outlined in detail below, this hypothesis is based on the observed similarities of the enzymes encoded in the \textit{yngABC} and \textit{ykcBC} operons to a multicomponent transmembrane glycosylation system required for the extracellular glycosylation of LTA. In \textit{B. subtilis}, WTA is glycosylated intracellularly by TagE, which adds the sugar residue directly onto the WTA backbone using a nucleotide-activated sugar as precursor, whereas LTA is glycosylated extracellularly using a

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\textbf{Figure 7. Detection of glucose modification on WTA and glycolipid analysis of WT and mutant \textit{B. subtilis} strains after growth under anaerobic conditions.} \textit{WT} \textit{B. subtilis} (WT P\textsubscript{hyper}), the \textit{gtaB} and \textit{yngB} single- and double-mutant strains \textit{ΔgtaB P\textsubscript{hyper} ΔyngB P\textsubscript{hyper}} and \textit{ΔgtaBΔyngB P\textsubscript{hyper}} were grown in an anaerobic chamber under fermentative growth condition on 2 x YT plates. \textbf{A}, bacteria were prepared for microscopy analysis and stained with the fluorescently labeled Alexa Fluor 594 concanavalin A lectin to detect glucose modifications on WTA as described in the \textit{Experimental procedures} section. The experiment was performed 3 times and representative phase-contrast, fluorescence, and merged images are shown for each strain. Scale bars represent 10 μm. \textbf{B}, total membrane lipids were isolated from the different \textit{B. subtilis} strains after growth in an anaerobic chamber, separated by TLC and glycolipids visualized by spraying the plates with α-naphthol and 95% sulfuric acid and heating. As control, a lipid sample isolated from the WT strain grown aerobically was run alongside the other samples. The experiment was performed 3 times, and a representative TLC plate is shown. WTA, wall teichoic acid; YT, yeast extract tryptone.
multicomponent transmembrane glycosylation system (21). Glycosylation of LTA starts when a nucleotide-activated sugar, in the case of *B. subtilis*, UDP-GlcNAc, is linked by the glycosyltransferase CsbB to the C55-P lipid carrier in the cytosol of the cell (Fig. 8B). The C55-P-sugar intermediate is subsequently flipped across the membrane, likely by the GtrA-type membrane protein GtcA, and the sugar is finally added onto the LTA polymer on the outside of the cell by the multimembrane–spanning GT-C–type glycosyltransferase YfhO (Fig. 8B) (19–21). Proteins with similarity to CsbB, GtcA, and YfhO are encoded in the YclJ-controlled yngABC and ykcBC operons. YkcC shows similarity to the glycosyltransferase CsbB and could therefore function to produce a C55-P-sugar intermediate (Fig. 8C). YngA is similar to GtcA, which is predicted to mediate the transport of C55-P-sugar intermediates across the membrane (Fig. 8C). Finally, YkbC is predicted to be a multimembrane spanning GT-C-fold glycosyltransferase similar to YfhO and could therefore transfer the sugar residue from the exported C55-P-sugar intermediate onto an extracellular cell wall component (Fig. 8C). By analogy to the function of CsbB, GtcA, and YfhO, we therefore speculate that YkcC, YngA, and YkbC constitute a multicomponent transmembrane glycosylation system that adds sugar decorations onto a cell wall component on the extracellular side of the membrane (Fig. 8C). We have shown here that YngB is a functional UGPase; hence, we speculate that UDP-glucose is the likely substrate for the proposed YkcC-YngA-YkbC multicomponent transmembrane glycosylation system (Fig. 8C). Although the glycosylation target in the bacterial cell envelope is currently unknown, it will be interesting to investigate this further in future studies. As the transcription of the genes in the yngABC and ykcBC operons is predicted to be activated during anaerobic growth conditions, the YkcC-YngA-YkbC system likely functions only during specific growth conditions and therefore may mediate glycosylations that form a specific adaptation to these growth conditions. Furthermore, while UDP-glucose produced by YngB is utilized by TagE and UgtP, it will also be interesting to determine if, through specific protein–protein interactions, the UDP-glucose produced by YngB can be more efficiently fed toward the YkcC-YngA-YkbC system likely functions only during specific growth conditions and therefore may mediate glycosylations that form a specific adaptation to these growth conditions. In conclusion, we provide the first experimental evidence that the *B. subtilis* YngB protein is a functional UGPase that is produced under anaerobic growth conditions. The UDP-glucose synthesized by YngB is utilized for the glycosylation of WTA and glycolipid production. However, we speculate that the UDP-glucose produced by YngB might also enter other glycosylation pathways, leading to the decorating of other cell envelope components with glucose residues under anaerobic or other growth conditions, under which genes forming part of the YclJ regulon are activated.

**Experimental procedures**

**Bacterial strains and growth conditions**

All strains used in this study are listed in Table S2. *E. coli* and *B. subtilis* strains were grown in LB medium at 37 °C unless otherwise stated. The LB medium for *B. subtilis* was supplemented with 0.2% D-glucose for aerobic growth condition. For anaerobic fermentative growth of *B. subtilis*, a single colony was first inoculated in LB medium and grown at 37 °C aerobically until reaching an absorbance of 1.0 at 600 nm. Then, 100 μl of the bacteria culture was spread on...
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2 × yeast extract tryptone agar plates (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 1% glucose, 20-mM K₃PO₄ pH 7.0, 15 g/L Bacto agar). The plates were incubated for 18 h at 37 °C in an anaerobic cabinet (Don Whitley Scientific) with an atmosphere of 10% CO₂, 10% H₂, and 80% N₂. Where appropriate, the growth medium was supplemented with antibiotics at the following final concentrations: *E. coli* cultures, ampicillin at 100 μg/ml and kanamycin at 30 μg/ml; for *B. subtilis* cultures, kanamycin at 10 μg/ml, erythromycin at 5 μg/ml, and spectinomycin at 100 μg/ml.

**Strain and plasmid construction**

All primers used in this study are listed in Table S3. For the construction of plasmids for the expression and purification of the C-terminally His-tagged *B. subtilis* GtaB and YngB proteins, the *gtaB* (BSU_35670) and *yngB* (BSU_18180) genes were amplified by PCR from *B. subtilis* 168 genomic DNA using primer pairs ANG3161/ANG3162 and ANG3163/ANG3164, respectively. The PCR products were digested with NcoI and XhoI and ligated with plasmid pET28b cut with these restriction enzymes. The resulting plasmids pET28b-*gtaB*-His and pET28b-*yngB*-His were recovered in *E. coli* strain XL1-Blue, yielding strains ANG5206 and ANG5207, respectively. The sequence of the inserts in the pET28b plasmids was confirmed by sequencing using primers ANG111 and ANG112. For protein expression, the plasmids pET28b-*gtaB*-cHis and pET28b-*yngB*-cHis were introduced into *E. coli* strain BL21(DE3), yielding strains ANG5208 and ANG5209.

For the construction of *B. subtilis* expressing *gtaB* or *yngB* from the IPTG-inducible hyperspank promoter (P<sub>hyper</sub>), the *gtaB* (BSU_35670) and *yngB* (BSU_18180) genes were amplified by PCR using *B. subtilis* 168 genomic DNA as template and primer sets ANG3203/ANG3204 and ANG3205/ANG3206, respectively. The PCR products were digested with NcoI and Xhol and ligated with plasmid pDR111 cut with the same restriction enzymes. The resulting plasmids pDR111-*gtaB* and pDR111-*yngB* were recovered in *E. coli* strain XL1-Blue, yielding strains XL1-Blue-pDR111-*gtaB* and XL1-Blue-pDR111-*yngB*. The sequences of the inserts in pDR111 plasmids were confirmed by sequencing using primers ANG1671 and ANG1672. Plasmids pDR111, pDR111-*gtaB*, and pDR111-*yngB* were linearized with Scal and introduced into the WT *B. subtilis* strain 168 yielding strains 168 ampy::spec P<sub>hyper</sub> (ANG5675), 168 ampy::spec P<sub>hyper</sub>-gtaB, and 168 ampy::spec P<sub>hyper</sub>-yngB, respectively. Next, the chromosomal DNA of strain 168ΔgtaB::kan (ANG5277) was introduced to strains 168 ampy::spec P<sub>hyper</sub> 168 ampy::spec P<sub>hyper</sub>-gtaB, and 168 ampy::spec P<sub>hyper</sub>-yngB yielding strains 168ΔgtaB::kan ampy::spec P<sub>hyper</sub> (ANG5676), 168ΔgtaB::kan ampy::spec P<sub>hyper</sub>-gtaB (ANG5677), and 168ΔgtaB::kan ampy::spec P<sub>hyper</sub>-yngB (ANG5678), respectively. The chromosomal DNA of strain 168ΔyngB::kan (ANG5263) was introduced into 168 ampy::spec P<sub>hyper</sub> and 168 ampy::spec P<sub>hyper</sub>-yngB yielding strains 168ΔyngB::kan ampy::spec P<sub>hyper</sub> (ANG5679) and 168ΔyngB::kan ampy::spec P<sub>hyper</sub>-yngB (ANG5680). The chromosomal DNA of strain 168ΔyngB::erm (ANG5659) was introduced into strain ANG5677, yielding strain 168ΔgtaB::kan ΔyngB::erm ampy::spec P<sub>hyper</sub>-gtaB (ANG5682). The chromosomal DNA of strain 168ΔgtaB::erm (ANG5658) was introduced into strains ANG5679 and ANG5680, yielding strains 168ΔgtaB::erm ΔyngB::kan ampy::spec P<sub>hyper</sub>-gtaB (ANG5681) and 168ΔgtaB::erm ΔyngB::kan ampy::spec P<sub>hyper</sub>-yngB (ANG5683). The deletion of the *gtaB* and *yngB* genes were confirmed by PCR using primer sets ANG3197/ANG3198 and ANG3199/ANG3200, respectively. The integration of the *gtaB* gene at the ampyE site was confirmed by PCR using primer sets ANG1663/ANG3204 and ANG1664/ANG3203. The integration of the *yngB* gene at the ampyE site was confirmed by PCR using primer sets ANG1663/ANG3206 and ANG1664/ANG3205. The integration of plasmid pDR111 at the ampyE site for strain 168 ampy::spec P<sub>hyper</sub> was confirmed by PCR using primer sets ANG1664/ANG1671 and ANG1663/ANG1672.

**Expression and purification of GtaB and YngB**

*E. coli* strain BL21(DE3) pET28b-*gtaB*-cHis (ANG5208) was grown in LB medium at 30 °C with shaking until reaching an absorbance of 600 nm of 0.6. Protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM, and the cultures were incubated overnight at 16 °C with agitation. Bacterial cells were harvested by centrifugation and washed once with cold 500-mM NaCl, 50-mM Tris buffer, pH 7.5, and the bacterial pellets were stored at −20 °C for future use. For the protein purification, the bacterial cells were suspended in 20-mL cold buffer A (500-mM NaCl, 50-mM Tris, pH 7.5, 5% glycerol, 10-mM imidazole) supplemented with cComplete protease inhibitor cocktail (Roche), 100 μg/ml lysozyme, and 10 μg/ml DNase, followed by passing the cell suspension twice through a French press cell at 1100 psi. For the purification of GtaB-cHis, the cell lysate was loaded onto a 5-mL HisTrap column equilibrated with buffer A. The column was washed with 5 column volumes of buffer A followed by elution using a linear gradient of 10 column volumes from buffer A to buffer B (500-mM NaCl, 50-mM Tris, pH 7.5, 5% glycerol, 10-mM imidazole). Elution fractions containing GtaB-cHis were pooled, and thrombin was added to cleave off the C-terminal His-tag. The protein was dialyzed at room temperature (RT) against 1L buffer C (500-mM NaCl, 50-mM Tris, pH 7.5, 5% glycerol) for 1 h, followed by overnight dialysis against 1L of fresh buffer C. The protein solution was then loaded onto a Superdex 10/60 HiLoad size-exclusion column (GE Healthcare) equilibrated with buffer C. After size-exclusion chromatography, the purified protein was concentrated using a PES 10-kDa cutoff Pierce protein concentrator.
37 °C in the minimal medium containing 42 mg/L methionine. The next day, bacterial cells were washed with the minimal medium and grown in the minimal medium containing 42 mg/L selenomethionine at 37 °C until reaching an absorbance at 600 nm of 0.5 to 0.6. At this point, 2.9 g/L additional glucose and IPTG to give a final concentration of 0.5 mM were added. The bacterial culture was incubated at 16 °C overnight with agitation. Cells were harvested, washed, and stored as described above. For the production of native YngB-cHis used in the kinetic assay, methionine was added to the medium in place of selenomethionine. For the purification of SeMet YngB-cHis or native YngB-cHis, the cell lysate was loaded by gravity flow onto a column containing 1-ml Ni-NTA resin (Qiagen) equilibrated with buffer A. The column was washed with 30 ml of buffer A and 30 ml of buffer D (500-mM NaCl, 50-mM Tris, pH 7.5, 5% glycerol, 50-mM imidazole). The proteins were eluted in 5 × 1-ml fractions using buffer B. The elution fractions were pooled and subjected to size-exclusion chromatography, and the purified protein was concentrated as described above.

**Protein crystallization, structural solution, and analysis**

SeMet YngB-cHis crystals were obtained by the sitting-drop method in 0.2 M potassium citrate tribasic monohydrate, 0.05 M lithium citrate tribasic tetrahydrate, 0.1 M sodium phosphate monobasic monohydrate, 25% PEG6000, using a protein concentration of 6 mg/ml. SeMet YngB-cHis crystals were cryoprotected with 30% ethylene glycol and flash-frozen in liquid nitrogen. Datasets were collected at the I03 Beamline at the Diamond Light Source (Harwell Campus, Didcot, UK). Data indexing, integration, scaling, and merging was performed using the xia2 3dii pipeline (58). The selenium sites and initial phases were solved using CRANK2 (59). Structure refinement was performed with Refmac (60) and model building with Coot (61). Data collection and refinement statistics are summarized in Table 1. The structure was validated through the Validation Pipeline wwpdb-VP and the geometry outliers assessed using MolProbity (62). The figures with the structure were generated using PyMOL.

**Enzyme kinetic analysis**

The steady-state kinetics assays with purified GtaB and YngB-cHis proteins were performed using a previously described method with some modifications (52). Briefly, enzyme assays were performed in 96-well plates in 100-μl reaction volumes. The reactions contained 50-mM Tris, pH 7.5, 500-mM NaCl, 5% glycercol, 10 mg/ml MgCl2, 0.05U of E. coli pyrophosphatase (New England Biolabs), 100-nM GtaBβα or 100-nM YngBβα-cH. For measuring the K_m value for Glc-1-P, the reactions contained 200-μM UTP and Glc-1-P at a concentration of 200 μM, 150 μM, 100 μM, 50 μM, 12.5 μM, 6.3 μM, or 3.1 μM. For measuring the K_m value of UTP, the reactions contained 200-μM Glc-1-P and UTP at a concentration of 200 μM, 150 μM, 100 μM, 50 μM, 12.5 μM, 6.3 μM, or 3.1 μM. Reactions were performed in triplicate and reactions without GtaB and YngBβα-cH were used as negative controls. Reactions were incubated at RT for 1 min for GtaBβα and 4 min for YngBβα-cH so that less than 20% of the substrates were converted in the reactions. The reactions were terminated by adding 100 μl of Biolum Green (Enzo Life Sciences) and after a 20-min incubation at RT, the absorbance was measured at 620 nm. The number of UDP-glucose formed per molecule of GtaB or YngB per second is plotted versus substrate concentration, followed by Michaelis–Menten nonlinear fitting using Prism.

**Detection of glucose residues on WTA using fluorescently labeled lectin concanavalin A**

Glucose modifications on WTA were detected by fluorescence microscopy using a previously described method with minor modifications (34). Single colonies of the different *B. subtilis* strains were used to inoculate 5-ml LB medium, and the cultures grown overnight at 37 °C. The overnight cultures were back diluted 1:100 into 25 ml of fresh LB medium supplemented with 1-mM IPTG and the cultures grown at 37 °C until reaching the mid-log growth phase (absorbance of 600 nm between 0.4 and 0.6). Cells equivalent to 100 μl of a culture with an absorbance of 600 nm of 0.5 were pelleted by centrifugation at 17,000g for 1 min and washed once with PBS, pH 7.4. Bacterial cells were subsequently suspended in 80-μl PBS and mixed with 20 μl of 1 mg/ml concanavalin A AlexaFluor 594 conjugate dissolved in 0.1 M sodium bicarbonate, pH 8.3, followed by incubation at RT for 30 min in the dark. The bacterial cells were subsequently washed three times with 100-μl PBS, subjected to 1-min centrifugation steps to pellet cells, and finally suspended in 100 μl PBS. Microscopic analysis was performed as described previously (20). Briefly, samples were spotted on microscope slides coated with a thin agarose film (1.2% agarose in distilled water). Phase-contrast and fluorescence images were taken using a 100x objective and a Zeiss Axios Imager.A1 microscope coupled to the AxioCam MRm and processed using the Zen 2012 (blue edition) software. The Zeiss filter set 00 was used for the detection of fluorescence signals. For the microscopy experiment using bacteria grown under anaerobic fermentative growth conditions, colonies obtained on agar plates incubated for 18 h at 37 °C in an anaerobic cabinet were suspended in 100-μl PBS to an absorbance at 600 nm of 0.5. The staining and microscopic analysis was performed as described above. Representative data from three independent experiments are shown.

**Isolation of WTA and its analysis by NMR and UPLC-MS**

*B. subtilis* strains were grown in 2 L LB medium supplemented with 1-mM IPTG at 37 °C. Once the cultures reached an absorbance at 600 nm of 0.6, the cells were harvested by centrifugation and WTA isolated using a previously described method (19). The NMR analysis of WTA was performed as described previously (19, 20). Briefly, 2 mg of WTA from each strain was suspended and lyophilized twice in 500-μl D_2O of 99.96% purity. Lyophilized WTA at the final step was suspended in 500-μl D_2O of 99.96% purity and NMR spectra were recorded on a 600-MHz Bruker Advance III spectrometer equipped with a TCI CryoProbe. NMR spectra were recorded

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at 303 K with a total recycling time of 5 s and a 1H flip angle of approximately 30°. Two independent experiments were performed, and very similar spectra were obtained and one spectrum for each strain is shown. For the ultra performance liquid chromatography (UPLC)-MS analysis of the purified WTA, the method used was adapted from previously described protocols (20, 63). Briefly, 2 mg of the purified WTA was lyophilized in deionized distilled water. The lyophilized WTA was then depolymerized into monomeric units by hydrolysis of the phosphodiester bonds using 48% hydrofluoric acid for 20 h at 0 °C. The depolymerized WTA material was subjected to UPLC-MS analysis as described previously (63). All data were collected and processed using the MassLynx software, version 4.1 (Waters Corp.).

Isolation of membrane lipids and TLC analysis

For the isolation of total membrane lipids, the different B. subtilis strains were grown overnight at 37 °C in 100-ml LB medium supplemented with 1-mM IPTG. Cells were harvested by centrifugation, and total membrane lipids isolated as described previously (64). TLC analysis and detection of glycolipids were performed as described (64). Briefly, isolated lipids were suspended in chloroform and 0.5 mg were spotted on Ä60 silica gel plates (Macherey-Nagel). Lipids were separated using a developing solvent of chloroform:methanol:water (65:25:4). Plates were sprayed with 0.5% α-naphthol in 50% methanol and then with 95% sulfuric acid. Glycolipids were visualized as purple bands by a final heating step. For the glycolipid analysis of bacteria grown under anaerobic fermentative conditions, colonies obtained on agar plates after incubating at 37 °C in an anaerobic cabinet were scraped off the plates and suspended in 0.1 M sodium citrate, pH 4.7, for subsequent membrane lipid isolation and TLC analysis as described above. Representative data from three independent experiments are shown.

MALDI-TOF MS and MALDI-TOF MS/MS analysis of glycolipids

MALDI-TOF analysis of glycolipids was performed using a previously described method with some minor modifications (64). A total of 5 × 0.5 mg of lipids were spotted on silica plates and separated by TLC as described above. The silica matrix with the lipids from appropriate areas of the TLC plates were scraped into glass tubes and extracted overnight at RT with 6 ml of a 1:1 methanol:chloroform mix for each sample. Next day, the silica matrix was removed by filtering the solutions through classic Sep-Pak silica cartridges (Waters) pre-equilibrated with 6 ml methanol followed by 6-ml chloroform. The filtered samples with the extracted lipids were dried under a stream of nitrogen. Dried lipids were suspended in 50-μl chloroform and aliquots mixed 1:1 with matrix. The matrix consisted of a 9:1 mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid (super-DHB, Sigma-Aldrich) at a final concentration of 10 mg/ml dissolved in chloroform:methanol at a ratio of 9:1. One μl sample was spotted onto a disposable MSP 96 polished steel plate. As calibration standard, the peptide calibration standard II (Bruker) in 0.1% TFA was mixed 1:1 with IVD Matrix α-Cyano-4-hydroxycinnamic acid. The samples were analyzed on a MALDI Biotyper Sirius system (Bruker Daltonics, Germany). The mass profiles were acquired using the FlexControl 3.4 software (Bruker Daltonics, Germany) with mass spectra scanned in the m/z range of 600 to 2000. Spectra were recorded in the reflector positive-ion mode (laser intensity 95%, ion source 1 = 10.00 kV, ion source 2 = 8.98 kV, lens = 3.00 kV, detector voltage = 2652 V, pulsed ion extraction = 150 ns). Each spectrum corresponded to an ion accumulation of 5000 laser shots randomly distributed on the spot. Representative spectra from two independent experiments are shown. The obtained spectra were processed with default parameters using the FlexAnalysis v.3.4 software (Bruker Daltonics, Germany). For the MALDI-TOF MS/MS analysis, MS/MS fragmentation profiles were acquired on a 4800 Proteomics Analyzer (with TOF-TOF Optics, Applied Biosystems, plate: 384 Opti-TOF 123 mm × 84 mm AB Sciex NCO318050, 1016629) using the reflectron mode. Samples were analyzed operating at 20 kV in the positive-ion mode. MS/MS data were analyzed using the Data Explorer software, version 4.9, from Applied Biosystems.

Data availability

The atomic coordinates and structure factors have been deposited in the Protein Data Bank under the accession code 7B1R. https://doi.org/10.2210/pdb7B1R/pdb

Supporting information—This article contains supporting information.

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Conflict of interest—The authors declare no conflicts of interest in regard to this article.
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Abbreviations—The abbreviations used are: C21-P, undecaprenyl phosphate; Glc-1-P, α-glucose-1-phosphate; Glc2-DAG, diglucosyl-diacylglycerol; LTA, lipoteichoic acid; TCS, two-component system; UGPase, UTP-glucose-1-phosphate uridylyltransferase; UPLC, ultra performance liquid chromatography; WTA, wall teichoic acid.

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