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LETTER

In Vitro Reconstitution of Two Essential Steps in Wall Teichoic Acid Biosynthesis

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ABSTRACT Wall teichoic acids (WTAs) are anionic polymers that decorate the cell walls of many Gram-positive bacteria. These structures are essential for survival or virulence in many organisms, which makes the enzymes involved in their biosynthesis attractive targets for the development of new antibacterial agents. We present a strategy to obtain WTA biosynthetic intermediates that involves a combination of chemical and enzymatic transformations. Using these intermediates, we have reconstituted the first two committed steps in the biosynthetic pathway. This work enables the exploration of WTA-synthesizing enzymes as antibiotic targets.

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The emergence of antibiotic resistance poses a major threat to human health, prompting interest in the exploration of new antibiotic targets (*1*). Many antibiotics inhibit the biosynthesis of peptidoglycan, the cross-linked carbohydrate polymer that comprises the major structural component of the cell wall and prevents the cell from bursting under high internal osmotic pressure. Peptidoglycan is not the only important cell wall component, however. The peptidoglycan layers in many Gram-positive organisms are functionalized with wall teichoic acids (WTAs), anionic polymers that are attached to peptidoglycan via a phosphodisaccharide core (Figure 1; *2*). Although their exact functions are unknown, wall teichoic acids play important biological roles. They have been shown to be essential for survival in *Bacillus subtilis* (*3, 4*) and to function as virulence factors that promote *Staphylococcus aureus* infections (*5*, *6*). The enzymes involved in WTA synthesis are, therefore, potential targets for the development of new antibiotics to treat Gram-positive bacterial infections. Here we report the preparation of synthetic substrate analogues and their use in reconstituting the activities of TagA and TagB, two essential enzymes in the pathway for wall teichoic acid biosynthesis in *B. subtilis*, which is the major Gram-positive model organism. This work verifies the proposed functions of the enzymes and lays the foundation for detailed mechanistic and structural studies.

WTAs are synthesized as lipid-linked precursors on the cytoplasmic surface of the bacterial membrane (Figure 1; *2*). The first membrane-bound intermediate, undecaprenyl-diphospho-*N*-acetylglucosamine (GlcNAc-pp-und) (Figure 1), which is formed by the enzyme TagO, is utilized in the synthesis of several different anionic cell wall polymers in *B. subtilis* (*7*). Therefore, the first committed step in the biosynthesis of the major WTA in *B. subtilis* 168 involves the transfer of *N*-acetylmannosamine (ManNAc) from UDP to the C4 hydroxyl of GlcNAc-pp-und to form the ManNAc-β-(1,4)-GlcNAc disaccharide core of WTA (Figure 1). This disaccharide core is elaborated to a charged polymer by a series of enzymatic modifications that are proposed to begin with the addition of a glycerol phosphate ester to the C4 position of the ManNAc sugar. The enzymes proposed to catalyze these first two committed steps in teichoic acid biosynthesis, TagA and TagB, were identified based on data from transcriptional fusions (*8*), thermosensitive mutations (*9*, *10*), and sequence homology (*11*). However, the putative functions of TagA and TagB have not been demonstrated *in vitro* because the lipid-anchored substrates needed to monitor activity cannot be isolated in useful quantities from bacterial cells. Moreover, these substrates contain a 55-carbon 'carrier' lipid that is expected to hamper both substrate isolation and kinetic investigations.

Problems related to substrate availability and lipid chain length can potentially be addressed by synthesizing alternative substrates containing shorter lipid chains. However, chemical synthesis cannot provide ready access to the substrates down-

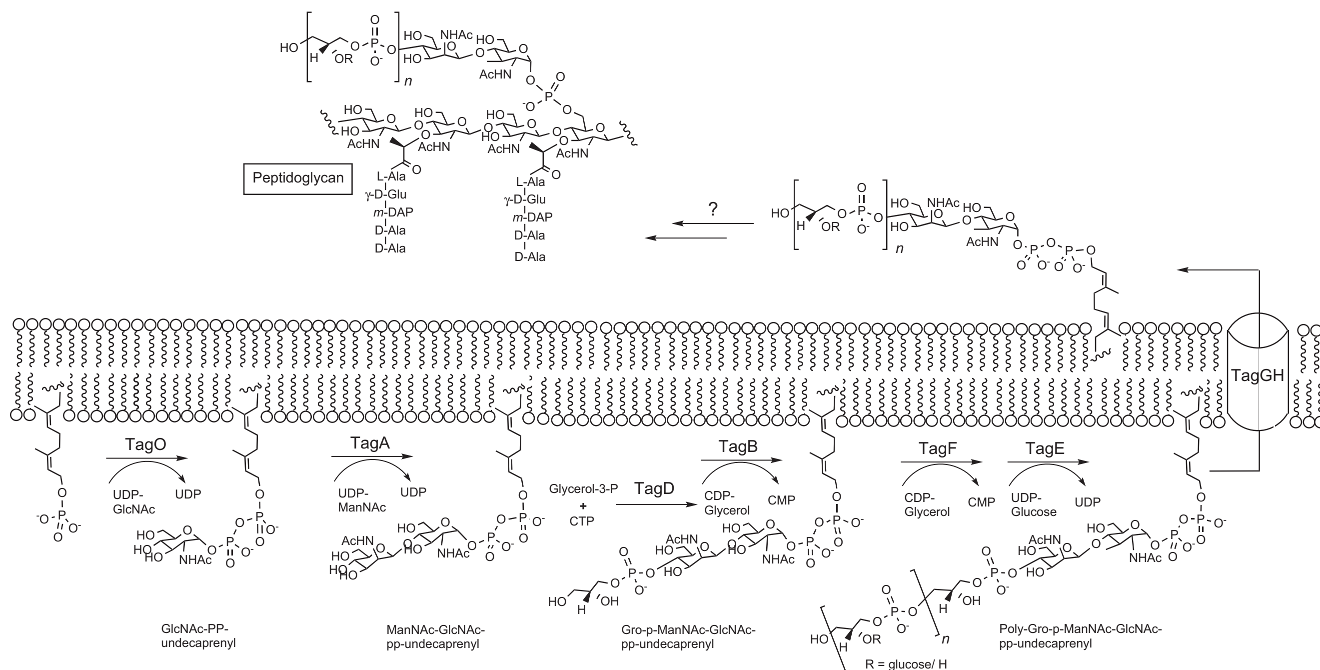


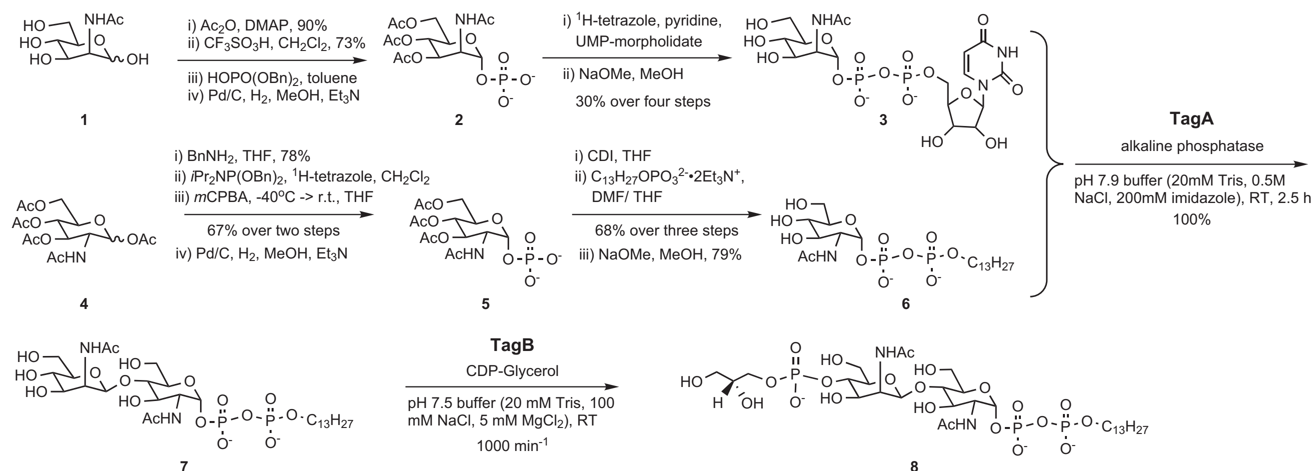
Figure 1. Polyglycerol phosphate WTA biosynthesis in *B. subtilis* 168.

stream of TagA, which contain β linkages to *N*-acetyl mannosamine. 1,2-*cis*-*O*-Glycosidic linkages to mannosamine are notoriously challenging to construct, and the ManNAc-β-(1,4)-GlcNAc linkage found in WTA has never been made chemically. Furthermore, synthetic routes to related compounds are lengthy and involve cumbersome functional group interconversions (*12, 13*; see Supporting Information.) Therefore, we decided to explore a chemoenzymatic strategy in which TagA itself is used to make the ManNAc-β-(1,4)-GlcNAc linkage found in all downstream substrates in the WTA pathway.

The natural TagA acceptor contains a 55-carbon undecaprenyl chain that is embedded in the bacterial membrane. Sequence analysis suggests that TagA does not contain any membrane spanning regions, and we surmised that the undecaprenyl chain is not specifically recognized by TagA. To simplify the synthesis and subsequent kinetic studies, we prepared an acceptor analogue containing a 13-carbon saturated lipid chain (Scheme 1, **6**; *14–17*). Following a known route, we also prepared the required UDP-ManNAc donor sugar **3**, which is not commercially available (Scheme 1; *18*, *19*).

The ability of TagA to utilize acceptor analogue **6** was evaluated by incubating the recombinant enzyme with synthetic substrates **3** and **6** overnight and analyzing the reaction mixture by liquid chromatography/mass spectrometry (LC/MS). A large peak at $m/z = 765$ was observed, consistent with the formation of the ManNAc-GlcNAc-pp-lipid product. To assess the efficiency with which TagA uses **6**, we measured the reaction rate using a continuous coupled enzyme assay that links the production of UDP to the oxidation of NADH (*15, 17, 20, 21*). The apparent K_m of **6** at a UDP-ManNAc concentration of 1.5 mM was found to be 190 ± 30 μM, and the turnover number was calculated to be 410 ± 50 min^{-1}, consistent with the role of this glycosyltransferase in the biosynthesis of a primary metabolite. The kinetic measurements confirm the proposed function of TagA as a ManNAc transferase and demonstrate that this synthetic acceptor analogue is a reasonable substrate to use in further characterization of TagA.

The high turnover number of TagA suggested that it would have utility for preparing the substrate for the next enzyme in the pathway, TagB. Unlike TagA, TagB has previously been overexpressed and efforts to reconstitute activity have been reported (*22*). Because the disaccharide acceptor of TagB was not available, however, activity was monitored by following the incorporation of radioactivity from CDP-[^{32}P]-glycerol into bacterial membranes thought to contain the TagB substrate. The reported turnover number, 0.004 min^{-1}, is far too low to keep pace with cell wall biosynthesis, indicating a problem with either the assay conditions or the enzyme itself. To obtain pure substrate to monitor TagB activity, we converted 2 mg of compound **3** to compound **7** using TagA and UDP-ManNAc (Scheme 1). In the presence of alkaline phosphatase to consume UDP, quantitative conversion to product was achieved within 2.5 h. The identity of the product was con-



Scheme 1. Preparation of substrates for TagA and TagB.

firmed by NMR, with TOCSY spectra used to make resonance assignments and the expected β stereochemistry of the linkage confirmed by large ROESY cross peaks between H^1, H^3, and H^5 of the ManNAc sugar (See Supporting Information). Because the chemical synthesis of **3** and its subsequent enzymatic transformation to **7** are straightforward, it should be possible to obtain more material simply by scaling up the enzymatic reaction; however, we note that milligram quantities of substrates are typically sufficient for thousands of kinetic assays.

The purified recombinant enzyme was incubated with substrate **7** and commercially available CDP-glycerol, and the reaction mixture was analyzed by LC/MS. After a 3 h incubation, the starting material **7** was virtually gone and new peaks were observed at $m/z = 459$ and 919, consistent with the formation of the Gro-p-ManNAc-β-(1,4)-GlcNAc-pp-lipid product **8** (Scheme 1). The appearance of this product was found to depend on the presence of active TagB and both substrates, thus verifying the proposed function of the enzyme.

The activity of TagB was evaluated by separating quenched reactions on a high-performance liquid chromatography column and quantitating CMP formation by UV absorbance. The reaction rate was found to be 1 μM product min^{-1} nM^{-1} enzyme, providing an estimated turnover number of 1000 min^{-1} under the reaction conditions. This turnover number is more than 5 orders of magnitude greater than that reported previously, highlighting the advantages of using discrete synthetic substrates rather than crude bacterial membrane preparations in studying WTA synthesizing enzymes. Since both TagA and TagB are functional in the absence of detergents and utilize substrates containing short lipid chains, we conclude that neither enzyme requires a membrane interface for activity even though their natural substrates are membrane anchored.

TagA and TagB are potential antibiotic targets, but they are also of interest for other reasons. For example, TagA catalyzes a transformation—attachment of a sugar to a lipid-anchored monosaccharide—which is analogous to that performed by the glycosyltransferase MurG in the biosynthesis of bacterial peptidoglycan (*23–24*) and also by the second enzyme in the dolichol pathway for N-linked glycosylation (*25*). Sequence alignments suggest that TagA is unrelated to these or other glycosyltransferases (Gtfs) for which structural information exists, and TagA may thus represent an uncharacterized Gtf superfamily. TagB belongs to a class of glycerophosphotransferases that contains both processive and nonprocessive enzymes. There is no detailed structural or mechanistic information for any member of this class of enzymes, and information on TagB may shed light on the broader family. Furthermore, we anticipate that TagB can be used to make acceptor substrates for TagF, an unusual processive glycerophosphotransferase belonging the same enzyme superfamily as TagB (*26, 27*). Detailed structural and mechanistic studies of TagA and TagB are now underway.

METHODS

General Methods. Chemicals and solvents were from Sigma-Aldrich. Silica gel (60 Å, 32–63 μm) was from Sorbent Technologies. NMR spectra were recorded on Varian Inova 400 or 500 MHz spectrometers. Mass spectra (ESI) were recorded using an Agilent 1100 series LC/MSD instrument with an electrospray ionization (ESI) source in negative ion mode. LC/MS analysis of enzymatic reactions was also performed on this instrument, using a Zorbax 300-SB-C18 column for LC separation.

Synthesis of Compound 3. The triethylammonium salt of **2** (Scheme 1; 0.15 g, 0.239 mmol), prepared following published methods (*18*, *19*), and uridine 5´-monophosphomorpholidate (0.534 g, 0.774 mmol) were dissolved in 6 mL of dry pyridine. 1H-Tetrazole (0.050 g, 0.717 mmol) was added, the reaction was stirred for 48 h at room temperature (RT), the solvent was removed, and the residue was purified over a C-18 column (0–10% EtOH in 0.1% aqueous NH_4HCO_3) to give the peracylated precursor of **3.** (Spectral details are provided in Supporting Information.) This compound (0.06 g, 0.077 mmol) was dissolved in 4 mL of dry MeOH, and 0.46 mL of NaOMe (0.228 mmol) was added. The reaction was stirred for 1 h at RT and quenched with CH_3COONH_4 (1 mL, 0.5 M in H_2O), the solvent was removed, and the residue was purified over a C-18 column (0–10% EtOH in 0.1% aqueous NH_4HCO_3) to give **3** in 30% total yield from **8**.

Synthesis of Compound 6. The peracylated precursor of **6** was prepared following a published route (Scheme 1; *14-17*). This compound was dissolved in 6 mL of dry MeOH, and 1.4 mL of NaOMe (0.705 mmol) was added. After 1 h at RT, the reaction was quenched with CH_3COONH_4 (2.8 mL, 0.5 M in H_2O), the solvent was removed, and the residue was purified over a C-18 column using a gradient of 0–45% EtOH in 0.1% aqueous NH_4HCO_3 to give **6** in 79% yield.

Preparation of Compound 7. To a solution of TagA (1.5 mL, 1 mg mL^{-1}) in 20 mM Tris buffer (pH 7.9, 0.5 M NaCl, 200 mM imidazole) was added compound **6** (1.5 mg, 2.5 mmol), UDP-ManNAc (3.3 mg, 5.0 mmol), and 40 μL of alkaline phosphatase (20 U $μL^{-1}$). After 2.5 h at RT, the reaction was purified over a C-18 column 0–50% EtOH in 0.1% aqueous NH_4HCO_3 to give **7**.

Cloning, Expression, and Purification of TagA. The *tagA* gene was amplified from *Bacillus subtilis* strain PY79 genomic DNA using two rounds of PCR. Primers 5´-TGATTTTGCTTTTAGAAACTCTCG-3´

and 5´-TTTCAGGTTTCACCCATTGC-3´ were used to amplify a fragment containing *tagA*. The gene was then amplified using primers 5´-GTGAGAATTCGATGCAAACAGAGACTATTCAC-3´ and 5´-GTGACTCGAGAATCTGTTTTGTATGATCTTTTTC-3´ and cloned into the *Eco*RI and *Xho*I sites (underlined) of pET-24b(+) (Novagen). TagA was expressed in mid-log phase cultures of *E. coli* strain Rosetta(DE3)pLysS (Novagen), induced with 0.5 mM IPTG for 3 h at 37 °C. Cells were lysed by freeze–thaw and resuspended in 40 mL of buffer (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 0.5 M NaCl) containing 20 μL of Benzonase (25 U μL^{-1}), 40 μL of protease inhibitor cocktail, and 40 μL of Triton X-100. The clarified lysate was purified over a Ni^{2+} affinity column (Novagen His·Bind resin) yielding 4 mg L^{-1} TagA.

Cloning, Expression, and Purification of TagB. The *tagB* gene was amplified from *Bacillus subtilis* PY79 genomic DNA using primers 5´-GAGCATGTCGCTAGCATGAAAATAAGATCACTACTGG-3´ and 5´-CACTGCAGTCTCGAGGCTTATTAAATTTTCGATGAAATT-3´. *TagB* was cloned into the *Nhe*I and *Xho*I sites pET-24b(+) (Novagen). TagB was expressed in mid-log phase cultures of the *E. coli* strain BL21(DE3) (Novagen), induced with 0.5 mM IPTG for 22 h at 16 °C. Cells were lysed with Bugbuster (Novagen) supplemented with Benzonase (Novagen), protease inhibitor cocktail, and lysozyme following the Novagen protocol. Clarified lysate was purified over a Ni^{2+} column (Novagen IDA His·Bin resin) yielding 12 mg L^{-1} of TagB.

Continuous Coupled Enzyme Assay for TagA. This assay couples the production of UDP to the oxidation of NADH, leading to a drop in NADH fluorescence (*15*, *17*, *20*, *21*). Reactions were carried out in 96-well plate microplates (Costar 3603) in a volume of 100 μL. NADH fluorescence was monitored at 465 nM using a Perkin-Elmer HTS 7000 Plus BioAssay reader. Reactions contained buffer (20 mM Tris, pH 7.9, 5 mM $MgCl_2$, 450 mM NaCl), 0.4 U μL^{-1} PK, 0.2 U μL^{-1} NDPK, 0.5 mM PEP, 0.2 U μL^{-1} LDH, 0.25 mM NADH, 0.5 mM ATP, and 0.1 mg mL^{-1} BSA. Reaction mixtures containing substrates were incubated at RT for 15 min prior to adding TagA (100 nM). Kinetic parameters for TagA with substrate analogue **6** were calculated as described in the Supporting Information.

HPLC Assay for TagB. TagB (10 nM) was incubated at RT with 200 mM **7** and 400 mM CDP-glycerol in buffer (20 mM Tris, pH 7.5, 100 mM NaCl, and 5 mM $MgCl_2$). Reactions were quenched with an equal volume of cold methanol, loaded onto an analytical anion exchange HPLC column (Phenosphere SAX), and eluted with a gradient of 0–56% buffer B over 17 min (buffer A, 5 mM $NH_4H_2PO_4$ pH 2.8; buffer B, 750 mM NH_4PO_4 pH 3.7). CMP was monitored at 260 nm.

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Supporting Information Available: Complete synthetic and spectral data for compounds **3**, **6**, and **7**, and enzyme assay details. This material is available free of charge via the Internet.

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