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The Wall Teichoic Acid Polymerase TagF Efficiently Synthesizes Poly(glycerol phosphate) on the TagB Product Lipid III

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Our understanding of the function of cell-wall teichoic acid polymerases such as TagF from *Bacillus subtilis* has been limited by the tools available for a functional assay. Teichoic acid polymerase activity has previously been studied by using crude membrane preparations as a source of substrate(s). Thus, an understanding of the most basic features of the teichoic acid polymerization has eluded characterization. Here we make use of a soluble synthetic glycolipid to provide the first demonstration that TagF polymerizes glycerol phosphate directly on the product of TagB—teichoic acid lipid III—at a rate approximately 100 times higher than observed with crude membrane preparations. Interestingly, polymer length was determined by the ratio of glycolipid acceptor to CDP-glycerol, implying that polymerization occurs in a distributive manner. This work provides new insights into the reaction catalyzed by TagF, a prototypic teichoic acid polymerase.

The bacterial cell wall has been a popular target for the design of antibacterial agents. Nevertheless, cell wall-active antibiotics have exclusively targeted peptidoglycan synthesis and thus overlook other cell wall components. In Gram-positive bacteria, cell wall teichoic acids are a chemically diverse group of phosphate-rich polymers that are covalently linked to peptidoglycan. Wall teichoic acid accounts for up to 60% of the Gram-positive cell-wall dry weight.[1] Indeed, wall teichoic acid has recently been shown to be essential to the proper rod-shaped morphology of *Bacillus subtilis*[2] and a key virulence determinant for the human pathogen *Staphylococcus aureus*.[3,4] Wall teichoic acid synthesis is thus an emerging target for the development of new cell wall-active antibiotics.

Teichoic acid polymers in *B. subtilis* 168 consist of repeats of glycerol phosphate linked through a phosphodiester bond from the 1-position carbon to the terminal phosphate.[5] The polymer is covalently attached to the cell wall peptidoglycan through a disaccharide phosphate "linkage unit" that bridges the 6-hydroxyl of muramic acid and the poly(glycerol phosphate) polymer (Scheme 1 A). Collectively, sequence-based homology analysis of teichoic acid gene clusters, studies of recombinant Tag proteins, and analyses of the chemical structure of wall teichoic acid have begun to describe the biosynthetic pathway for wall teichoic acid biogenesis.[6] Synthesis is initiated on the cytoplasmic face of the membrane on an undecaprenyl phosphate molecule, through a stepwise addition at the non-reducing end, of *N*-acetylglucosamine-1-phosphate (TagO), *N*-acetylmannosamine (TagA),[7,8] and a single residue of glycerol phosphate (TagB)[7,9] to produce an undecaprenyl glycolipid that has been dubbed the teichoic acid linkage unit[10] or lipid III, following a more recent naming scheme for teichoic acid intermediates proposed by Ginsberg et al. (Scheme 1 B).[7] The remaining and still enigmatic functions in teichoic acid synthesis are the assembly of a complete poly(glycerol phosphate) polymer on lipid III and its transfer to peptidoglycan. While there are no candidate genes for the latter transferase activity, *tagF* has long been associated with the poly(glycerol phosphate) polymerase function.[11,12] The TagF protein, however, has no significant sequence homology to proteins of known function, and so characterization of the role of this novel protein in poly(glycerol phosphate) synthesis remains a key landmark in understanding of teichoic acid biogenesis.

Advancement of the study of cell wall teichoic acid polymerases such as TagF has been hindered by a lack of the necessary chemical tools for a functional assay. The association of TagF to the intracellular face of the membrane bilayer[13] along with the intermediates of teichoic acid biosynthesis has complicated purification of assay components. Previously, membrane preparations were necessary as a source of substrate(s) to investigate the function of teichoic acid polymerases. Using this crude substrate source, our laboratory has collected considerable evidence suggesting that the TagF enzyme plays a role in the formation of the glycerol phosphate polymer.[12,14] In that work, we showed that recombinant purified TagF protein could potentiate the incorporation of a glycerol phosphate polymer into *B. subtilis* membrane preparations when incubated with CDP-glycerol.[12] While these experiments failed unequivocally to define the protein and substrate complement required for teichoic acid polymer synthesis, they were nevertheless consistent with a role for TagF as the poly(glycerol phosphate) polymerase. Because of the undefined natures of the substrates, however, we could not rule out other possible factors in polymer synthesis. TagF may have formed a polymer of glycerol phosphate directly on lipid III or perhaps onto a separate undecaprenyl phosphate moiety with subsequent transfer of the polymer to lipid III. Similarly, soluble assembly of a glycerol phosphate polymer before incorporation into a membrane precursor was also a plausible scenario. The possible mechanisms of TagF polymerization and incorporation of

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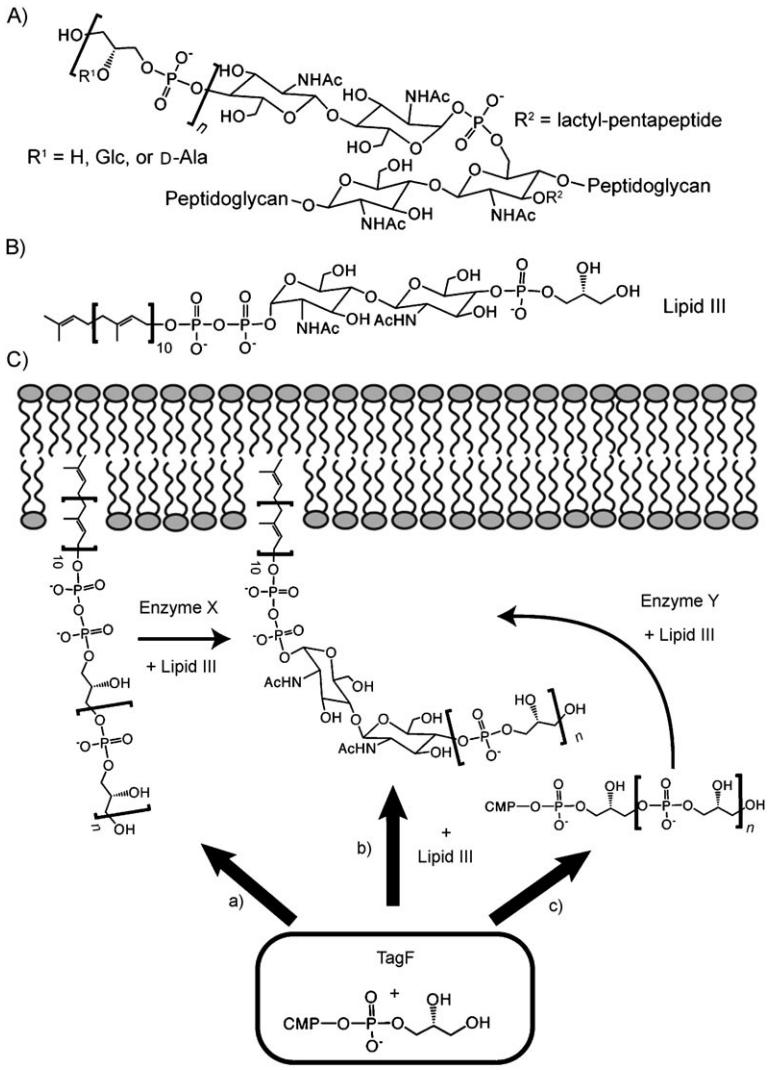

Scheme 1. Reaction catalyzed by TagF. A) Structure of wall teichoic acid linked to the 6-hydroxyl of muramic acid in peptidoglycan. B) Structure of TagB product lipid III. C) Possible mechanisms of polymerization by TagF: a) polymerization of glycerol phosphate onto an undecaprenyl phosphate carrier with subsequent addition to lipid III by an unknown protein, b) direct polymerization of glycerol phosphate onto the product of TagB, lipid III, and c) soluble polymerization on a nucleotide activated carrier with subsequent enzymatic addition to lipid III.

glycerol phosphate into a membrane fraction are summarized in Scheme 1 C.

Soluble substrate analogues have been opportune for the study of cell wall enzymes with substrates that contain isoprenoid membrane anchors. Peptidoglycan lipid I analogues have been created to study the glycosyltransferase activity of MurG,[15,16] and more recently peptidoglycan lipid II analogues have been adapted to a soluble assay of PBP1A transglycosylase activity from *Escherichia coli* and *Aquifex aeolicus*.[17,18] These molecules have been instrumental in the study of substrate specificity for peptidoglycan glycosyltransferase/polymerases. Here we describe the use of a teichoic acid lipid III analogue for the study of TagF in a defined assay system.

In testing the hypothesis that lipid III is the substrate for the TagF protein we employed a substrate analogue in which the C_{55} undecaprenyl group of lipid III was substituted with a 13-unit saturated hydrocarbon. Previous work had shown that this poly-isoprenoid mimic significantly increased the solubility of the glycolipid and maintained the chemical reactivity of the associated carbohydrate functionality.[7,8] Figure 1 shows our efforts to test the dependence of the transfer of glycerol phosphate from CDP-glycerol to this pseudosubstrate both on time and on TagF concentration through an HPLC assay that monitors CMP production. The assay showed a strict linear time dependence with saturating substrates, and the rates of TagF-catalyzed glycerol phosphate transfer were directly proportional to the TagF protein added to this reaction mixture (Figure 1). Indeed, this relationship allowed for the estimation of a turnover number of 27 s^{-1} for TagF with this substrate. This rate represents a turnover 100 times greater than that determined previously by use of inverted membrane vesicles (16 min^{-1})[12] as a crude, undefined substrate. Furthermore, the experiments here have employed a chemically defined system containing only TagF, CDP-glycerol, and a lipid III analogue, implying that neither accessory membrane components nor proteins were required for this activity.

To investigate the acceptor specificity of TagF we have tested a wide variety of acceptor substrates including farnesol, farnesyl phosphate, geranylgeranyl phosphate, glycerol, and glycerol phosphate. Incubation of TagF and CDP-glycerol with these compounds

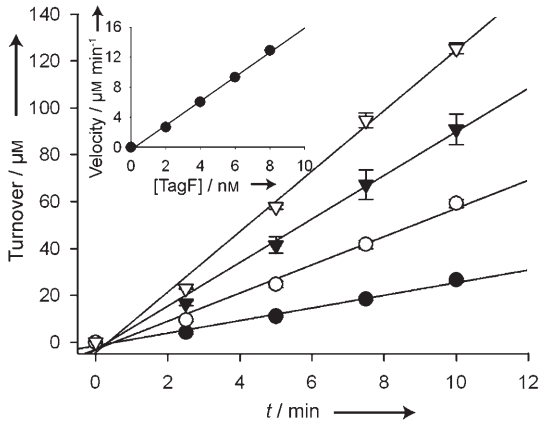

Figure 1. Dependence of TagF polymerase activity on time and enzyme concentration. TagF activity was assayed by monitoring CMP release from CDP-glycerol during polymerization. In a reaction mixture containing Tris (pH 7.5, 50 mM) and $MgCl_2$ (30 mM), TagF [2 nM (●), 4 nM (○), 6 nM (▼), and 8 nM (▽)] was incubated for 2.5–10 min with CDP-glycerol (1 mM) and lipid III (20 μM). Reactions were quenched with urea (4 M). Turnover of CDP-glycerol to CMP was monitored by absorbance at 271 nm after separation by paired-ion HPLC. Inset: Derived initial velocities plotted as a function of TagF concentration. The calculated turnover from the slope of the regression line was 27 s^{-1}. Reaction rates were linear as a function of time and as a function of the concentration of the TagF protein.

did not produce any detectable glycerol phosphate transfer. We also tested the product of the TagA reaction as a potential substrate and found no evidence of glycerol phosphate transfer to this substrate by TagF (Figure S3). These data suggest that TagF has a strict specificity for an acceptor substrate that has been primed by the addition of a single glycerol phosphate residue. Interestingly, this is consistent with the genetic indispensability of the *tagB* gene in teichoic acid biosynthesis.[2]

The suitability of the lipid III analogue as a substrate for TagF was further evaluated through the determination of steady-state Michaelis constants. Figure 2 outlines the dependence of

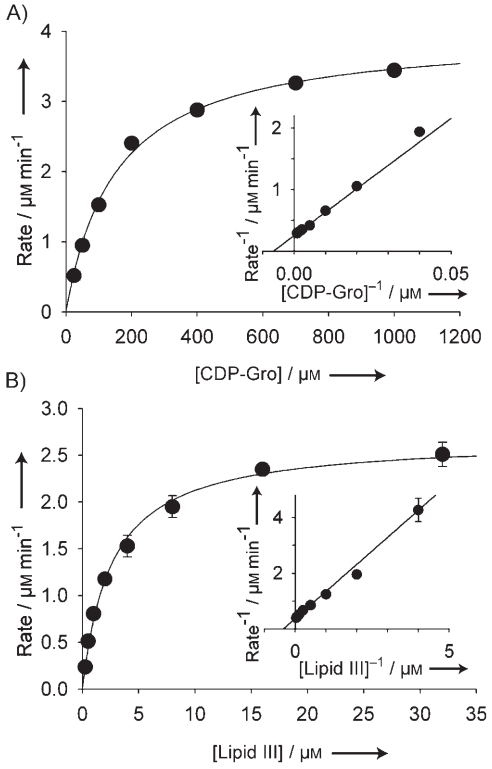

Figure 2. Dependence of TagF polymerase initial velocity rates on concentration of CDP-glycerol and lipid III. All reactions were carried out with TagF (2.5 nM), Tris (pH 7.5, 50 mM) and $MgCl_2$ (30 mM). A) The apparent K_m for CDP-glycerol was determined to be 152 ± 8 μM by plotting of initial rates as a function of CDP-glycerol concentrations; lipid III was maintained at the saturating concentration of 20 μM, and CDP-glycerol was varied from 25 μM to 1000 μM. B) The apparent K_m for lipid III was determined to be 2.6 ± 0.2 μM by plotting of initial rates as a function of lipid III concentrations. CDP-glycerol was maintained at 400 μM, and lipid III was varied from 0.25 μM to 32 μM. Insets: double reciprocal plots of $1/v$ versus 1/[S] for each substrate. All data were fitted by nonlinear least-squares regression to the equation $v = V_{max}[S]/(K_m + [S])$ by use of Sigmaplot 8.0 (SPSS Inc., Chicago, IL).

TagF reaction velocity on the concentrations both of CDP-glycerol and of lipid III. The apparent K_m of 152 ± 8 μM for CDP-glycerol (lipid III fixed at 20 μM) was consistent with our published observations (K_m = 340 μM) made with recombinant TagF and crude membrane preparations.[12] That the K_m value for CDP-glycerol was largely unchanged in our chemically defined system provides some comfort that the reaction catalyzed by TagF with the lipid III analogue was comparable to that observed when TagF was assayed with the use of membrane vesicles. Perhaps most importantly, we now have, for the first time, a sense of the Michaelis parameters for the acceptor lipid III. We determined an apparent K_m value for the lipid III analogue of 2.6 ± 0.2 μM (CDP-glycerol fixed at 400 μM), a value 60 times lower than that of CDP-glycerol. The high specificity constant for the lipid III analogue ($k_{cat}/K_m = 1.1 \times 10^7\ M^{-1} s^{-1}$) is a strong argument that lipid III is indeed the physiological substrate for TagF. Similarly, these findings suggest that the lipid III analogue used in this work is a suitable synthetic pseudosubstrate for lipid III. We cannot rule out the possibility that the kinetic parameters may differ in catalysis of polymer formation on the membrane-embedded physiological substrate. It has previously been shown, however, that enzymes involved in teichoic linkage unit synthesis fractionated with undecaprenyl-linked intermediates in sucrose density centrifugation, while enzymes involved in the main chain teichoic acid synthesis (TagF) were not associated with these lipid-tethered molecules.[19] Hence enzymes involved in lipid III synthesis may be more sensitive to modification in the lipid region than TagF. For example, the enzyme TagA showed a preference for longer aliphatic chains.[8] Conversely, the undecaprenyl tether of lipid III and the peripheral membrane association of TagF likely function to colocalize enzyme and substrate at the membrane; chemical recognition of lipid III by TagF is therefore expected to be mediated by the dissacharide-glycerol phosphate moiety. Substitution of the natural undecaprenyl moiety with a 13-unit saturated hydrocarbon may therefore have had little impact on substrate binding and specificity. In sum, the work here provides strong evidence for a model in which TagF catalyzes the polymerization of poly(glycerol phosphate) directly onto lipid III (Scheme 1C, model b.).

Size exclusion chromatography was used to evaluate the poly(glycerol phosphate) polymer length formed by the TagF enzyme on lipid III. A ^{14}C radiolabel present exclusively on the ManNAc of the glycolipid acceptor or on the glycerol of CDP-glycerol was used to differentiate between the lipid III substrate and the poly(glycerol phosphate) components of teichoic acid during chromatography. The use either of [^{14}C]lipid III or of [^{14}C]CDP-glycerol produced a radiolabeled polymer in the TagF reaction, confirming that glycerol phosphate is polymerized on lipid III. Varying ratios of lipid III analogue (2.5–250 μM) to CDP-glycerol (1 mM) were incubated until only trace amounts of CDP-glycerol remained (1 h). Figure 3 shows the retention times for TagF reactions with the different ratios of lipid III to [^{14}C]CDP-glycerol on a Waters ProteinPak SW300 (Mississauga, ON) size exclusion column (0.1% NH_4HCO_3/10% MeCN, 0.5 mL min^{-1}). Single-stranded deoxyribonucleic acids (ssDNAs) were used as calibration standards. A single nucleotide in the phosphodiester backbone of the ssDNA represents an appropriate metric for a single glycerol phosphate unit of teichoic acid as the two repeating units contain an identical number of bonds between the phosphodiester linkages.

From calibration with polynucleotide standards it is clear that the polymer length can be controlled by varying the ratios of substrates. A ratio of four CDP-glycerol molecules per

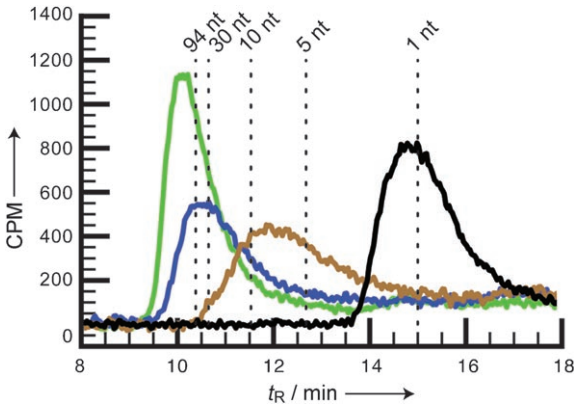

Figure 3. Size exclusion analysis of the TagF polymer product with varying ratios of CDP-glycerol to the lipid III analogue. CDP-glycerol was varied from four to 400 equiv of lipid III, and the reaction was left to go to completion. All reaction mixtures contained CDP-glycerol (1 mM) and TagF (100 nM). The black trace indicates the elution for [^{14}C]CDP-glycerol, the brown trace indicates the polymer elution for a reaction with a 4:1 ratio of CDP-glycerol to lipid III (250 μM), the blue trace for a reaction with a 40:1 ratio of CDP-glycerol to lipid III (25 μM), and the green trace for a reaction with a 400:1 ratio of CDP-glycerol to lipid III (2.5 μM). Vertical dotted lines indicate retention times for single stranded nucleotide (nt) calibration standards.

lipid III analogue forms a polymer eluting between the 5-unit and 10-unit standards. This retention time is consistent with the expected size of four glycerol phosphate units plus lipid III analogue; the lipid III analogue is approximately the length of four glycerol phosphate units as judged by the number of intramolecular bonds in the linear molecule. Similarly, the incubations of 40 CDP-glycerol molecules per lipid III analogue eluted between the 30-unit and the 94-unit ssDNA standards, a retentio time consistent with polymers containing approximately 40 glycerol phosphate residues. Incubations of 400 CDP-glycerol molecules per lipid III eluted in the column void. This would be consistent with the migration of a large polymer approximately 400 units in length. Control of polymer length by substrate availability as seen with TagF is indicative of a distributive mechanism of polymerization. A distributive polymerase repeatedly dissociates from and reassociates to the polymer, adding only as few as one unit in each binding event, while a processive polymerase will remain associated with the growing polymer and will catalyze many additions in a single binding event.[20] A processive mechanism would form equivalently long polymers from both high and low ratios of CDP-glycerol to lipid III as the ratio of the effective concentration of TagF-lipid III complexes to CDP-glycerol would be identical and independent of lipid III concentration, provided that TagF concentration is limiting. Further biochemical studies are necessary to determine the factors controlling polymer length and whether TagF exhibits any degree of processivity.

The large polymers seen in our in vitro sizing studies with recombinant TagF and defined substrates are curious in light of the well established polymer size of 45–60 glycerol phosphate units for teichoic acid in the cell wall of *B. subtilis* in vivo.[21] Perhaps length regulation is determined by membrane association of the TagF enzyme, or a chemical modification of the terminal residue by a proteinaceous factor as seen in LPS O-antigen biosynthesis.[22] Indeed, poly(glycerol phosphate) teichoic acid is thought to be modified at the 2-position on the cytoplasmic face of the cell membrane by a glucosyltransferase encoded in *tagE*.[23] Glucosylation of the terminal residue of the teichoic acid chain by the TagE enzyme possibly provides a length-determining modification to prevent re-binding and further polymerization by TagF. Alternatively, length may be determined by intracellular substrate concentrations. It has been shown in vivo and in vitro that capsular polysaccharide length can be determined by availability of nucleotide activated precursors in *Streptococcus pneumoniae*.[24,25] In the experiments reported here for the TagF reaction, we have shown that the relative proportion of substrates CDP-glycerol to the lipid III analogue could determine polymer length in a reaction that went largely to completion. Where the next step in wall teichoic acid biogenesis is export by the ABC transporter TagGH, it is conceivable that the kinetics of poly(glycerol phosphate) synthesis and the general flux of metabolites through this biosynthetic pathway govern substrate availability and play a dominant role in polymer length.

Wall teichoic acid synthesis is an emerging target for the development of new cell-wall-active antibiotics. Sequence-based homology analysis of teichoic acid gene clusters, studies of recombinant Tag proteins, and analyses of the chemical structure of wall teichoic acid have begun to describe the biosynthetic pathway for wall teichoic acid biogenesis. The remaining and still enigmatic functions in teichoic synthesis are the assembly of a complete poly(glycerol phosphate) polymer on lipid III and its transfer to peptidoglycan. The TagF enzyme has long been associated with the poly(glycerol phosphate) polymerase function, but has no significant sequence homology to proteins of known function. Previously our laboratory has shown that recombinant purified TagF protein was able to potentiate the incorporation of a glycerol phosphate polymer into *B. subtilis* membrane preparations when incubated with CDP-glycerol. While these experiments were consistent with a role for TagF as the poly(glycerol phosphate) polymerase, we could not define the protein or substrate complement necessary for polymer synthesis. Here we describe the use of a teichoic acid lipid III analogue for the study of TagF in a chemically defined assay system. We have shown for the first time that the TagF protein alone polymerizes glycerol phosphate onto the lipid III analogue without a requirement for exogenous protein factors and establishes lipid III and CDP-glycerol as the sole substrates required for this reaction. Finally we have shown that TagF catalyzes the poly(glycerol phosphate) synthesis in a distributive reaction in which the size of the polymer is governed by the ratio of the substrates CDP-glycerol and lipid III. The assay system presented here for TagF opens the door for more detailed studies of the kinetics and chemical mechanism of this enigmatic polymerase and related enzymes. Furthermore, the chemically defined assay system shows promise for high-throughput screening for the discovery of small molecule inhibitors that may serve as probes for TagF mechanism and function, and also as leads for new antibacterial drugs.

Experimental Section

General methods: All reagents were purchased from Sigma–Aldrich (Mississauga, ON) unless otherwise specified. Scintillation fluid and [^{14}C]-UDP-GlcNAc (288 mCi mmol^{-1}) were purchased from Perkin–Elmer Life Sciences (Boston, MA). [^{14}C]CDP-glycerol (20 mCi mmol^{-1}) was synthesized as previously described.[12] TagB and TagF were purified as previously described.[9,12] Chromatography was performed on a Waters HPLC system (Mississauga, ON). All cloning was performed in *E. coli* Novablues cells (EMB Biosciences, Mississauga, ON). For plasmid selection, kanamycin (50 μg mL^{-1}) and ampicillin (50 μg mL^{-1}) were used.

Cloning, expression, and purification of *Staphylococcus aureus* TagA: *S. aureus tagA* was PCR amplified by use of the Roche High Fidelity PCR System™ (Roche, Laval, QC) with primers *tagA* For (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACCA-TGACTGTTGAAGAAAGATCC-3′) and *tagA* Rev (5′-GGGGACCACTT-TGTACAAGAAAGCTGGGTCCTAGTGATGGTGATGGTGATGTTTCGCCTT-TTTTATTTTTCTTTTTGC-3′). The PCR product was subsequently cloned into pDONR201 followed by pDEST14 by use of the GATEWAY PCR and Expression Cloning Systems (Invitrogen, Burlington, ON). The resulting plasmid pDEST14-TagAHis was transformed into a BL21-AI cell line (Invitrogen, Burlington, ON) for protein expression, resulting in strain EB1245.

For isolation of His-tagged TagA, LB medium (4 L) was inoculated (1:100) with an overnight culture of EB1245 and grown at 37 °C (250 rpm). The cultures were allowed reach an OD_{600} of 0.4 before induction with arabinose (0.2%). After 3 h of induction (37 °C, 250 rpm), the cells were harvested by centrifugation (7000 *g* for 15 min) and washed with saline. The resulting pellet was suspended in buffer A [25 mL, $NaH_2(PO_4)$ (20 mM), NaCl (500 mM), and glycerol (10%), pH 7.4] with imidazole (15 mM) containing EDTA-free Complete™ protease inhibitor (Roche, Laval, QC) and passaged three times through the French press (69 MPa). The debris was separated by centrifugation (40000 *g* for 30 min) followed by filtration through a 0.45 μm filter. The resulting solution was purified over a HiTrap Ni^{2+} chelating column (1 mL, Amersham, Baie d'Urfe, QC) with use of a gradient from 15–100% buffer B [$NaH_2(PO_4)$ (20 mM), NaCl (500 mM), glycerol (10%), and imidazole (500 mM), pH 7.4]. The resulting fractions were analyzed by SDS-PAGE, and fractions containing TagA were pooled at a concentration of 2.58 μM.

Cloning, expression, and purification of *Bacillus subtilis* MnaA: *B. subtilis mnaA* was amplified by polymerase chain reaction with Vent DNA polymerase (New England Biolabs, Beverly, MA) and primers mnaAfor1 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAAA-AAAACTAAAAGTGATGACCG-3′) and mnaArev1 (5′-GGGGACCACTT-TGTACAAGAAAGCTGGGTCTTATTTGCCTGTAAATGAATCCGG-3′). The PCR product was subsequently recombined into pDONR201 followed by pDEST17 with the aid of the GATEWAY™ PCR and Expression Cloning Systems (Invitrogen, Burlington, ON). All cloning procedures were carried out in *Escherichia coli* Novablue strain (EMD Biosciences, Mississauga, ON). The resulting plasmid pDEST17-mnaA has an amino-terminal hexahistidine fusion and has the additional residues MSYYHHHHHHLESTSLYKKAGL at the amino terminus. This plasmid was transformed into the *E. coli* BL21 (DE3) cell line for protein expression resulting in strain EB594.

For isolation of hexahistidine-tagged MnaA, LB medium (4 L) was inoculated (1:100) with an overnight culture of EB594 and grown at 37 °C at 250 rpm for 3 h. The cultures were induced with isopropyl β-D-thiogalactoside (1 mM) and incubated for 16 h at 16 °C and 200 rpm. The cells were harvested by centrifugation (7000 *g* for 15 min) and washed with saline. The resulting pellet was suspended in lysis buffer [30 mL, $NaH_2(PO_4)$ (20 mM), pH 7.4, NaCl (500 mM), glycerol (10%), DNAse (10 μg mL^{-1}), RNAse (10 μg mL^{-1}), EDTA-free Complete™ protease inhibitor (Roche, Mississauga, ON)] and lysed by passage through a French Pressure cell. The debris was separated by centrifugation (40000 *g* for 30 min), and the resulting supernatant was loaded onto a HiTrap Ni^{2+} chelating column (1 mL, Amersham Biosciences, Baie D'Urfe, PQ) with use of a discontinuous gradient of imidazole (15 mM to 500 mM). Fractions were analyzed by SDS-PAGE, and those containing MnaA were pooled at a concentration of 61.76 μM.

Synthesis of teichoic acid lipid III: A soluble analogue of the product of the TagO reaction (C_{13}PP-GlcNAc) was synthesized as previously described.[7,18,26–28] Further details of the chemical synthesis can be found in the Supporting Information. UDP-ManNAc was enzymatically synthesized from UDP-GlcNAc by use of the enzyme MnaA.[29] Synthesis of the TagA product and the TagB product lipid III was monitored either by absorbance (210 nm) or by incorporation of radiolabeled ManNAc and glycerol phosphate onto the lipid carrier by in-line scintillation counting after separation by a linear gradient of NH_4HCO_3 (0.1%, Fisher Scientific, Ottawa, ON) and MeCN on a 30 cm C18 Symmetry column (Waters, Mississauga, ON). A one-pot coupled reaction was used to synthesize the lipid III analogue. UDP-GlcNAc (1 mM), C_{13}PP-GlcNAc (1 mM), alkaline phosphatase (20 units, Roche, Indianapolis, IN), MnaA (1.2 μM), and TagA (0.25 μM) were all placed in a single reaction vessel. Without further purification the intermediate (C_{13}PP-GlcNAc-ManNAc) was converted into the TagF substrate by addition of TagB (1.3 μM) and CDP-glycerol (1 mM). The lipid III analogue was synthesized to a final concentration of 700 μM. The reaction scheme is depicted in the Supporting Information. Proteins were removed by passing the reaction through an Ultrafree-MC 5000 NMWL centrifugal filter (Fisher Scientific, ON). Radiolabeled C_{13}PP-GlcNAc-ManNAc or lipid III was made by the addition of [^{14}C]-UDP-GlcNAc or [^{14}C]-CDP-glycerol.

TagF assay: Substrates and products of the TagF reaction were separated by reversed-phase chromatography on a Waters Novapak C–18 column (Mississauga, ON) with the ion pairing agent tetrabutylammoniumhydrogen sulfate (TBAHS). Product CMP eluted after a short retention in buffer PicA [potassium phosphate (15 mM), TBAHS (10 mM), pH 7] and the substrate CDP-glycerol eluted at the start of a 4 min linear gradient to PicB [potassium phosphate (35 mM), TBAHS (10 mM), acetonitrile (30%), pH 7]. Substrates and products were detected by absorbance at 271 nm, and turnover was calculated on the basis of the ratio of the integrated peaks. Reactions were quenched by addition of urea (4 M).

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Keywords: enzyme catalysis · glycerol phosphate polymer · glycolipids · lipid III · teichoic acid

[1] I. C. Hancock, *Biochem. Soc. Trans.* **1997**, *25*, 183–187.
[2] M. A. D'Elia, K. E. Millar, T. J. Beveridge, E. D. Brown, *J. Bacteriol.* **2006**, *188*, 8313–8316.

[3] M. A. D'Elia, M. P. Pereira, Y. S. Chung, W. Zhao, A. Chau, T. J. Kenney, M. C. Sulavik, T. A. Black, E. D. Brown, *J. Bacteriol.* **2006**, *188*, 4183–4189.
[4] C. Weidenmaier, J. F. Kokai-Kun, S. A. Kristian, T. Chanturiya, H. Kalbacher, M. Gross, G. Nicholson, B. Neumeister, J. J. Mond, A. Peschel, *Nat. Med.* **2004**, *10*, 243–245.
[5] W. W. Navarre, O. Schneewind, *Microbiol. Mol. Biol. Rev.* **1999**, *63*, 174–229.
[6] A. P. Bhavsar, E. D. Brown, *Mol. Microbiol.* **2006**, *60*, 1077–1090.
[7] C. Ginsberg, Y. H. Zhang, Y. Yuan, S. Walker, *ACS Chem. Biol.* **2006**, *1*, 25–28.
[8] Y. H. Zhang, C. Ginsberg, Y. Yuan, S. Walker, *Biochemistry* **2006**, *45*, 10895–10904.
[9] A. Bhavsar, R. Truant, E. Brown, *J. Biol. Chem.* **2005**, *280*, 36691–36700.
[10] C. R. Harrington, J. Baddiley, *Eur. J. Biochem.* **1985**, *153*, 639–645.
[11] H. M. Pooley, F. X. Abellan, D. Karamata, *J. Bacteriol.* **1992**, *174*, 646–649.
[12] J. W. Schertzer, E. D. Brown, *J. Biol. Chem.* **2003**, *278*, 18002–18007.
[13] A. Formstone, R. Carballido-Lopez, P. Noirot, J. Errington, D. J. Scheffers, *J. Bacteriol.* **2008**, *190*, 1812–1821.
[14] J. W. Schertzer, A. P. Bhavsar, E. D. Brown, *J. Biol. Chem.* **2005**, *280*, 36683–36690.
[15] P. Cudic, D. C. Behenna, M. K. Yu, R. G. Kruger, L. M. Szewczuk, D. G. McCafferty, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 3107–3110.
[16] H. Liu, T. K. Ritter, R. Sadamoto, P. S. Sears, M. Wu, C. H. Wong, *ChemBioChem* **2003**, *4*, 603–609.
[17] D. Barrett, T. Wang, Y. Yuan, Y. Zhang, D. Kahne, S. Walker, *J. Biol. Chem.* **2007**, *282*, 31964–31971.
[18] X. Y. Ye, M. C. Lo, L. Brunner, D. Walker, D. Kahne, S. Walker, *J. Am. Chem. Soc.* **2001**, *123*, 3155–3156.
[19] J. Leaver, I. C. Hancock, J. Baddiley, *J. Bacteriol.* **1981**, *146*, 847–852.
[20] L. P. Bloom, M. F. Goodman, *Polymerase Processivity: Measurement and Mechanisms, in Encyclopedia Of Life Sciences*, Wiley, Chichester, **2001**.
[21] J. Pollack, F. Neuhaus, *J. Bacteriol.* **1994**, *176*, 7252–7259.
[22] C. Raetz, C. Whitfield, *Annu. Rev. Biochem.* **2002**, *71*, 635–700.
[23] C. Mauël, M. Young, D. Karamata, *J. Gen. Microbiol.* **1991**, *137*, 929–941.
[24] W. T. Forsee, R. T. Cartee, J. Yother, *J. Biol. Chem.* **2000**, *275*, 25972–25978.
[25] C. L. Ventura, R. T. Cartee, W. T. Forsee, J. Yother, *Mol. Microbiol.* **2006**, *61*, 723–733.
[26] L. Chen, H. Men, S. Ha, X. Y. Ye, L. Brunner, Y. Hu, S. Walker, *Biochemistry* **2002**, *41*, 6824–6833.
[27] L. Danilov, T. Chojnacki, *FEBS Lett.* **1981**, *131*, 310–312.
[28] M. M. Sim, H. Kondo, C. H. Wong, *J. Am. Chem. Soc.* **1993**, *115*, 2260–2267.
[29] B. Soldo, V. Lazarevic, H. M. Pooley, D. Karamata, *J. Bacteriol.* **2002**, *184*, 4316–4320.

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