Chemical Science

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Cite this: *Chem. Sci.*, 2014, **5**, 3823

Reconstituting poly(glycerol phosphate) wall teichoic acid biosynthesis *in vitro* using authentic substrates†

Robert T. Gale,[a] Edward W. Sewell,[a] Teresa A. Garrett[b] and Eric D. Brown*[a]

Received 18th March 2014
Accepted 16th June 2014

DOI: 10.1039/c4sc00802b

www.rsc.org/chemicalscience

Wall teichoic acids (WTAs) are phosphate-rich anionic polymers that constitute a substantial portion of the Gram-positive cell wall. Recent work has demonstrated the importance of WTAs in cell shape, virulence and antibiotic resistance. These findings highlight WTA biosynthetic enzymes as attractive targets for novel antimicrobial agents. Due to challenges involved in the isolation of natural substrates, *in vitro* studies of the recombinant enzymes have largely employed soluble substrate analogues. Herein we present a semisynthetic approach to obtain the authentic precursor for WTA biosynthesis, Lipid α, complete with its polyisoprenoid lipid moiety. We show that this material can be used to reconstitute the activities of four enzymes involved in poly(glycerol phosphate) WTA biosynthesis in a detergent micelle. This work enables the creation of chemically defined and realistic systems for the study of interfacial catalysis by WTA biosynthetic machinery, which could aid efforts to discover and develop novel agents against WTA biosynthesis.

Introduction

The bacterial cell wall has been targeted with spectacular success in the development of antibiotics. Indeed, β-lactams and glycopeptides that inhibit cell wall biosynthesis are the most widely used chemotherapeutic agents to treat bacterial infections.[1] Nevertheless, increasing resistance to these agents is a serious threat to their continued use and to public health.[2] Thus, the search is on for new agents of unique chemical class that are unsusceptible to existing resistance mechanisms and are effective perturbants of this celebrated and well-validated target.

In addition to peptidoglycan, the Gram-positive bacterial cell wall is largely composed of long, phosphate-rich, anionic polymers called wall teichoic acids (WTAs).[3] These polymers are typically comprised of repeating polyol phosphate residues modified with D-alanyl and glycosyl substituents.[4,5] WTAs have emerged as attractive antibacterial targets[6] due to their important roles in cell shape determination,[7] virulence[8–10] and antibiotic resistance.[11,12] While studies of the genetics and physiology of WTA biosynthesis have provided strong validation for this biosynthetic pathway as a target for new antibiotics, a lack of tools and understanding of the biochemistry of WTA synthesis remains an obstacle to its effective utility in modern antibiotic drug discovery.

Despite ready access to recombinant enzymes involved in WTA biosynthesis, challenges in the isolation of natural substrates from bacterial sources have hindered detailed studies. Efforts to date have involved the synthesis of soluble analogues of the substrates to study WTA biosynthesis *in vitro*.[13,14,17] These soluble analogues substitute a short aliphatic or prenyl chain for the C_{55} undecaprenyl moiety of the authentic precursor glycolipid, GlcNAc-PP-undecaprenol. These analogues provided ready access to pure substrates for the functional characterization of several WTA biosynthetic enzymes,[13–17] and have largely underpinned our current understanding of the biosynthetic pathway. These soluble analogues, however, fail to recapitulate the interfacial nature of catalysis of WTA glycolipids. Indeed, the many glycosyltransferase enzymes and the flippase transporter involved in WTA synthesis are membrane bound and thus the membrane interface is central to synthesis and assembly of WTA.[6]

Here we present a semisynthetic strategy to obtain the authentic WTA glycolipid precursor GlcNAc-PP-undecaprenol, also known as Lipid α (Table 1 outlines the nomenclature for WTA intermediates). Further we show that this molecule, in a detergent micelle, is a capable substrate for the activities of four consecutive recombinant enzymes – TagA, the ManNAc transferase, TagB, the poly(glycerol phosphate) primase, TagF, the poly(glycerol phosphate) polymerase and TagE, the poly(glycerol phosphate) glucosyltransferase – in the synthesis of poly(glycerol phosphate) WTA *in vitro*. This work provides an important new avenue for the characterization of WTA enzymes at interfaces using authentic and chemically defined substrates.

[a]*Michael G. DeGroote Institute of Infectious Disease Research and Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario L8N 3Z5, Canada. E-mail: ebrown@mcmaster.ca*

[b]*Department of Chemistry, Vassar College, Poughkeepsie, New York 12604, USA*

† Electronic supplementary information (ESI) available: Supplementary figures, synthetic protocols and characterization data. See DOI: 10.1039/c4sc00802b

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Table 1 Nomenclature for undecaprenyl-linked WTA intermediates[a]

Intermediate	Chemical composition
Lipid α	GlcNAc-1-P-P-Und
Lipid β	ManNAc-β-(1–4)-GlcNAc-1-P-P-Und
Lipid ϕ.*n*	$(GroP)_n$-ManNAc-β-(1–4)-GlcNAc-1-P-P-Und
Lipid γ	$(GroP^*)_n$-ManNAc-β-(1–4)-GlcNAc-1-P-P-Und

[a] WTA intermediates are named according to the enzyme utilizing the molecule as a substrate. *n* represents the number of repeating *sn*-glycerol-3-phosphate units. The asterisk (*) denotes oligomeric and polymeric species bearing α-linked glucose residues. Abbreviations: GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; GroP, *sn*-glycerol-3-phosphate; P, phosphate; Und, undecaprenol.

Results and discussion

Semisynthetic preparation of Lipid α

WTA biosynthesis utilizes undecaprenyl phosphate (**4**) as a lipid carrier to build glycosyl intermediates (Fig. 1 details the biosynthetic pathway). This lipid carrier is essential for transport of oligosaccharides across the bacterial membrane during synthesis of many other important polysaccharides including lipopolysaccharides,[18,19] peptidoglycans[20] and capsular polysaccharides.[21] Undecaprenyl phosphate (**4**) was required in quantity for use in chemical transformations to generate Lipid α (**5**) and, while this molecule is commercially available, its cost is prohibitive for purchase in useful quantities. It can be obtained from bacterial sources, however it represents a relatively minor species relative to other membrane lipids[22] and thus it is challenging to isolate and purify. Compound **4** is also available through total synthesis, however the methodology involves a cumbersome multistep procedure.[23] Thus, we elected to obtain undecaprenyl phosphate (**4**) through a semisynthetic approach, whereby the precursor undecaprenol (**3**) was obtained from a plant source, namely from *Laurus nobilis* leaves (bay leaves), according to published methodology.[24–26] Subsequent phosphorylation of the polyprenol was achieved with tetra-*n*-butylammonium dihydrogen phosphate and trichloroacetonitrile according to published methods,[27] to afford undecaprenyl phosphate (**4**) in 40% yield over the phosphorylation step.

Lipid α (**5**) represents a challenging synthetic target due to the acid sensitivity of the anomeric diphosphate linkage. For this reason, our synthetic strategy to the undecaprenyl-linked glycosyl diphosphate involved a late stage introduction of the diphosphate linkage, and use of base-cleavable protecting groups that allowed for a final global deprotection step. Thus, our strategy involved (a) peracetylation of commercially available *N*-acetylglucosamine (**1**), (b) anomeric acetate deprotection, (c) hemiacetal α-phosphorylation, (d) undecaprenyl phosphate (**4**) coupling, and (e) base-mediated global deprotection (Scheme 1). The synthesis began with the preparation of α-phosphate **2** in 25% yield over five steps following published methodology.[13,14,28] Coupling of undecapenyl phosphate (**4**) with the α-phosphate **2** was conducted with 1,1′-carbonyldiimidazole (CDI) and tin(II) chloride. Global acetate deprotection using sodium methoxide in methanol transformed the resulting peracetyl diphosphate intermediate to desired target Lipid α (**5**) in 20% yield over

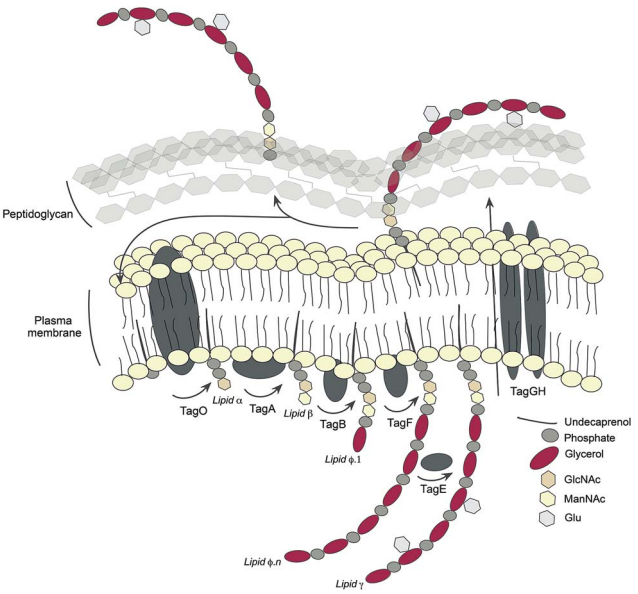

Fig. 1 Poly(glycerol phosphate) WTA biosynthesis in *B. subtilis* 168. WTA biosynthesis takes place at the inner leaflet of the cytoplasmic membrane. The first reaction in the pathway is mediated by the transmembrane protein TagO, wherein a UDP-GlcNAc precursor is coupled to a membrane-anchored undecaprenyl lipid carrier to produce undecaprenylpyrophosphoryl-GlcNAc, also known as Lipid α (**5**).[49] In a second reaction, TagA catalyzes the transfer of ManNAc from a UDP-ManNAc precursor to the C(4) hydroxyl group of the lipid-linked GlcNAc, forming Lipid β.[13] TagB catalyzes the transfer of glycerol phosphate from a CDP-glycerol precursor to the lipid-linked disaccharide.[13,31] An additional 25–35 glycerol phosphate monomers are added to this product, Lipid ϕ.1, by the polymerase TagF utilizing CDP-glycerol precursors.[14,15,34] The polymer, Lipid ϕ.*n*, is tailored with α-linked Glu residues by the glycosyltransferase TagE through use of UDP-Glu precursors, forming Lipid γ.[16] The polymer is translocated to the extracellular leaflet through the action of the two-component ABC transporter TagGH.[50] The last step in *B. subtilis* 168 poly(glycerol phosphate) WTA biosynthesis is mediated by currently uncharacterized transferases, which are predicted to cleave the polymer from its lipid anchor and attach it to the C(6) hydroxyl group of peptidoglycan muramic acid.[47] Abbreviations: GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; Glu, glucose; UDP, uridine diphosphate; CDP, cytidine diphosphate.

coupling and deprotection steps and in 5% yield over the entire synthetic procedure. We found that yields of the coupling reaction were higher with the use of tin(II) chloride, which agree with recent findings highlighting the effectiveness of this agent for promoting phosphate–phosphate coupling.[29] In addition, our modest yield is commensurate with those reported for other CDI-mediated coupling reactions yielding lipid-linked glycosyl diphosphates.[29,30] Detailed synthetic procedures and chemical characterization can be found in the ESI.†

In vitro reconstitution of TagA and TagB activities

Access to Lipid α (**5**) allowed us to begin studying WTA biosynthesis using authentic substrates. We first reconstituted the activities of two *B. subtilis* 168 enzymes involved in poly(-glycerol phosphate) biosynthesis *in vitro*. These enzymes, TagA and TagB, transform Lipid α (GlcNAc-PP-Und) to Lipid β

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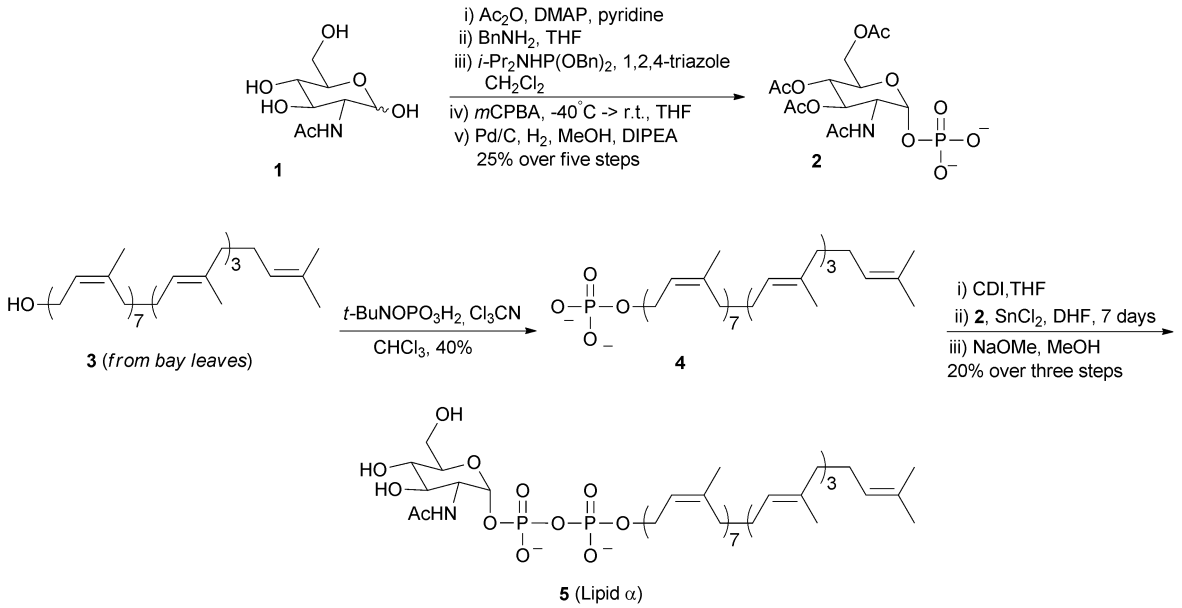

Scheme 1 Semisynthetic preparation of Lipid α (5).

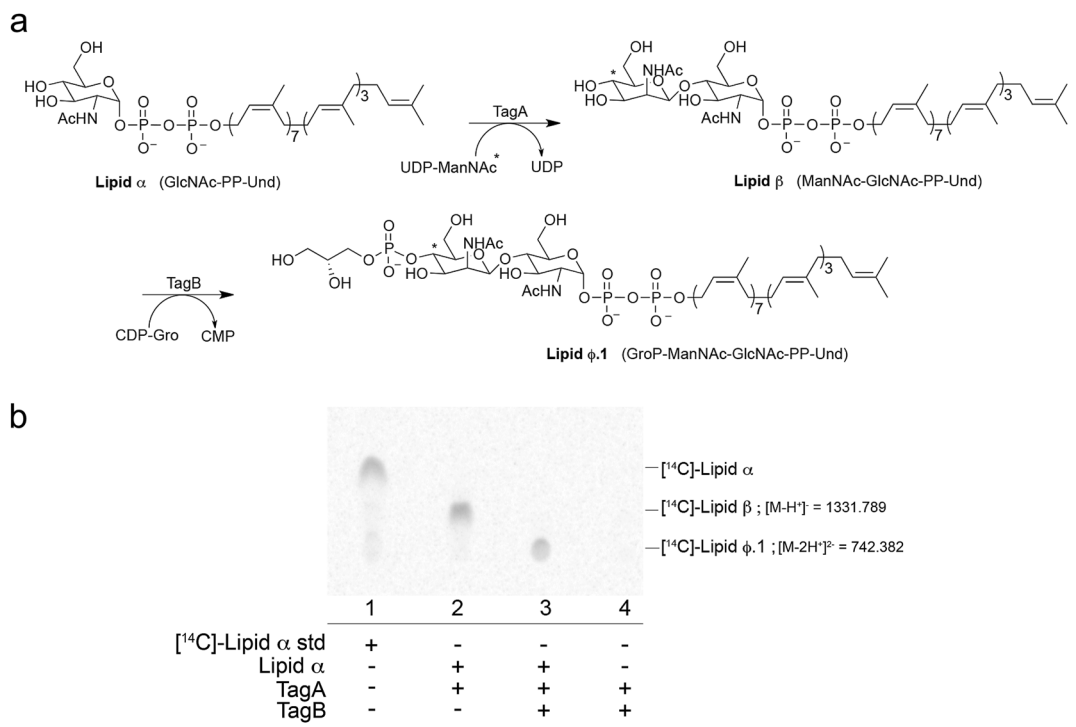

Fig. 2 Semisynthetic Lipid α (5) can be used to reconstitute the activities of pure, recombinant WTA biosynthetic enzymes TagA and TagB *in vitro*. (a) Reactions catalyzed by TagA and TagB *in vivo* and in our *in vitro* system. The asterisk (*) denotes ^{14}C-labelled compounds in assays employing semisynthetic Lipid α (5). Nomenclature and chemical composition for each WTA intermediate is shown in bold and in brackets respectively. (b) Product characterization by TLC autoradiography and ESI-MS of WTA intermediates synthesized *in vitro*. Relevant reaction components are indicated (+/−) below the appropriate lane and reaction products confirmed by ESI-MS are indicated to the right of the frame with experimental *m/z* values shown (for associated MS spectra, see Fig. S1†). In reactions involving semisynthetic derived Lipid α (5) (lanes 2–4), radioactivity was incorporated into reaction products from a UDP-[^{14}C]ManNAc precursor (see panel a). Lipid-linked reaction products were extracted according to published methodology.[12] Lane 1, [^{14}C]-Lipid α standard prepared from membranes of *Escherichia coli* cells expressing recombinant TarO from *Staphylococcus aureus* following known methods;[12] lane 2, TagA-mediated reaction of semisynthetic Lipid α (5) to Lipid β; lane 3, TagA and TagB-mediated reaction of semisynthetic Lipid α (5) to Lipid ϕ.1; lane 4, control reaction without either sources of Lipid α (5).

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(ManNAc-GlcNAc-PP-Und) and Lipid β to Lipid ϕ.1 (GroP-ManNAc-GlcNAc-PP-Und), respectively.[13,31]

We reconstituted Lipid α (5) into detergent micelles containing Triton-X100 to prevent substrate aggregation and to provide an interface for catalysis. This approach has previously proved effective for reconstituting functional enzyme activities in assays containing undecaprenyl-linked substrates.[30,32] We assessed the ability of TagA and TagB to make their respective products in this system through a combination of analytical TLC and high-resolution mass spectrometry (HRMS) (Fig. 2). The radioactive precursor UDP-[^{14}C]ManNAc was used to incorporate radioactivity into products after TagA-mediated catalysis (Fig. 2a). This allowed us to monitor product formation following lipid extraction[12,33] by analytical TLC autoradiography (Fig. 2b). Overnight incubation of Lipid α containing micelles with TagA and UDP-[^{14}C]ManNAc led to the formation of a radioactive lipid species more polar than a radiolabelled Lipid α standard derived from a bacterial membrane source[12] as visualized by normal-phase TLC. LC/MS analysis of this species showed a peak at m/z = 1331.789 corresponding to the $[M - H^+]^-$ ion of Lipid β (Fig. S1a†). Negative ion collision-induced dissociation mass spectrometry (MS/MS) analysis of the *in vitro* product (m/z 1331.8, Fig. S1a†) showed the formation of two predominant product ions at m/z 485.116 and 907.601, which corresponds to the ManNAc-GlcNAc-P and undecaprenyl diphosphate[51] respectively from Lipid β (Fig. S2a,† inset).

Incubation of Lipid α mixed-micelles with TagA, TagB, UDP-[^{14}C]ManNAc and CDP-glycerol produced a radioactive lipid species more polar than both Lipid α and Lipid β (Fig. 2b). LC/MS analysis of this species showed a peak at m/z = 742.382 corresponding to the $[M - 2H^+]^{2-}$ ion of Lipid ϕ.1 (Fig. S1b†). MS/MS analysis of this $[M - 2H^+]^{2-}$ ion (Fig. S2b†) showed the formation of several predominant product ions consistent with the structure of Lipid ϕ.1. The ion at m/z 152.996 corresponds to $C_3H_6O_5P^-$ and is typical of lipids containing glycerol phosphate.[52] The product ions at m/z 639.123, 577.157 and 359.043 correspond to ions derived from the GroP-ManNAc-GlcNAc-P portion of Lipid ϕ.1 as indicated in Fig. S2b inset.† The ion at m/z 907.614 is indicative of undecaprenyl diphosphate.[51]

In vitro reconstitution of TagF and TagE activities

The formation of Lipid β and Lipid ϕ.1 intermediates in our *in vitro* assays demonstrated that semisynthetic Lipid α was a capable substrate for the WTA machinery. Therefore, using Lipid ϕ.1 generated *in vitro*, we sought to reconstitute the remaining steps involved in the intracellular biosynthesis of poly(glycerol phosphate) WTA. To accomplish this, Lipid ϕ.1 was further transformed to Lipid ϕ.*n* ([GroP]$_n$-ManNAc-GlcNAc-

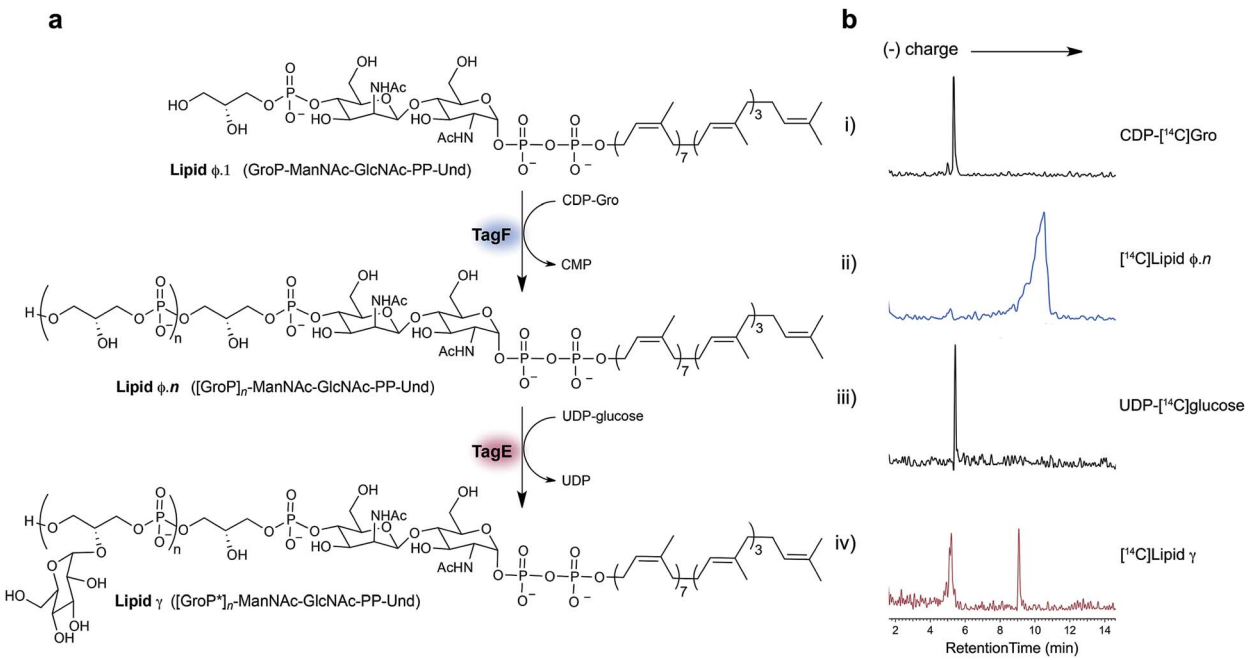

Fig. 3 Authentic late-stage WTA intermediates are substrates for polymerization and glucosylation. (a) *In vivo* and *in vitro* reactions mediated by the poly(glycerol phosphate) polymerase TagF and the glucosyltransferase TagE. Nomenclature and chemical composition for each late-stage wall teichoic acid intermediate is shown in bold and in brackets respectively. (b) Product characterization by anion exchange HPLC of late-stage WTA intermediates synthesized *in vitro*. In reactions involving TagF (shown in blue), Lipid ϕ.1 was prepared through TagA and TagB-mediated reactions starting with semisynthetic Lipid α (5) and purified by lipid extraction. Radioactivity was incorporated into polymeric products using a CDP-[^{14}C]Gro precursor. In reactions involving TagE (shown in red), Lipid ϕ.*n* polymer was prepared in a non-radioactive assay using Lipid ϕ.1 and TagF. Radioactivity was incorporated into this substrate following incubation with TagE and UDP-[^{14}C]glucose. Radioactive reaction components were separated by anion exchange HPLC and visualized with in-line scintillation counting. (i) TagF reaction mixture prior to the addition of enzyme (T = 0); (ii) NaOH-treated (0.5 M NaOH, 37 °C, 25 min) TagF reaction mixture after 4 hours of incubation with enzyme; (iii) TagE reaction mixture prior to addition of enzyme (T = 0); (iv) NaOH-treated (0.5 M NaOH, 37 °C, 25 min) TagE reaction mixture after 4 hours of incubation with enzyme. Predicted radioactive components prior to mild alkali treatment are indicated to the right of the panel.

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PP-Und) using the poly(glycerol phosphate) polymerase TagF.[34] We then tailored this polymer with α-linked glucose residues using the glucosyltransferase TagE[16] to generate Lipid γ ([GroP*]$_n$-ManNAc-GlcNAc-PP-Und) (Fig. 3a). We developed a radioactive anion exchange HPLC-based assay to monitor product formation during enzyme-mediated reactions (see Methods). Radioactive precursors CDP-[^{14}C]glycerol and UDP-[^{14}C]GlcNAc were used to incorporate radioactivity into products following incubation with TagF and TagE respectively. Our initial attempts with this approach failed to reveal radioactive products that could be detected by HPLC after incubation of TagF with non-radioactive Lipid ϕ.1 and CDP-[^{14}C]glycerol (data not shown). We hypothesized that the lipid moiety of the TagF product may have interfered with effective chromatographic separation, likely through aggregation and resulting heterogeneity. Accordingly, we treated the reaction products with mild alkali conditions (0.5 M NaOH, 37 °C, 25 min), well known to liberate poly(glycerol phosphate) polymers from their disaccharide anchors (Fig. S4a†).[35] Anion exchange HPLC analysis of this sample revealed a broad peak with a greater retention time than its radioactive precursor (Fig. 3b). This result suggested that the TagF product was highly anionic. To confirm this, we synthesized previously characterized poly(glycerol phosphate) polymers[14,15,36] (Fig. S3a†) of distinct chemical composition and compared their elution profiles to the alkali-treated TagF reaction. We found that all polymers had a similar retention time to the TagF product (Fig. S3b†).

Treatment of poly(glycerol phosphate) WTAs with mild acid conditions is known to hydrolyze phosphodiester linkages between glycerol phosphate residues (Fig. S4a†).[34,35] Thus, we further subjected the alkali-treated TagF reaction mixture to mild acid conditions (1 N HCl, 100 °C, 3 h) and monitored hydrolysis by anion exchange HPLC. Under these conditions, we observed a complete loss of signal from the polyanionic species (Fig. S4b†). Hydrolytic products coincided with the retention time of [^{3}H]glycerol (Fig. S5†), a well-known product of WTA hydrolysis.[34] Indeed, we subjected a previously characterized poly(glycerol phosphate) polymer[15] to these treatments and observed similar degradation (Fig. S4†). Taken together, the characteristic WTA lability patterns of the TagF product demonstrate TagF's ability to make Lipid ϕ.*n* in this interfacial system.

We have previously shown that recombinant TagE can glucosylate soluble poly(glycerol phosphate) polymers.[16] Likewise, using the authentic Lipid ϕ.*n* substrate and UDP-[^{14}C]glucose, we observed a TagE-dependent signal at a longer retention time than the radioactive precursor using anion exchange HPLC (Fig. 3b). This retention time was similar to that of other well-characterized WTA polymers and alkali-treated Lipid ϕ.*n* produced from TagF (Fig. S3†). These findings are consistent with the conclusion that TagE is able to α-glucosylate poly(-glycerol phosphate) polymers assembled on the authentic undecaprenyl-linked substrate.

Conclusions

WTAs constitute a significant component of the Gram-positive bacterial cell wall and have emerged as attractive antibiotic targets owing to the crucial roles they play in *Staphylococcus aureus* host colonization,[8,39] peptidoglycan synthesis co-ordination[40] and β-lactam resistance.[11,12] These structures have been targeted with success recently in both academe[12,41] and the pharmaceutical sector,[42] where biosynthetic inhibitors have been shown to restore the efficacy of β-lactams against methicillin-resistant *S. aureus* (MRSA). While progress in the discovery of inhibitors using cell-based approaches has been promising, many biochemical details of WTA synthesis remain obscure.

Biochemical study of WTA enzymes has been hampered by the limited availability of natural substrates. Recent use of soluble substrate analogues of WTA biosynthetic intermediates has facilitated the study of WTA enzymes *in vitro*, away from the membrane. These studies have culminated in defining the functions of WTA enzymes;[13,14,16] obtaining kinetic parameters for enzyme-mediated reactions;[13–17] discerning substrate preferences;[17] elucidating steps involved in WTA assembly;[43,44] describing mechanism[15,16] and mode[15] of catalysis; and helping to unveil WTAs unique role in antibiotic resistance.[11] Despite the utility these analogues have displayed in enhancing understanding of WTA biosynthesis, they are unable to mimic the natural membrane environment where WTA biosynthetic enzymes function.[31,34,45] Thus, even basic features of interfacial WTA synthesis and assembly have eluded characterization.

In the work presented here, we detail a robust strategy to obtain in quantity both undecaprenyl phosphate (**4**) and a valuable intermediate in WTA biosynthesis, Lipid α (**5**). We show that Lipid α is a capable substrate for poly(glycerol phosphate) WTA by reconstituting the activities of WTA enzymes TagA, TagB, TagF and TagE *in vitro*. This work contributes to other efforts broadly focused on the application of authentic undecaprenyl-linked substrates for the *in vitro* study of bacterial cell envelope biosynthesis, namely *O*-polysaccharide biosynthesis in *Escherichia coli*[30] and peptidoglycan assembly in pathogenic *Streptococcus pneumonia*.[32] Studies of this nature may provide new mechanistic information that is otherwise unobtainable through employment of soluble analogues and significantly enhance our limited understanding of the synthesis and assembly of bacterial cell surface structures.

Access to Lipid α and other WTA intermediates reported here lays an important foundation to probe the molecular details of interfacial WTA biosynthesis. We envision the employment of these compounds in mixed-micelle or unilamellar vesicle systems to properly assess the influence of the membrane interface on catalytic efficiency, substrate binding/dissociation, processivity and length regulation by WTA enzymes. Further, our methods are not limited to the model Gram-positive bacterium *B. subtilis*. The synthetic approach described here could be used to study WTA biosynthesis in MRSA and other pathogens, as Lipid α is a common precursor for the assembly of WTA. These authentic substrates could also be used in crystallographic studies to further understand the molecular structures and interactions between lipid acceptors and the WTA machinery. Indeed, the recent crystal structure of the TagF polymerase from *Staphylococcus epidermidis* was obtained for

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the apo-enzyme.[46] Finally, synthetic poly(glycerol phosphate) WTA may well be of utility for the study of currently uncharacterized enzyme(s), for example, those responsible for anchoring WTA to the peptidoglycan meshwork.[47]

Experimental

General methods

Chemicals and solvents were purchased from Sigma-Aldrich (Oakville, ON, Canada) or Fisher Scientific (Whitby, ON, Canada) unless otherwise stated. Analytical thin layer chromatography (TLC) was carried out on silica gel 60 F254 aluminium-backed plates from EMD Chemicals (Gibbstown, NJ, USA) or glass-backed C18 plates from Silicycle (Quebec City, QC, Canada). TLC plates were visualized by exposure to ultraviolet light and/or exposure to iodine vapour (I_2) or an acidic solution of *p*-anisaldehyde. UDP-[^{14}C]GlcNAc (0.1 mCi mL^{-1}) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). *Sn*-[U-^{14}C]glycerol-3-phosphate (0.05 mCi mL^{-1}), UDP-[^{14}C]glucose (0.02 mCi mL^{-1}) and Ultima Gold liquid scintillation cocktail were purchased from PerkinElmer (Woodbridge, ON, Canada). [2-^{3}H]glycerol (1 mCi mL^{-1}) was purchased from Amersham Biosciences (Piscataway, NJ, USA). Recombinant TagA, TagB, TagF, TagE, TarD and MnaA were prepared as previously described.[14,16,31,34,37] Anion exchange HPLC was conducted on a Waters (Mississauga, ON, Canada) ACQUITY UPLC H-Class using a ThermoScientific (Waltham, MA, USA) DNAPac PA 200 column. Radioactive components were separated using a linear gradient from 0 → 100% 1.25 M NaCl in 20 mM Tris (pH 8) and visualized with in-line scintillation counting using a PerkinElmer Radiomatic 150TR.

Semisynthetic preparation of Lipid α (5)

Full synthetic procedures and product characterization can be found in the ESI.†

Compound synthesis

UDP-ManNAc and UDP-[^{14}C]ManNAc were prepared enzymatically from UDP-GlcNAc and UDP-[^{14}C]GlcNAc respectively using MnaA.[38] CDP-glycerol and CDP-[U-^{14}C]glycerol were synthesized enzymatically using *Staphylococcus aureus* TarD following previously described methods.[37] Lipid ϕ.1 analogue was prepared chemoenzymatically as described previously[14] and subsequently purified over a Waters Sep-Pak C18 Plus Cartridge using 95% MeCN in 0.1% aqueous NH_4OH for compound elution. [^{14}C]Lipid ϕ.10 analogue was prepared through incubation of Lipid ϕ.1 analogue (3.6 μM) with TagF (2.4 μM) and CDP-[U-^{14}C]glycerol (32.4 μM, 0.3 μCi) for 4 hours in 50 mM Tris–HCl (pH 8) and 30 mM $MgCl_2$ at ambient temperature.[15] [^{14}C]Lipid ϕ.40 analogue was prepared in a similar manner using 140 μM CDP-[U-^{14}C]glycerol (0.3 μCi). [^{14}C]CDP-glycerol-linked polymer was prepared enzymatically through incubation of TagF (2.4 μM) and CDP-[U-^{14}C]glycerol (36 μM, 0.3 μCi) for 4 hours in 50 mM Tris–HCl (pH 8), 30 mM $MgCl_2$ and 0.13% (v/v) Triton X-100 at ambient temperature.[36] [^{14}C]-Lipid α standard was prepared following previously described TarO activity assays of *E. coli* cell membranes.[12] Briefly, TarO-enriched membranes (500 μg protein) were incubated with UDP-GlcNAc (300 μM), UDP-[^{14}C]GlcNAc (0.1 μCi), 0.1% TritonX-100 and reaction buffer (50 mM Tris pH = 8, 10 mM $MgCl_2$, 1 mM EDTA) for 2 hours at ambient temperature. Lipid-linked products were extracted following known methods,[12] and analyzed by TLC.

High-resolution mass spectrometry

High-resolution mass spectra and collision-induced dissociation mass spectra (MS/MS) were collected on an Agilent 6520 quadrupole time-of-flight (Q-TOF) mass spectrometer following previously described methods.[48] All compounds were lyophilized and resuspended in $CHCl_3/CH_3OH$ (2 : 1) prior to analysis.

TagA and TagB *in vitro* reconstitution

To reconstitute the activity of TagA, dried semisynthetic Lipid α (0.22 mM) was resuspended in a buffer containing 0.13% (v/v) Triton X-100, 50 mM Tris (pH 8.0) and 220 mM NaCl. The mixture was vortexed vigorously and sonicated for 5 min at ambient temperature. TagA (11.2 μM), MnaA (6 μM) and UDP-[^{14}C]GlcNAc (1 mM, 0.1 μCi) were added to initiate the reaction. The reaction mixture incubated at ambient temperature for 4 hours then overnight at 5 °C. TagB reconstitution assays were performed identically, with the exception that CDP-glycerol (2 mM) and TagB (6 μM) were added to the reaction mixture after 4 hours of incubation. A control reaction containing no Lipid α was also set up under these conditions. Reactions were quenched using $CHCl_3/CH_3OH$ (3 : 2) and lipid-linked products were extracted according to published methods.[12,33] Reaction extracts and [^{14}C]-Lipid α standard were separated by normal phase TLC using a $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4) solvent system. The plates were dried and exposed overnight at ambient temperature to a storage phosphor screen (GE Healthcare). Radioactive compounds were visualized using a Typhoon Trio Variable Mode Imager (GE Healthcare). Identical TagA and TagB reconstitution reactions were set up using nonradioactive UDP-GlcNAc for MS analysis.

TagF and TagE *in vitro* reconstitution

Lipid ϕ.1 was obtained from lipid extracts of non-radioactive TagB reconstitution reactions. To reconstitute the activity of TagF, dried Lipid ϕ.1 was resuspended in a buffer containing 0.13% (v/v) Triton X-100, 50 mM Tris (pH 8.0) and 30 mM $MgCl_2$. This solution was vortexed vigorously and sonicated for 5 minutes at ambient temperature. TagF (2.4 μM) and CDP-[^{14}C]glycerol (36 μM, 0.3 μCi) were added to initiate the reaction. The reaction mixture incubated at room temperature for 4 hours. An identical reaction was set up using CDP-glycerol in place of CDP-[^{14}C]glycerol to generate non-radioactive Lipid ϕ.*n* for TagE reconstitution assays. To reconstitute the activity of TagE, UDP-[^{14}C]glucose (36 μM, 0.4 μCi) and TagE (1 μM) were added to a non-radioactive TagF reaction after 4 hours of incubation. The reaction mixture proceeded for an additional 4 hours at ambient temperature. TagF and TagE reactions were treated with mild alkali conditions to liberate poly (glycerol phosphate) polymers from disacharide anchors[35] for anion

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exchange HPLC analysis. Briefly, each reaction mixture was treated to 0.5 M NaOH for 25 min at 37 °C. The reaction mixtures were neutralized with the addition of 0.5 M HCl prior to anion exchange HPLC analysis. For WTA lability experiments, these reactions underwent further treatment in 1 N HCl for 3 hours at 100 °C (ref. 35) prior to anion exchange HPLC analysis.

Acknowledgements

We thank Dr Kalinka Koteva and Dr Mehdi Keramane for assistance during chemical synthesis. We also thank Soumaya Zlitni and Dr Sebastian Gehrke for helpful discussions in the preparation of this manuscript. We are grateful for the guidance provided by Dr Eefjan Breukink for the preparation of undecaprenyl phosphate. Support to EDB for this work included a salary award (Canada Research Chair) and an operating grant from the Canadian Institutes of Health Research (MOP-15496). This work was also supported by National Science Foundation Major Research Instrumentation Award 1039659 to TAG.

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Published on 17 June 2014. Downloaded by Northern Illinois University on 09/09/2014 15:36:10.

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