Studies of the Genetics, Function and Kinetic Mechanism of TagE – the Wall Teichoic Acid Glycosyltransferase in Bacillus subtilis 168

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The biosynthetic enzymes involved in wall teichoic acid biogenesis in Gram-positive bacteria have been the subject of renewed investigation in recent years with the benefit of modern tools of biochemistry and genetics. Nevertheless, there have been only limited investigations into the enzymes that glycosylate wall teichoic acid. Decades-old experiments in the model Gram-positive bacterium, Bacillus subtilis 168, using phage resistant mutants implicated tagE (also called gtaA and rodD) as the gene coding for the wall teichoic acid glycosyltransferase. This study and others have provided only indirect evidence to support a role for TagE in wall teichoic acid glycosylation. In this work, we showed that deletion of tagE resulted in the loss of α-glucose at the C-2 position of glycerol in the poly(glycerol phosphate) polymer backbone. We also reported the first kinetic characterization of pure, recombinant wall teichoic acid glycosyltransferase using clean synthetic substrates. We investigated the substrate specificity of TagE using a wide variety of acceptor substrates and found that the enzyme had a strong kinetic preference for the transfer of glucose from UDP-glucose to glycerol phosphate in polymeric form. Further, we showed that the enzyme recognized its polymeric (and repetitive) substrate with a sequential kinetic mechanism. This work provides direct evidence that TagE is the wall teichoic acid glycosyltransferase in B. subtilis 168 and provides a strong basis for further studies of the mechanism of wall teichoic acid glycosylation, a largely uncharted aspect of wall teichoic acid biogenesis.

Wall teichoic acids are anionic, phosphate rich polymers that constitute a substantial portion of the cell wall of Gram-positive bacteria. Although the precise function of these polymers is unknown, they have been shown to play a role in critical cellular processes, namely cell shape determination in Bacillus subtilis (1) and pathogenesis in Staphylococcus aureus (2-3). Of the Gram-positive organisms studied to date, most produce either a poly(glycerol phosphate) or poly(ribitol phosphate) polymer as the major wall teichoic acid (4). The main chain hydroxyl groups on both of these polymers are subject to modification with D-alanine and glycosyl residues. The D-alanylation modification of teichoic acids has been extensively studied and has been shown to play an important role in modulating the properties of the bacterial cell envelope, for example, in regulating resistance to certain antimicrobial molecules (4-6). By contrast, there have been limited investigations into wall teichoic acid glycosylation and its functional significance is unknown.

The wall teichoic acid biosynthetic pathway has largely been elucidated in the Gram-positive model organism, B. subtilis 168. This organism produces a linear 1,3-linked poly(glycerol phosphate) polymer that is modified at position 2 of glycerol with D-alanine or glucose (4). Classical genetic experiments in B. subtilis led to the isolation of the tag gene cluster for wall teichoic acid synthesis (7-8) and studies over the past decade using recombinant proteins have assigned biochemical functions to nearly all of the proteins involved in poly(glycerol phosphate) synthesis (9-13). In addition, the pathway responsible for teichoic acid D-alanylation has been characterized in B. subtilis (14), as well as in other bacteria such as S. aureus (5). Together...
these studies have begun to describe a model for wall teichoic acid biosynthesis and modification in *B. subtilis* 168. Synthesis occurs through the sequential action of several enzymes on the cytoplasmic face of the cell membrane on an undecaprenyl phosphate molecule. TagO and TagA add N-acetylglucosamine-1-phosphate and N-acetylmannosamine residues, respectively. TagB primes the undecaprenyl-pyrophosphoryl disaccharide with a single unit of glycerol-3-phosphate to complete formation of the linkage unit. The polymerase, TagF, then catalyzes the addition of 30-50 units of glycerol-3-phosphate – a substrate that is provided by TagD in the activated form, CDP-glycerol. Once synthesis is complete, the polymer is exported by TagGH to the outside of the cell where it is attached to the 6-hydroxyl of N-acetylmuramic acid of peptidoglycan by an unknown transferase and modified with cationic D-alanyl esters (Figure 1) (4,13). Significant gaps still remain, however, in our understanding of wall teichoic acid synthesis, most notably in relation to wall teichoic acid glycosylation.

The putative gene coding for the wall teichoic acid glycosyltransferase in *B. subtilis* 168 was first identified using phage resistant mutants. Mutations in *tagE* have been shown to be associated with resistance to bacteriophages φ25 and φ29, which recognize glucose residues on teichoic acid as a receptor (15-17). A similar approach involving phage resistant mutants was recently used to identify the wall teichoic acid glycosyltransferase in *S. aureus*. An elegant transposon mutagenesis screen for resistance to phage 80 led to the isolation of *tarM* (18). Disruption of this gene led to wall teichoic acid that completely lacked N-acetylmuramic. The wall teichoic acid glycosyltransferase activity of TarM was subsequently confirmed by demonstration that crude extracts containing recombinant enzyme catalyzed the transfer of N-acetylmuramic onto an uncharacterized membrane acceptor in vitro (18).

Glaser and Burger conducted the first and only in vitro study of poly(glycerol phosphate) polymer glycosylation in *B. subtilis* nearly fifty years ago (19). This was a traditional study of multi-step purification of glycosyltransferase activity from wild-type cells where the poly(glycerol phosphate) acceptor was provided in the form of membrane vesicles derived from *B. subtilis*. Thus, neither the enzyme nor the acceptor substrate was homogenous or unambiguously identified. Nevertheless, the TagE protein was later ascribed this activity following work using phage resistant mutants that linked mutations in the encoding gene to the loss of glucose associated with the poly(glycerol phosphate) polymer (16).

In this work, we have demonstrated that precise deletion of *tagE* results in the absence of α-glucose at the C-2 position along the poly(glycerol phosphate) polymer backbone. Furthermore, we have conducted the first biochemical study of purified, recombinant TagE with pure synthetic acceptor substrates and have shown that the enzyme catalyzes the transfer of glucose from UDP-glucose onto a poly(glycerol phosphate) polymer acceptor at an appreciable rate in vitro. Using a robust HPLC-based assay to monitor wall teichoic acid glycosyltransferase activity, we have explored the sugar donor and acceptor specificity of the enzyme and have investigated its steady state kinetic mechanism. TagE showed a strong kinetic preference for UDP-glucose as its sugar donor and utilized a sequential (ternary complex) kinetic mechanism to catalyze the addition of glucose onto acceptor substrates. This study unambiguously establishes TagE as the wall teichoic acid glycosyltransferase in *B. subtilis* 168.

**EXPERIMENTAL PROCEDURES**

*General Methods* – Strains, plasmids and oligonucleotides used in this work are listed in Table 1. *Escherichia coli* and *B. subtilis* strains were grown in Luria–Bertani (LB) medium. Ampicillin was used at a concentration of 50 µg/mL (*E. coli*) while spectinomycin was used at a concentration of 150 µg/mL (*B. subtilis*). HotStar Taq PCR reagents, gel extraction and plasmid mini-prep kits were purchased from Qiagen (Mississauga, ON, Canada). Vent polymerase was obtained from New England Biolabs (Beverly, MA, U.S.A), the Expand PCR system was purchased from Roche Diagnostics (Laval, PQ, Canada), and the Gateway™ cloning system was from Invitrogen (Burlington, ON, Canada). Cloning was performed in the *E. coli* strain Novablue (Novagen, Madison, WI, U.S.A.) according to established protocols (20). *B. subtilis* competent cells were prepared and transformed as
were incubated overnight at 37 °C and then bacteriophage was spotted onto the plate. Plates were cotton-swab and then 10 µL of SPO1 onto a LB-agar plate to form a lawn using a sterile medium. An aliquot of both cultures was streaked assay (25). 1D and 2D 1H and 31P NMR spectra was accomplished using a phenol-sulphuric acid dextrin. The detection of carbohydrate material using a Bio-Gel P-6 column calibrated with blue carboxylic acid was carried out by size exclusion chromatography (95 °C, 1 h). Subsequent purifications were accomplished using a peptidoglycan by treatment with 1 % acetic acid described (24). Teichoic acid was released from determination were carried out as previously described (21). SPO1 phage was obtained from the Bacillus Genetic Stock Center (Ohio State University, Columbus, OH, U.S.A.). All chemicals were purchased from Sigma (Oakville, ON, Canada) unless otherwise specified. UDP-[14C]-glucose and scintillation fluid were purchased from Perkin-Elmer Life Sciences (Boston, MA, U.S.A). CDP-glycerol was synthesized according to established methods (22). MnaA, TarA, TagB, TarD and TagF were purified as previously described (10,12,22-23). Chromatography was performed on a Waters HPLC system (Mississauga, ON, Canada). Construction of a ΔtagE Strain – To create a clean ΔtagE strain, primers tagE-a and tagE-b, tagE-c and tagE-d, and spec-F and spec-R were used with Vent polymerase to amplify chromosomal DNA or plasmid DNA in the latter case. The PCR products were purified and used as templates in a final reaction with primers tagE-a and tagE-d to create a product wherein a spectinomycin resistance cassette beginning at its translational start site and lacking a terminator was flanked by 1kb regions surrounding the tagE locus. The 3kb PCR product was transformed into EB6 to create a tagE deletion strain (EB2252). The resulting strain was confirmed by PCR with spectinomycin cassette specific primers and primers designed to anneal to sequences outside the region of recombination. The ΔtagE strain was also examined for resistance to bacteriophage SPO1. A liquid culture of wild-type B. subtilis 168 and the ΔtagE strain was grown overnight at 30 °C in LB medium. An aliquot of both cultures was streaked onto a LB-agar plate to form a lawn using a sterile cotton-swab and then 10 µL of SPO1 bacteriophage was spotted onto the plate. Plates were incubated overnight at 37 °C and then examined for a clear zone of lysis. Cell Wall Isolation and Analysis – Strains were grown overnight in 100 mL of LB medium at 30 °C. Cell wall isolation and phosphate content determination were carried out as previously described (24). Teichoic acid was released from peptidoglycan by treatment with 1 % acetic acid (95 °C, 1 h). Subsequent purifications were carried out by size exclusion chromatography using a Bio-Gel P-6 column calibrated with blue dextrin. The detection of carbohydrate material was accomplished using a phenol-sulphuric acid assay (25). 1D and 2D 1H and 31P NMR spectra were recorded on a Bruker NSC 600 spectrometer. The temperature was kept at 300 K in all experiments. Prior to performing the NMR experiments, the samples were lyophilized three times with D2O (99.9%). TSP (δH 0.00, δC 0.0) in D2O was used as reference for both 1H and 31C experiments. Orthophosphoric acid (δP 0.0) was used as the external reference for the 31P NMR experiments. Cloning, Expression and Purification of B. subtilis 168 TagE – The Gateway™ recombination-based cloning system and primers tagE-F and tagE-R were used to create a pDEST17-tagE vector for the expression of N-terminal hexahistidine-tagged TagE. The plasmid was transformed into E. coli BL21(AI) cells (Invitrogen, Burlington, ON, Canada). The sequence of tagE inserted into pDEST17-tagE was confirmed by sequencing. E. coli BL21(AI) cells harbouring pDEST17-tagE were grown at 37 °C in LB medium supplemented with 50 µg/mL ampicillin to an OD600 of 0.8. The culture was then induced with 0.2 % (w/v) arabinose and grown for 20 hours at 16 °C. The cells were harvested by centrifugation (8000 × g for 15 minutes) and then washed with 0.85 % NaCl. Cells were resuspended in purification buffer (20 mM sodium phosphate, pH 7.2, 500 mM NaCl, and 5 % glycerol) containing 0.1 mg/mL DNase I, 0.1 mg/mL RNase A, and Calbiochem Protease Inhibitor Cocktail Set III (Roche, Laval, QC, Canada). Cells were lysed by passage through a cell disruptor and then the lysate was spun at 20000 × g for 15 minutes. The pellet was resuspended in purification buffer and CHAPS was added to a final concentration of 2 % (w/v). The resuspended pellet was then incubated for 1 hour at 4 °C with gentle rocking. Following centrifugation at 20000 × g for 15 minutes, the supernatant was filtered through a 0.45 µM filter and applied to a 5-ml Hi-Trap His column (Amersham, Baie d’Urfe, QC, Canada) pre-equilibrated in purification buffer containing 15 mM imidazole. TagE was eluted in a stepwise manner in purification buffer containing 25, 50, and 400 mM imidazole. Fractions were visualized by Coomassie-stained SDS-PAGE and pure fractions of TagE were pooled and dialyzed overnight in dialysis buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM DTT and 10 % glycerol. Following dialysis, the purified protein was separated into aliquots and stored at -80 °C.
Lipid φₙ Analogue Synthesis – Soluble lipid α, β and φ.1 analogues were synthesized according to established methods (9,23). Reactions for the synthesis of the lipid φ.40 analogue contained 50 mM Tris, pH 8.0, 30 mM MgCl₂, 100 nM TagF, and 39 equivalents of CDP-glycerol per lipid φ.1 analogue. Lipid φₙ analogues ranging from 5 to 80 glycerol phosphate units were synthesized by varying the ratio of CDP-glycerol molecules to the lipid φ.1 analogue. Reaction progress was determined by monitoring the conversion of CDP-glycerol to CMP at 271 nm by previously described methods (23). All reactions were allowed to proceed to near completion before being filtered through a 30,000 MWCO centrifugal filter (Millipore, Billerica, MA, U.S.A).

Wall Teichoic Acid Glycosyltransferase Assay – Reactions were conducted at room temperature in buffer containing 50 mM Tris, pH 7.4, 30 mM MgCl₂ and TagE (1-50 nM) with UDP-glucose as the sugar donor and the lipid φ.40 analogue as the acceptor unless otherwise specified. Reactions were quenched by the addition of urea to a final concentration of 6 M. Substrates and products of the TagE reaction were separated by reversed-phase chromatography on an Inertsil ODS-3 column (Canadian Life Sciences, Peterborough, ON, Canada) with the ion pairing agent tetrabutylammoniumhydrogen sulfate (TBAHS). Each sample was eluted at a flow rate of 1 mL/min using a linear gradient of buffer PicA (15 mM potassium phosphate, pH 7.0, 10 mM TBAHS) to PicB (15 mM potassium phosphate, pH 7.0, 10 mM TBAHS, 30 % (v/v) acetonitrile). UDP-Glucose and UDP were detected by absorbance at 262 nm and turnover was calculated on the basis of the ratio of the integrated peaks. For reactions containing UDP-[¹⁴C]-glucose, substrates and products were separated on a Waters Shodex KW-803 column in buffer containing 0.1 % ammonium hydrogen carbonate and 10 % acetonitrile and detected by inline scintillation counting. All initial rate data were fitted by non-linear least-squares regression to the equations in either SigmaPlot 8.0 or the Enzyme Kinetics Module 1.1 (SPSS Inc, Chicago, IL, U.S.A). The Michaelis-Menten equation (Equation 1) and equations that describe sequential (Equation 2) and ping-pong (Equation 3) mechanisms are given below:

Equation 1:
\[ v = \frac{V_{\text{max}} [S]}{(K_m + [S])} \]

Equation 2:
\[ v = \frac{V_{\text{max}} [A][B]}{(K_{ia}K_{mb} + K_{mb}[A] + K_{ma}[B] + [A][B])} \]

Equation 3:
\[ v = \frac{V_{\text{max}} [A][B]}{(K_{mb}[A] + K_{ma}[B] + [A][B])} \]

A and B are the reactants, \( K_{ma} \) and \( K_{mb} \) are the Michaelis constants for A and B and \( K_{ia} \) is the dissociation constant for A from the enzyme complex EA (27).

Poly(glycerol phosphate) Polymerization Assay – A [¹⁴C]-lipid φ.5 analogue was synthesized by incubating a lipid φ.1 analogue (100 µM) with a mixture of CDP-glycerol (300 µM) and [¹⁴C]-CDP-glycerol (100 µM at 0.01 µCi/µL) in a reaction buffer containing 50 mM Tris, pH 8.0, 30 mM MgCl₂ and 100 nM TagF. Reactions were allowed to proceed to completion and the [¹⁴C]-lipid φ.5 analogue (30 µM) was subsequently incubated with 4 mM UDP-glucose and 50 nM TagE for 3 hours. Reaction progress was determined by PIC-HPLC at 262 nm. The non-glycosylated and glycosylated [¹⁴C]-lipid φ.5 analogues were filtered through a 30,000 MWCO centrifugal filter (Millipore, Billerica, MA, U.S.A) and then incubated with 4 mM unlabeled CDP-glycerol and 100 nM TagF for 5 hours at room temperature. Reaction substrates and products were separated by size exclusion chromatography using a Waters Shodex KW-803 column in buffer containing 0.1 % ammonium hydrogen carbonate and 10 % acetonitrile at 0.5 mL/min. All injections contained 0.1 µCi of radiolabeled substrate and reaction products were visualized by inline scintillation counting.

RESULTS

The tagE Gene Codes for the Wall Teichoic Acid Glycosyltransferase in B. subtilis 168. To
determine whether tagE codes for the wall teichoic acid glycosyltransferase, we created a ΔtagE strain by allelic replacement of tagE with a spectinomycin resistance cassette. Given that tagDEF are encoded in an operon, we left the last 26bp of the tagE coding sequence intact to ensure not to disrupt the native ribosome binding site of tagF located in the 3′ end of tagE. Phosphate analysis showed that the ΔtagE strain contained wild-type levels of phosphate in its cell wall (Figure 2A), confirming that there were no polar effects on tagF. Furthermore, the ΔtagE strain exhibited no major changes in morphology or growth kinetics compared to the wild-type parental strain (data not shown). We then tested both strains for resistance to bacteriophage SPO1, which recognizes glycosylated teichoic acid as a receptor (28). B. subtilis 168 was susceptible to SPO1 phage while the ΔtagE strain was resistant (Figure 2B). This strongly suggests that deletion of tagE leads to the loss of glucose along the wall teichoic acid polymer. To confirm this, we isolated wall teichoic acid from B. subtilis 168 and the ΔtagE strain and analyzed the polymers by 1H NMR. As shown in Figure 2C, the 1H NMR spectrum revealed an anomeric proton signal at δ5.07 (J1,2 = 2.1 Hz) that could be assigned to α-glucose at the C-2 position in wall teichoic acid isolated from wild-type B. subtilis 168. This finding is consistent with the previous stereochemical assignment of the glucose linkage (19). By contrast, this signal was absent in the 1H NMR spectrum of wall teichoic acid from the ΔtagE strain (Figure 2C). Taken together, these results demonstrate that tagE is involved in wall teichoic acid glycosylation in B. subtilis 168.

Assaying the Glycosyltransferase Activity of TagE in vitro. Having confirmed a role for TagE in wall teichoic acid glycosylation, we sought to investigate the activity of the enzyme in vitro. The reaction catalyzed by TagE in our in vitro assay is depicted in Scheme 1. Recombinant TagE that had been purified to homogeneity (Figure S1) was incubated with the activated sugar donor UDP-glucose and a soluble analogue of lipid ϕ.40, the product of the TagF reaction. The nomenclature of lipid-linked teichoic acid intermediates is summarized in Table 2 (29). The lipid ϕ.40 analogue consists of 40 glycerol phosphate units that are in a 1,3-linkage and attached to a lipid ϕ.1 analogue. We chose to synthesize a poly(glycerol phosphate) polymer of this length given that wall teichoic acid polymers in the cell wall of B. subtilis 168 typically contain 30-50 units of glycerol phosphate (12). The transfer of glucose from UDP-glucose onto the lipid ϕ.40 analogue was monitored using an HPLC-based assay that measures UDP production. Using radiolabeled UDP-[14C]-glucose, we confirmed that the production of UDP in this assay was stoichiometric with the transfer of glucose from UDP-glucose onto the acceptor (data not shown). We took great care to ensure that, under our assay conditions, the lipid ϕ.40 analogue-dependent production of UDP was linear with both time and the amount of enzyme added (Figure 3). By analyzing the dependence of the reaction velocity on enzyme concentration, we estimated a turnover of 16 s⁻¹ for TagE under conditions where both substrates were saturating.

Sugar Donor and Acceptor Specificity of TagE. We investigated the activity of TagE in the presence of UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, UDP-N-acetylgalactosamine and UDP-gluconic acid. Preliminary work suggested that TagE could utilize a number of these UDP-sugars as substrates. To investigate this further, we determined the specificity constants for these donors by measuring UDP formation. TagE catalyzed the transfer of glucose from UDP-glucose onto the lipid ϕ.40 analogue acceptor with a kcat of 17 ± 0.20 s⁻¹ and with a Kₘ for UDP-glucose of 770 ± 52 μM (kcat/Kₘ = 2.2 × 10⁶ M⁻¹s⁻¹) at saturating concentrations of the lipid ϕ.40 analogue acceptor. The specificity constants for UDP-galactose, UDP-N-acetylgalactosamine and UDP-gluconic acid were at least 60-fold lower than the specificity constant for UDP-glucose (Table 3). TagE showed no activity with UDP-N-acetylgalactosamine. Taken together, these data indicate that TagE has a large preference for UDP-glucose as the sugar donor.

We then examined the acceptor specificity of TagE to determine the minimum unit required for the glycosyltransfer reaction. TagE showed no activity when incubated with UDP-glucose and precursors for poly(glycerol phosphate) synthesis: glycerol; glycerol phosphate; CDP-glycerol; and lipid α, β and ϕ.1 analogues (Table 3). These findings indicate that polymer synthesis by TagF must occur prior to glycosylation by TagE. The effect of TagF polymer synthesis on TagE activity
was examined by testing different lipid φₙ analogues, ranging from 5 to 80 glycerol phosphate units. As shown in Table 3, the turnover numbers were comparable for all of the lipid φₙ analogues tested while the \( K_m \) values (reported in terms of polymer concentration) decreased as polymer length increased. Consequently, the specificity constants increased from \( 6.1 \times 10^5 \) M\(^{-1}\)s\(^{-1}\) to \( 1.7 \times 10^7 \) M\(^{-1}\)s\(^{-1}\) as polymer length was varied from 5 to 80 glycerol phosphate units. These data suggest that TagE has a kinetic preference for longer poly(glycerol phosphate) polymers. However, when the \( K_m \) values are adjusted to account for the number of glycerol phosphate units per polymer, the specificity constants for all of the lipid φₙ analogues are similar (approximately \( 1.0 \times 10^5 \) M\(^{-1}\)s\(^{-1}\)). For instance, if the \( K_m \) value of the lipid φ₄₀ analogue is adjusted to account for the 40 glycerol phosphate units in the polymer, the \( K_m \) value increases from 4.4 µM to 180 µM. Consequently, the specificity constant for the lipid φ₄₀ analogue decreases from \( 4.1 \times 10^6 \) to \( 1.0 \times 10^5 \) M\(^{-1}\)s\(^{-1}\) (Table 3). The similar specificity constants of the lipid φₙ analogues (reported in terms of glycerol phosphate units) indicates that TagE activity is independent of polymer length, implying that the enzyme recognizes glycerol phosphate units, albeit in the context of a repeating glycerol phosphate polymer. Further, incubating TagE with a poly(glycerol phosphate) polymer lacking the linkage unit analogue, the ManNAc-β-(1-4)-GlcNAc-1-P-P-tridecane portion, revealed that TagE modified the polymer containing only glycerol phosphate at a rate similar to that of the lipid φₙ analogues (Table 3). Indeed, the dependence of the kinetic parameters on glycerol phosphate concentration and not on the length of the polymer suggested that TagE followed a distributive mechanism, binding and releasing the polymer with each round of catalysis.

**Initial Rate Analysis of TagE.** We proceeded to determine whether TagE reaction kinetics could be accurately described by initial velocity expressions developed for non-processive bi-bi enzyme systems. We explored TagE reaction rates at varying concentrations of UDP-glucose and the lipid φ₄₀ analogue acceptor by monitoring the conversion of UDP-glucose to UDP. Double reciprocal plots of the initial rate data are shown in Figure 4. The data fit very well to a sequential (ternary complex) kinetic mechanism (Equation 2) and further suggest that TagE follows a distributive, non-processive reaction with its acceptor substrate. The kinetic constants from this experiment are summarized in Table 4. The \( K_m \) values were \( 3.7 \pm 0.61 \) mM for UDP-glucose and \( 0.72 \pm 0.10 \) µM for the lipid φ₄₀ analogue. The \( k_{cat}/K_m \) constants were \( 6.8 \times 10^3 \) M\(^{-1}\)s\(^{-1}\) and \( 3.5 \times 10^7 \) M\(^{-1}\)s\(^{-1}\) for UDP-glucose and the lipid φ₄₀ analogue, respectively. These data are consistent with the kinetic parameters determined for the two substrates under saturating conditions (Table 3).

**Glycosylation Impairs Polymer Extension by TagF.** Glycosylation has been proposed to be a length-determining modification that prevents further poly(glycerol phosphate) polymerization by TagF (23). To investigate this possibility, we examined the effect of glycosylation on TagF activity by incubating the enzyme with CDP-glycerol and either a \([^{14}C]\)-lipid φ₅ analogue or a glycosylated \([^{14}C]\)-lipid φ₅ analogue. The products of these reactions were subsequently separated by size exclusion chromatography. Under these conditions, all of the \([^{14}C]\)-lipid φ₅ substrate analogue was converted to a higher molecular weight product as indicated by the shift in retention time (Figure 5). In the reaction containing the glycosylated \([^{14}C]\)-lipid φ₅ substrate analogue, only a small percentage was converted to a larger glycerol phosphate containing product (Figure 5). This result indicates that TagF could not polymerize glycerol phosphate units onto a glycosylated substrate analogue as efficiently as it could onto the unmodified analogue. Glycosylation therefore impairs the poly(glycerol phosphate) polymerization reaction catalyzed by TagF in vitro.

**DISCUSSION**

The biosynthesis of wall teichoic acids has been well-characterized and the roles of many of the enzymes have been established with \textit{in vitro} biochemical assays using pure recombinant proteins and well defined substrates (9-12). Wall teichoic acid glycosylation is less well characterized. The TagE protein of \textit{B. subtilis} 168 has long been designated the wall teichoic acid glycosyltransferase in this organism despite any strong evidence to support this assignment. In this work, we have provided unambiguous genetic and
have clearly demonstrated a role for the glucose substituent (19). From this work, we glucosidases to elucidate the stereochemistry of this assignment is in agreement with the work that the glucose linkage is in the α-configuration. Using modern NMR techniques we have confirmed that the wall teichoic acid modification is dispensable for cell growth (15). We showed that deletion of tagE leads to resistance to bacteriophage SPO1 and to the loss of the α-glucose substitution at the C-2 position along the poly(glycerol phosphate) backbone. These findings validate the bacteriophage work that had linked mutations in tagE to a deficiency in wall teichoic acid glycosylation (15-16). In addition, using modern NMR techniques we have confirmed that the glucose linkage is in the α-configuration. This assignment is in agreement with the work from Glaser and Burger who used α- and β-glucosidases to elucidate the stereochemistry of the glucose substituent (19). From this work, we have clearly demonstrated a role for tagE in the glycosylation of wall teichoic acid.

Previous efforts in characterizing wall teichoic acid glycosyltransferases have employed partially purified enzymes and uncharacterized membrane acceptors to follow enzyme activity (18-19). We purified recombinant B. subtilis 168 TagE to homogeneity and developed an HPLC-based assay to examine the wall teichoic acid glycosyltransferase activity of the enzyme with clean synthetic substrates in vitro. The assay was robust as evidenced by the linearity of product formation with time and enzyme concentration. We have shown that TagE can catalyze the transfer of glucose from UDP-glucose onto a lipid α.40 substrate analogue acceptor, providing direct biochemical evidence that TagE is a wall teichoic acid glycosyltransferase. Having performed this characterization with pure recombinant protein and synthetic substrates, we have established that the TagE protein is active in the absence of any accessory components or proteins. Initial rate analysis of the reaction catalyzed by TagE revealed the following kinetic parameters: $k_{cat}$ of $25 \pm 0.56 \text{s}^{-1}$, $K_m$ of $3.7 \pm 0.61 \text{mM}$ for UDP-glucose and $K_m$ of $0.72 \pm 0.10 \mu\text{M}$ for the lipid φ.40 analogue. The high specificity of TagE for UDP-glucose provides strong evidence that this activated sugar donor is the physiological substrate of TagE. Given that UDP-glucose is not found in the cell envelope and that TagE is predicted to function in the cytoplasm, the glycosylation reaction likely occurs before the wall teichoic acid polymer is exported to the outside of the cell. The low micromolar affinity of TagE for the acceptor substrate is similar to the affinity of other wall teichoic acid biosynthetic enzymes for their respective substrates, including the TagF polymerase (23). The use of the lipid φ.40 substrate analogue in our work as a mimic of the natural acceptor for TagE is strengthened by the fact the enzyme does not recognize the linkage unit portion of its substrate.

We sought to determine whether TagE could glycosylate the precursors for polymer synthesis or if the enzyme only modified a polymer once it has been synthesized. Thus, we also examined the acceptor specificity of TagE to gain insight into the mechanism of wall teichoic acid glycosylation. We showed that TagE was only able to use a poly(glycerol phosphate) polymer as a substrate, demonstrating that polymer synthesis must be initiated by TagF prior to glycosylation. The similar specificity constants of both the short and long lipid φ.2n analogues, which have taken into account the number of glycerol phosphate units per polymer, indicate that polymer length has no effect on the activity of TagE. It is therefore conceivable that glycosylation could occur once TagF has primed the lipid φ.1 substrate with at least one unit of glycerol phosphate. However, we showed that TagF could not transfer glycerol phosphate units to a glycosylated substrate analogue as efficiently as it could to an unmodified analogue. Although it is unclear why glycosylation impairs polymer extension by TagF, these findings suggest that TagE must bind sufficiently upstream of TagF on a polymer to ensure that poly(glycerol phosphate) synthesis is not blocked. We therefore find it most likely that polymer synthesis is complete, or near complete, prior to modification by TagE (Figure 6). We also showed that TagE can glycosylate a polymer that lacks a lipid φ.2 analogue, indicating that the enzyme recognizes the repeating glycerol phosphate units of the polymer. This finding, in addition to the length-independence of the TagE
reaction, suggests that TagE is a distributive enzyme that catalyzes the addition of a single sugar residue with each binding event.

TagE reaction kinetics could be accurately described by initial velocity expressions developed for non-polymerase bi-bi enzyme systems. Steady state kinetic experiments showed that the enzyme utilizes a sequential mechanism. Many glycosyltransferases, including those that bind undecaprenyl-linked acceptors, utilize a sequential mechanism in which the sugar donor binds before the acceptor substrate (30-32). Product inhibition studies are required to determine whether the sequential mechanism is random or ordered with respect to substrate binding. Given that a glycosylated lipid φ.40 analogue reaction product also functions as a substrate, we were unable to elucidate the binding order of substrates to TagE. A lipid φ.40 product analogue that can inhibit TagE and not act as a substrate would be an opportune probe to determine the sequence of substrate binding and product release.

TagE catalyzed the transfer of α-glucose from UDP-glucose onto position 2 of the poly(glycerol phosphate) polymer with retention of stereochemistry at the anomeric reaction center of the sugar donor. TagE is thus a member of retaining GT-B fold glycosyltransferases. The mechanism of retaining glycosyltransferases has been proposed to be that of a double displacement mechanism wherein a covalently bound glycosyl-enzyme intermediate forms (33). An alternate S_n1-like mechanism has also been proposed wherein a short-lived oxocarbenium ion intermediate forms and a nucleophilic attack occurs from the same face from which the leaving group departs (33). Given that TagE reaction kinetics were most accurately described by a sequential mechanism instead of a ping-pong mechanism, we find it likely that the enzyme would utilize an S_n1-like pathway to retain the anomeric stereochemistry with respect to the donor substrate (Figure 6).

The physiological significance of wall teichoic acid glycosylation is unknown. This wall teichoic acid modification has been proposed to play a role in regulating the length of poly(glycerol phosphate) polymers by preventing re-binding and further polymerization by TagF (23). Indeed, we have shown that the addition of glucose near the terminal end of a polymer impairs polymer extension by TagF in vitro. Despite this, previous work from our research group showed length-regulation by TagF on association with heat- and proteinase-treated B. subtilis membranes (26). Further work is clearly required to understand the functional significance of this modification and the mechanism by which wall teichoic acid polymer length is regulated.

In this study, we have demonstrated that TagE is responsible for wall teichoic acid glycosylation in B. subtilis 168. For the first time, we have defined the kinetic parameters for a wall teichoic acid glycosyltransferase and presented evidence that TagE utilizes a distributive and sequential mechanism to transfer glucose from UDP-glucose onto a poly(glycerol phosphate) polymer. The robust assay that we developed to monitor wall teichoic acid glycosyltransferase activity should prove useful in future work elucidating the chemical mechanism of the glycosyltransfer reaction. Most importantly, our work has filled a critical gap in the understanding of wall teichoic acid biosynthesis.

REFERENCES
FOOTNOTES

We thank Edward Sewell, Justyna Troczynska, Trevor Mace-Brickman and Alyssa Grunwald (Department of Biochemistry and Biomedical Sciences and the Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, Ontario, Canada) for the construction of the pDEST17-tagE expression plasmid and assay development. This work was supported by the Natural Sciences and Engineering Research Council of Canada, the Canadian Institutes of Health Research Grant MOP-15496, a Canada Research Chair held by E.D.B. and an Ontario Graduate Scholarship held by S.E.A.

The abbreviations used are: tag, teichoic acid glycerol phosphate; HPLC, high pressure liquid chromatography

FIGURE LEGENDS

SCHEME 1. Reaction catalyzed by TagE in vivo. In the in vitro TagE activity assay, the lipid β portion of the poly(glycerol phosphate) polymer has been replaced by a soluble analogue of lipid β (ManNAc-β-(1-4)-GlcNAc-1-P-P-tridecane).

FIGURE 1. Proposed pathway for wall teichoic acid biosynthesis in B. subtilis 168. Wall teichoic acid polymers are composed of a disaccharide containing N-acetylglucosamine-1-phosphate (white oval with phosphate group) and N-acetylmannosamine (black oval) and ~40 repeating glycerol-3-phosphate units (square). These polymers are synthesized on the cytoplasmic face of the cell membrane on an undecaprenyl phosphate molecule (wavy line with phosphate group). Once synthesis is complete, the polymer is exported to the outside of the cell and covalently attached to peptidoglycan.

FIGURE 2. Deletion of tagE leads to the loss of α-glucose at the C-2 position of poly(glycerol phosphate) wall teichoic acid. (A) Phosphate analysis and (B) SPO1 phage susceptibility of the ΔtagE (EB2252) and the wild-type B. subtilis 168 parent strain (EB6). (C) 1H NMR spectra of wall teichoic acid isolated from B. subtilis 168 (top) and the ΔtagE strain (bottom). The α-glucose anemic resonance at δ5.07 is indicated by a dashed vertical line.

FIGURE 3. Dependence of TagE activity on time and enzyme concentration. Reactions contained 3 mM UDP-glucose, 15 µM lipid φ.40 analogue and 1 (●), 2.5 (∗), 5 (▲) or 10 nM (△) TagE. Reactions were quenched with urea to a final concentration of 6 M following 1, 3, 6 and 12 minute incubations. The conversion of UDP-glucose to UDP was monitored at 262 nm following separation by paired-ion HPLC. Inset: Plot of initial velocity versus TagE concentration. The slope of the plot represents the turnover of TagE under saturating conditions (16 s⁻¹).

FIGURE 4. TagE utilizes a sequential (ternary complex) mechanism. (A) Double reciprocal plot of 1/velocity versus 1/[UDP-glucose]. UDP-Glucose was varied from 1600-12800 µM while the lipid φ.40 concentration was fixed at 0.5 (●), 1 (∗), 2 (■) and 8 µM (∇). (B) Double reciprocal plot of initial rate data with varying lipid φ.40 concentrations (0.5-8 µM) at fixed UDP-glucose concentrations (1600 (●), 3200 (∗), 6400 (■) and 12800 µM (∇)). All experiments were conducted with 2.5 nM TagE and reaction rates were determined by monitoring the conversion of UDP-glucose to UDP at 262 nm. The data were fitted by non-linear least squares method to a sequential kinetic mechanism (Equation 2).
FIGURE 5. Glycosylation impairs poly(glycerol phosphate) polymerization by TagF in vitro. (A) The solid black trace indicates elution for the $[^{14}C]$-lipid φ.5 analogue. The dotted black trace indicates $[^{14}C]$-lipid φ.5 analogue elution for a reaction containing CDP-glycerol and TagF. Formation of a higher molecular weight product, whose elution is consistent with a polymer containing nearly 50 glycerol phosphate units, is indicated by an asterisk. (B) Elution of the glycosylated $[^{14}C]$-lipid φ.5 analogue following incubation with (dotted line) or without (solid line) TagF. Glycosylated and non-glycosylated $[^{14}C]$-lipid φ.5 analogues were incubated with 4 mM CDP-glycerol and 100 nM TagF for 5 hours. Reaction substrates and products were then separated by size exclusion chromatography on Waters Shodex KW-803 column in buffer containing 0.1 % ammonium hydrogen carbonate and 10 % acetonitrile at 0.5 mL/min.

FIGURE 6. Mechanism of wall teichoic acid glycosylation in B. subtilis 168 in vivo. (A) TagF synthesizes a polymer of ~40 units of glycerol-3-phosphate (square) from CDP-glycerol onto lipid φ.1. Once polymer synthesis is complete, or near complete, TagE transfers glucose (light grey oval) from UDP-glucose onto the poly(glycerol phosphate) polymer. The extent and distribution of glucose along the polymer is unknown. The modified polymer is then exported by the ABC-transporter, TagGH. (B) A proposed S_N1-like reaction for TagE involves formation of an oxocarbenium ion intermediate and nucleophilic attack from the acceptor substrate on the same face from which the leaving group departs. The products of the reaction are a glycosylated poly(glycerol phosphate) polymer and UDP. Abbreviations: R, an O or NH₂ group on an amino acid in the active site of the enzyme; R₁, the poly(glycerol phosphate) acceptor substrate.
Table 1. Strains, plasmids and oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Strain, Plasmid or Oligonucleotide</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Novablue</td>
<td>General <em>E. coli</em> cloning strain ((endA1 hsdR17(r_{K12}^{-} m_{K12}^{-})supE44 thi-1 recA1 gyrA96 relA1 lacF' [proA + B' lacI/ZΔM15::Tn10(TcR)])</td>
<td>Novagen</td>
</tr>
<tr>
<td>EB863</td>
<td><em>E. coli</em> strain used for protein over-expression ((F^- ompT hsdS_b(r_{B}^- m_{B}^-) gal dcm araB::T7RNAP-tetA))</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>EB6</td>
<td>Wild-type <em>B. subtilis</em> ((hisA1 argC2 metC3))</td>
<td>(34)</td>
</tr>
<tr>
<td>EB2252</td>
<td>tagE deletion strain derived from EB6 ((hisA1 argC2 metC3 tagE::spec))</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUS19</td>
<td>pUC19 derivative used as a source for a spec(^R) cassette</td>
<td>(35)</td>
</tr>
<tr>
<td>pDEST17-tagE</td>
<td>Expression plasmid for N-terminal His(_6)-tagged TagE</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Oligonucleotides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tagE-F</td>
<td>5’ – ggggacaagtttctacaaaaagcagacctttgctctcatcaggtgagttaaatc – 3’</td>
<td></td>
</tr>
<tr>
<td>tagE-R</td>
<td>5’ – ggggaccactttgacaaaaaagcagacctttgctctcatcaggtgagttaaatc – 3’</td>
<td></td>
</tr>
<tr>
<td>tagE-a</td>
<td>5’ – ggggaccactttgacaaaaaagcagacctttgctctcatcaggtgagttaaatc – 3’</td>
<td></td>
</tr>
<tr>
<td>tagE-b</td>
<td>5’ – ctataaactatttaaataacagattttttagtaaattttaatattgaagct – 3’</td>
<td></td>
</tr>
<tr>
<td>tagE-c</td>
<td>5’ – ctataaactatttaaataacagattttttagtaaattttaatattgaagct – 3’</td>
<td></td>
</tr>
<tr>
<td>tagE-d</td>
<td>5’ – ctataaactatttaaataacagattttttagtaaattttaatattgaagct – 3’</td>
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</tr>
<tr>
<td>spec-F</td>
<td>5’ – agtgaggaggatatattgaac – 3’</td>
<td></td>
</tr>
<tr>
<td>spec-R</td>
<td>5’ – tttttaatatttttaatatt – 3’</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Nomenclature for wall teichoic acid intermediates (29).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate or Substrate Analogue</th>
<th>Chemical Composition$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TagA</td>
<td>Lipid $\alpha$</td>
<td>GlcNAc-$\alpha$-P-P-und</td>
</tr>
<tr>
<td>TagB</td>
<td>Lipid $\beta$</td>
<td>ManNAc-$\beta$-(1-4)-GlcNAc-$\alpha$-P-P-und</td>
</tr>
<tr>
<td>TagF</td>
<td>Lipid $\phi$.n</td>
<td>$(\text{GroP})_n$-ManNAc-$\beta$-(1-4)-GlcNAc-$\alpha$-P-P-und</td>
</tr>
<tr>
<td>TagA</td>
<td>Lipid $\alpha$ analogue</td>
<td>GlcNAc-$\alpha$-P-tridecane</td>
</tr>
<tr>
<td>TagB</td>
<td>Lipid $\beta$ analogue</td>
<td>ManNAc-$\beta$-(1-4)-GlcNAc-$\alpha$-P-tridecane</td>
</tr>
<tr>
<td>TagF</td>
<td>Lipid $\phi$.n analogue</td>
<td>$(\text{GroP})_n$-ManNAc-$\beta$-(1-4)-GlcNAc-$\alpha$-P-tridecane</td>
</tr>
</tbody>
</table>

$^a$Lipid $\phi$.1 is the product of the TagB reaction and contains a single glycerol phosphate unit. Lipid $\phi$.1 serves as a substrate for TagF which catalyzes the addition of $n$ glycerol phosphate units. Und, undecaprenyl; P, phosphate; GlcNAc, $N$-acetylglucosamine; ManNAc, $N$-acetylmannosamine; GroP, glycerol phosphate.
Table 3. Kinetic parameters determined for sugar donor and acceptor substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sugar Donors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDP-Glc</td>
<td>770 ± 52</td>
<td>17 ± 0.20</td>
<td>$2.2 \times 10^4$</td>
</tr>
<tr>
<td>UDP-Gal</td>
<td>ND</td>
<td>ND</td>
<td>$2.9 \times 10^2$</td>
</tr>
<tr>
<td>UDP-GlcNAc</td>
<td>ND</td>
<td>ND</td>
<td>$3.5 \times 10^2$</td>
</tr>
<tr>
<td>UDP-GalNAc</td>
<td>---d</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>UDP-GlcA</td>
<td>ND</td>
<td>ND</td>
<td>$9.1 \times 10^1$</td>
</tr>
<tr>
<td><strong>Acceptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gro</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>GroP</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>CDP-Gro</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Lipid $\alpha$ analogue (TagA Substrate)</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Lipid $\beta$ analogue (TagB Substrate)</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Lipid $\varphi.1$ analogue (TagF Substrate)</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Lipid $\varphi.0.5$ analogue</td>
<td>23 ± 3.1</td>
<td>14 ± 0.72</td>
<td>$6.1 \times 10^5$</td>
</tr>
<tr>
<td>(120)²</td>
<td></td>
<td></td>
<td>(1.2 × 10⁵)</td>
</tr>
<tr>
<td>Lipid $\varphi.1.0$ analogue</td>
<td>24 ± 0.92</td>
<td>26 ± 0.45</td>
<td>$1.1 \times 10^6$</td>
</tr>
<tr>
<td>(240)</td>
<td></td>
<td></td>
<td>(1.1 × 10⁵)</td>
</tr>
<tr>
<td>Lipid $\varphi.40$ analogue</td>
<td>4.4 ± 0.19</td>
<td>18 ± 0.24</td>
<td>$4.1 \times 10^6$</td>
</tr>
<tr>
<td>(180)</td>
<td></td>
<td></td>
<td>(1.0 × 10⁵)</td>
</tr>
<tr>
<td>Lipid $\varphi.80$ analogue</td>
<td>1.2 ± 0.11</td>
<td>20 ± 0.35</td>
<td>$1.7 \times 10^7$</td>
</tr>
<tr>
<td>(96)</td>
<td></td>
<td></td>
<td>(2.1 × 10⁵)</td>
</tr>
<tr>
<td>Polymer (no linkage unit)</td>
<td>NA</td>
<td>23 ± 1.6</td>
<td>NA</td>
</tr>
</tbody>
</table>

²The lipid $\varphi.40$ analogue acceptor concentration was fixed at 20µM
³The concentration of UDP-Glc was held constant at 3mM
⁴Specific kinetic constants were not determined as the reaction rate was not saturable at experimentally practical sugar donor concentrations
⁵Denotes that there was no detectable production of UDP
⁶Values in brackets represent kinetic parameters that have been adjusted to account for the number of glycerol phosphate units per polymer
⁷Values are not reported as there was no linkage unit analogue present to determine polymer concentration
Table 4. Summary of Kinetic Parameters for TagE.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(K_m) (µM)</th>
<th>(k_{cat}) (s(^{-1}))</th>
<th>(k_{cat}/K_m) (M(^{-1})s(^{-1}))</th>
<th>Dissociation Constant(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-Glc</td>
<td>3700 ± 610(^b)</td>
<td>6.8 × 10(^3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid (\phi.40)</td>
<td>0.72 ± 0.10</td>
<td>25 ± 0.56</td>
<td>3.5 × 10(^7)</td>
<td>0.46 ± 0.098</td>
</tr>
</tbody>
</table>

\(^a\) Obtained from Equation (2)

\(^b\) Standard error in the value is based on the fit of data to a sequential model
FIGURES

SCHEME 1
FIGURE 2

A

\[
\begin{array}{c}
\text{μmol P/mg cell wall} \\
0.0 \\
0.2 \\
0.4 \\
0.6 \\
0.8 \\
\end{array}
\]

EB6  EB2252

B

C

EB6  EB2252

\[ \delta \]

5.5  5.0  4.5  4.0  3.5

[Image of bar graph comparing μmol P/mg cell wall for EB6 and EB2252, and a black and white image showing growth patterns, along with a line graph comparing δ values for EB6 and EB2252.]
FIGURE 3

![Graph showing turnover vs time and velocity vs [TagE] concentration](image-url)
FIGURE 5

A

B

CPM

Time (minutes)

CPM

Time (minutes)
FIGURE S1. Purification of N-terminally hexahistidine-tagged TagE. Protein samples were analyzed by discontinuous SDS-PAGE using 3% stacking and 15% separating gels and were visualized by staining with Coomassie Blue. Lanes contained a molecular weight marker (in kDa) (lane 1), total cell lysate (lane 2) and Ni²⁺-purified TagE (lane 3).
FIGURES

FIGURE S1
Regular Paper:
Studies of the genetics, function and kinetic mechanism of TagE - the wall teichoic acid glycosyltransferase in *bacillus subtilis* 168

Sarah E. Allison, Michael A. D'Elia, Sharif Arar, Mario A. Monteiro and Eric D. Brown

*J. Biol. Chem.* published online May 10, 2011

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