WALL TEICHOIC ACID GLYCOSYLATION

GLYCOSYLATION OF WALL TEICHOIC ACIDS IN GRAM-POSITIVE BACTERIA

By

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Abstract

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* results in the loss of α-glucose at the C-2 position of glycerol in the poly(glycerol phosphate) polymer backbone. We also report 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 showed that this enzyme has a strong kinetic preference for the transfer of glucose from UDP-glucose to glycerol phosphate in polymeric form. Further, we showed that the enzyme recognizes 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 on the mechanism of wall teichoic acid glycosylation, a largely uncharted aspect of wall teichoic acid biogenesis.

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List of abbreviations

CDP	Cytidine diphosphate
Ci	Curie
HPLC	High Performance Liquid Chromatography
LB	Luria-Bertani
PIC	Paired Ion Exchange
Tris	Tris(hydroxymethyl)aminomethane
UDP	Uridine diphosphate

Chapter One - Introduction

1.1 MODIFICATION OF TEICHOIC ACIDS IN GRAM-POSITIVE BACTERIA

The bacterial cell wall plays a critical role in cellular processes such as growth, division, maintenance of cell shape and protection against both mechanical and osmotic lysis (1). The cell wall of Gram-negative bacteria is comprised of a thin layer of peptidoglycan surrounded by an outer membrane. By contrast, the Gram-positive cell wall contains a much thicker layer of peptidoglycan and substantial amounts of glycopolymers. The best studied glycopolymers are arguably teichoic acids which account for up to 60% of the Gram-positive cell wall dry weight (1). Teichoic acids are polyol phosphate polymers that are often extensively modified with D-alanine and additional sugar residues (2). These polymers can be either anchored into the cytoplasmic membrane (lipoteichoic acids) or covalently attached to peptidoglycan (wall teichoic acids). Together, lipoteichoic acids and wall teichoic acids create a "continuum of anionic charge" that extends over the bacterial cell surface (2). Although the precise function of teichoic acids is unknown, these polymers have been shown to play a key role in cellular functions from cell shape determination in *Bacillus subtilis* (3) to pathogenesis in *Staphylococcus aureus* (4-5). Teichoic acids are thus an emerging target for the development of new cell wall active antibiotics (6).

The ability to create mutants completely devoid of either lipoteichoic acids or wall teichoic acids has sparked a renewed interest in the study of these glycopolymers. Most recently, a significant number of studies have focused on the modification of teichoic acids with D-alanine and glycosyl residues. This chapter summarizes the structure and biosynthesis of teichoic acids, the mechanisms by which D-alanine and

sugar residues are incorporated into teichoic acids and the physiological significance of these modifications.

1.2 STRUCTURE OF TEICHOIC ACIDS

The chemical structures of lipoteichoic acid and wall teichoic acid have been determined for a number of Gram-positive organisms. Of those studied to date, many produce poly(glycerol phosphate) lipoteichoic acid that is tethered to the membrane by a glycolipid anchor, diglucosyldiacylglycerol (7). Although the 1,3-linked glycerol phosphate units of the main chain may be modified at position 2 with α-*N*-acetylglucosamine or α-galactose, the polymer is often extensively esterified with D-alanine (2).

Wall teichoic acids share some structural similarity with lipoteichoic acids despite being synthesized by an entirely separate biosynthetic pathway. Most often, the repeating units of wall teichoic acids are either glycerol phosphate or ribitol phosphate. Both types of polymers are anchored to peptidoglycan through a linkage unit consisting of a disaccharide component, *N*-acetylglucosamine-β-(1–4)-*N*-acetylmannosamine, and 1 to 3 units of glycerol phosphate (7). Similar to lipoteichoic acids, the repeating units of wall teichoic acids are subject to modification. For instance, the major wall teichoic acid of *B. subtilis* 168 is a linear 1,3-linked poly(glycerol phosphate) polymer which is modified at position 2 with D-alanine and α-glucose (Figure 1.1A) (8). In *S. aureus*, the 1,5-linked poly(ribitol phosphate) polymer is modified at the 2 and 4 positions with D-alanine and α/β-*N*-acetylglucosamine, respectively (Figure 1.1B) (9-10). The extent of teichoic acid

modification is strain dependent and can also be affected by growth conditions such as pH, temperature and salt concentrations (2).

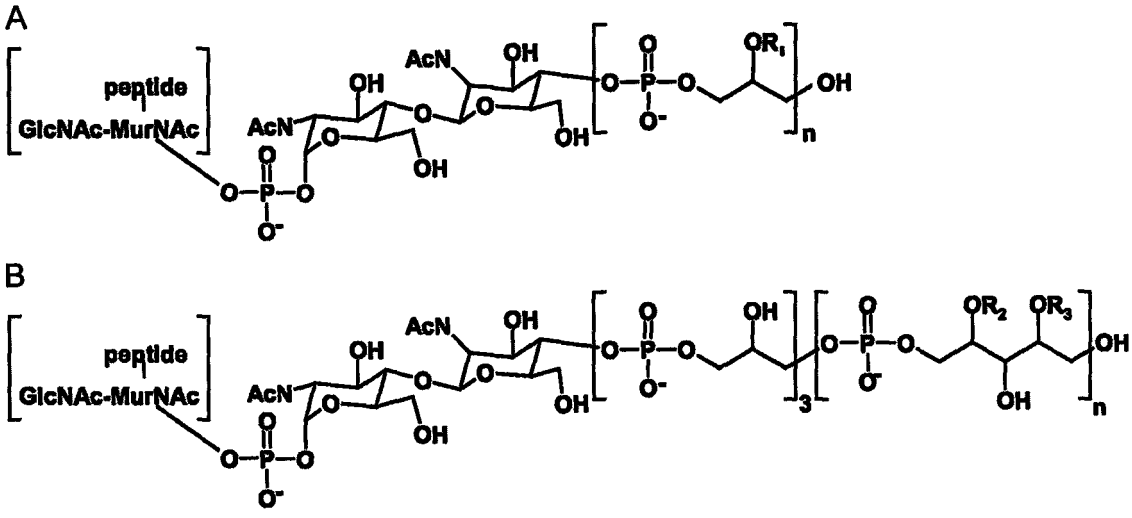

Figure 1.1 – Chemical structure of wall teichoic acid. Wall teichoic acids are covalently attached to peptidoglycan by a linkage unit. The wall teichoic acid in *B. subtilis* 168 contains repeating glycerol phosphate units (A) whereas the polymers in *S. aureus* contain ribitol phosphate units (B). Both types of wall teichoic acid are modified with D-alanyl esters and glycosyl residues at the indicated positions (R_1-R_3).

1.3 BIOSYNTHESIS OF TEICHOIC ACIDS

Lipoteichoic Acid Synthesis

Recently, great strides have been made in defining the genetic requirements for lipoteichoic acid synthesis. Lipoteichoic acid synthesis begins on the cytoplasmic face of the cell membrane. YpfP transfers two units of glucose onto diacylglycerol to form a glycolipid anchor that is then translocated by LtaA to the extracytoplasmic face of the cell membrane (11). The main chain polymerase, LtaS, catalyzes the addition of glycerol phosphate from phosphatidylglycerol onto the glycolipid anchor (Figure 1.2). Diacylglycerol, which is released during poly(glycerol phosphate) polymerization, is recycled back into phospholipid metabolism by a diacylglycerol kinase (11). The completed polymer is subsequently modified with cationic D-alanyl esters and glycosyl residues.

Wall Teichoic Acid Synthesis

The generation of temperature-sensitive mutants that were impaired for wall teichoic acid biosynthesis led to the isolation of the *tag* (*t*eichoic *a*cid *g*ene) cluster in *B. subtilis* 168 (12). Sequence-based analysis of teichoic acid gene clusters and studies using recombinant proteins have assigned functional roles to nearly all of the genes responsible for poly(glycerol phosphate) polymer synthesis. Synthesis occurs 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, to form a lipid β product (13). The lipid β product is then primed with a

single unit of glycerol phosphate by TagB to form the polymerization substrate, lipid φ.1 (14-15). The polymerase, TagF, adds 40-60 residues of glycerol-3-phosphate – a substrate that is provided by TagD in the activated form, CDP-glycerol (16-17). The completed polymer is believed to be glycosylated in the cytoplasm prior to export by the ABC transporter, TagGH (Figure 1.2) (18). Once the translocated polymer has been attached onto the 6-hydroxyl of *N*-acetylmuramic acid of peptidoglycan by an unknown transferase, the polymer is modified with D-alanine by an enzyme system that is encoded outside of the *tag* gene cluster.

In organisms such as *B. subtilis* W23 and *S. aureus*, which produce poly(ribitol phosphate) polymers, the first three steps of the biosynthetic pathway are identical to those in *B. subtilis* 168. However, the pathways for poly(glycerol phosphate) and poly(ribitol phosphate) polymer synthesis then diverge. In contrast to TagF, which catalyzes the addition of nearly 40-60 units of glycerol phosphate, TarF (*t*eichoic *a*cid *r*ibitol) only adds two units of glycerol phosphate. There are also two additional enzymes, TarK and TarL, which catalyze the addition of ribitol phosphate onto the growing polymer (6). In *B. subtilis* W23, these enzymes are proposed to function as a primase/polymerase pair with TarK adding a single unit of ribitol phosphate and TarL completing main chain polymer synthesis (19). By contrast, *S. aureus* TarK and TarL are functionally redundant enzymes, both of which are capable of poly(ribitol phosphate) polymerization (20). Ribitol phosphate is provided to these enzymes in the activated form, CDP-ribitol, by TarIJ. Once poly(ribitol phosphate) polymer synthesis is complete, the remaining steps in the pathway are similar to those in *B. subtilis* 168. The polymer is

glycosylated in the cytoplasm, exported by TarGH, attached to peptidoglycan and then esterified with D-alanyl residues (5).

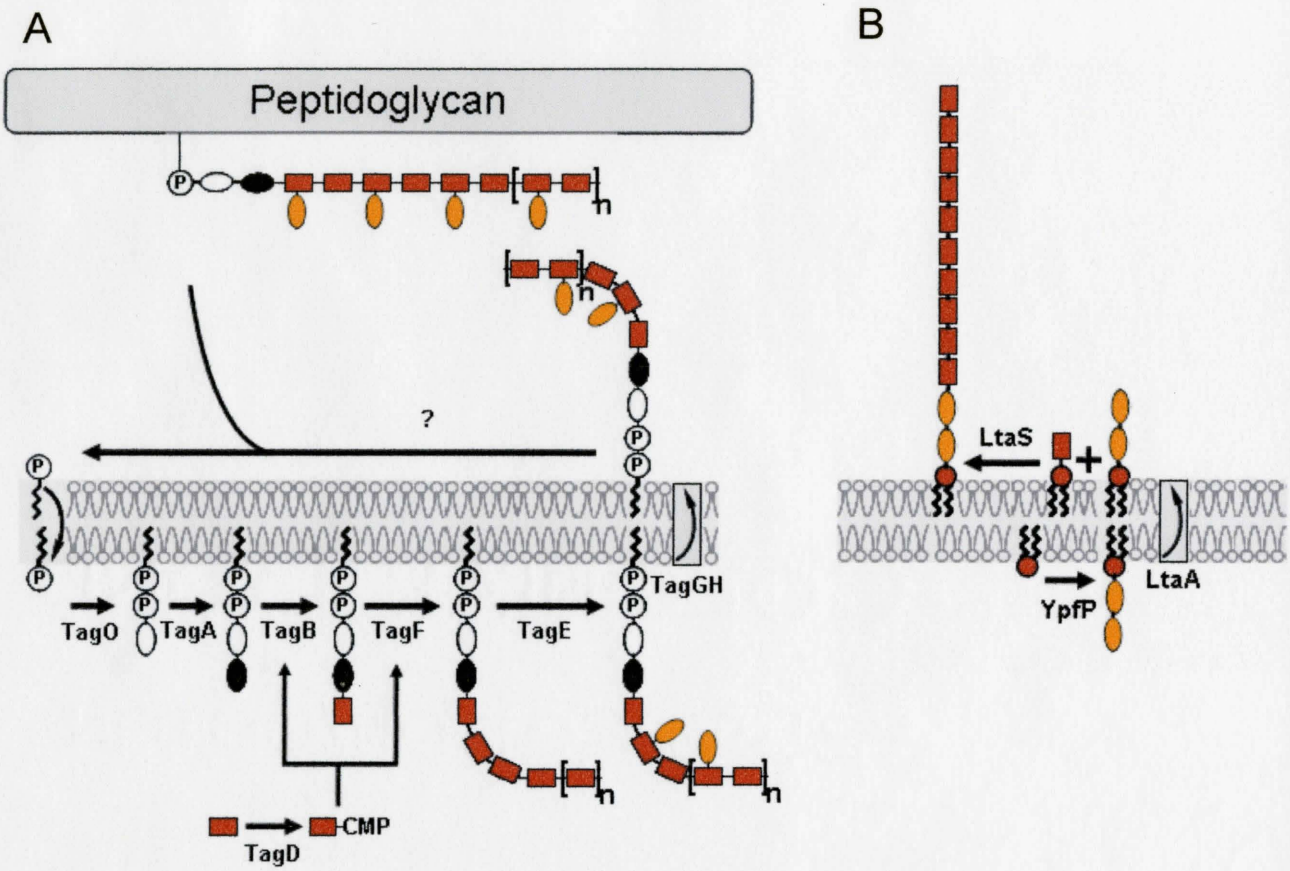

Figure 1.2 – Proposed pathway for poly(glycerol phosphate) polymer synthesis in *B. subtilis* 168. (A) Wall teichoic acid biosynthetic pathway. Symbols: wavy line with a phosphate unit, undecaprenyl phosphate; white oval, *N*-acetylglucosamine; black oval, *N*-acetylmannosamine; red box, glycerol phosphate; yellow oval, glucose. (B) Lipoteichoic acid biosynthetic pathway. Symbols: wavy line with red circle, diacylglycerol; yellow oval, glucose; red box, glycerol phosphate.

1.4 FUNCTIONS OF TEICHOIC ACIDS

Despite decades of investigations into the genetics and physiological significance of teichoic acids, a precise function for these polymers has remained elusive. However, the phenotypes associated with the loss of teichoic acids have led to a number of postulated functions. Teichoic acids have long been known to serve as a receptor for phage binding, a property that was exploited in the early isolation of mutant strains. Studies have also demonstrated a role for these highly anionic polymers in cation binding (2). Furthermore, a number of studies have highlighted the importance of teichoic acids in cell division, growth and morphology. For instance, the loss of lipoteichoic acids in *B. subtilis* leads to major defects in septum formation and cell separation while the loss of wall teichoic acids results in round and severely defective cells (8,21). Lipoteichoic acids have therefore been proposed to be involved in septum formation while wall teichoic acids are believed to be involved in elongation. Additional functions have also been proposed for teichoic acids in biofilm formation and host infection (22-23). A large number of these suggested functions are dependent or can be attributed to the specific tailoring modifications on teichoic acids. For this reason, the functions of teichoic acids are discussed later and in greater detail in the context of teichoic acid modification.

1.5 THE D-ALANYLATION MODIFICATION OF TEICHOIC ACIDS

Incorporation of D-Alanine into Teichoic Acids

To date, the D-alanylation modification of teichoic acids has been more extensively studied than glycosylation. Perego *et al.* were the first to characterize the

pathway responsible for D-alanine esterification of both lipoteichoic acids and wall teichoic acids (24). The *dlt* operon contains five genes, *dltA-dltE*, although only four gene products are required for the synthesis of D-alanyl lipoteichoic acid. DltA is a D-alanine-D-alanyl carrier protein ligase (Dcl) that activates D-alanine by ATP hydrolysis and transfers it to the 4'-phosphoantetheine prosthetic group of the D-alanine carrier protein (Dcp) encoded by *dltC* (25-26). DltB and DltD then facilitate the transport of D-alanyl-Dcp across the membrane and the incorporation of D-alanine into lipoteichoic acid (24). Once D-alanine has been incorporated into lipoteichoic acid, the D-alanyl esters are subsequently transferred to wall teichoic acid by an unknown protein which is located outside of the *dlt* operon (24,27).

The functional roles for DltA and DltC are well established whereas the roles for DltB and DltD are unclear. The functional assignment for DltB in the transport of D-alanyl-Dcp across the membrane is based on the hydropathy profile of the protein and its sequence similarity to transport proteins in the major facilitator superfamily and ATP-binding cassette family (28). DltD is a membrane-associated protein with a putative N-terminal signal peptide that does not have any significant similarity to known proteins. Some studies have suggested that the enzyme catalyzes the extracellular addition of D-alanine into lipoteichoic acid while others have postulated that DltD is anchored to the cytoplasmic face of the cell membrane to function in the binding of DltA and DltC to D-alanine (24,29). Establishing the topology of DltD will be of utmost importance in elucidating its role in the D-alanylation of lipoteichoic acid.

Functional Significance of Teichoic Acid D-Alanylation

The ability to create deletions of either single *dlt* genes or the entire *dlt* operon has established that the D-alanylation modification of teichoic acids is not required for cell viability (2). Despite this dispensability, the D-alanyl residues on teichoic acid are important for maintaining cation homeostasis and modulating the binding of some cell surface proteins (30-31). Furthermore, D-alanylation plays a role in biofilm formation, antibiotic resistance and virulence (23,31-32).

Given that teichoic acids are highly negatively charged, the incorporation of positively charged D-alanine alters the net charge of these polymers. As a result, there is a strong correlation between D-alanyl ester content and the binding of metal cations to teichoic acids (2). As the degree of D-alanylation increases, fewer Mg^{2+} and Ca^{2+} ions bind to the cell wall and those that do associate with only one phosphodiester anion along the backbone of a teichoic acid polymer. By contrast, the binding of metal cations increases as the D-alanyl ester content decreases and cations can bind two phosphodiester anions between neighbouring teichoic acid polymers (33). Although this reduces electrostatic repulsion between teichoic acids, bridging adjacent polymers can alter the binding of proteins to the cell surface. For instance, a *Streptococcus gordonii dltA* mutant had reduced levels of an adhesion protein on its cell surface which prevented coaggregation between this species and its intrageneric partners (34). By contrast, reduced levels of D-alanine have been shown to promote the binding of other cell surface proteins such as autolysins (35). The D-alanylation modification of teichoic acid

therefore plays an important role in regulating the composition of proteins on the surface of Gram-positive bacteria.

In addition to these functions, the D-alanyl residues of teichoic acids are necessary for the first step of biofilm formation wherein bacteria must adhere to a surface. *S. aureus dlt* mutants have been shown to be impaired in their ability to bind to polystyrene or glass surfaces and consequently cannot form biofilms (22). This phenotype has been attributed to the increased negative surface charge of the *dlt* mutant which leads to electrostatic repulsion of neighbouring teichoic acid polymers and thus reduces the capacity for colonization. A clear correlation has also been established between the extent of D-alanylation of teichoic acids and virulence for *L. monocytogenes, S. agalacitae,* and *S. aureus* (23,36-37). The virulence of a *L. monocytogenes* strain in which *dltA* had been inactivated was severely impaired in a mouse infection model (36). A similar phenotype has also been reported for Dlt$^-$ mutants in *S. aureus*. Collins *et al.* have shown that mice infected with a Dlt$^-$ strain have significantly lower rates of sepsis and fewer bacteria in their kidneys compared to mice infected with the wild-type strain (23). Thus, despite the dispensability of genes in the *dlt* operon, the pathway responsible for the D-alanylation modification of teichoic acids represents a promising anti-virulence target.

Dlt$^-$ mutant strains also exhibit an increased sensitivity to cationic antimicrobial peptides and glycopeptide antibiotics. *S. aureus* and *S. xylosus dlt* mutants are sensitized nearly 10-50 fold to cationic peptides such as defensins, protegrins, tachyplesins, and magainin II and gallidermin (32). *S. aureus* strains lacking D-alanyl esters also exhibit a

3-fold increase in sensitivity towards glycopeptide antibiotics such as vancomycin, teicoplanin and balhimycin (32). Given these results, the modification of teichoic acids with D-alanine has been proposed to be a common mechanism for resisting peptides produced by hosts and other Gram-positive bacteria (38).

1.6 THE GLYCOSYLATION MODIFICATION OF TEICHOIC ACIDS

Incorporation of Glycosyl Residues into Teichoic Acids

Investigations into teichoic acid glycosylation have been limited. Although the types of sugar residues that are attached to lipoteichoic acid and wall teichoic acid are known, the genes and gene products responsible for this modification have largely evaded characterization. To date, the genes that encode for lipoteichoic acid glycosyltransferases have not been identified. Recent progress has been made in defining the genetic basis for wall teichoic acid glycosylation in *S. aureus*. Xia *et al.* screened transposon mutant libraries for resistance to serogroup B bacteriophages which bind *N*-acetylglucosamine residues on the cell surface (39). By this approach, they found that integration of a transposon into *tarM* resulted in resistance to all tested serogroup B phages without affecting susceptibility to unrelated lytic phages. Structural analysis of wall teichoic acid isolated from the *tarM* mutant strain confirmed the absence of α-*N*-acetylglucosamine residues along the ribitol phosphate repeats. The wall teichoic acid glycosyltransferase activity of TarM was subsequently confirmed by demonstration that crude extracts containing recombinant enzyme catalyzed the transfer of *N*-acetylglucosamine onto an uncharacterized membrane acceptor *in vitro* (39). Despite this

study, there is still much work to be done in identifying and characterizing wall teichoic acid glycosyltransferases from other organisms.

Functional Significance of Teichoic Acid Glycosylation

The functional significance of wall teichoic acid glycosylation is unknown. Thus far, this modification has only been implicated in the binding of bacteriophages to *B. subtilis*, *S. aureus* and certain species of *Listeria* (40-42). Efforts to understand the physiological importance of teichoic acid glycosylation have been hindered by the lack of observable phenotypes for wall teichoic acid glycosyltransferase mutants. There are no major defects in growth kinetics, microscopic appearances or antibiotic susceptibility patterns in strains devoid of teichoic acid glycosyl substituents (39). However, it is conceivable that the addition of glycosyl residues could affect whether teichoic acid polymers can be modified with D-alanyl esters. If so, wall teichoic acid glycosylation could impact the net charge of these cell surfaces polymers thereby affecting processes such as biofilm formation and virulence.

1.7 AIM OF THESIS

Although great strides have been made in understanding wall teichoic acid biosynthesis in recent years, significant gaps still remain, most notably in relation to wall teichoic acid glycosylation. In the Gram-positive model organism, *B. subtilis* 168, the TagE protein has long been implicated as the wall teichoic acid glycosyltransferase

despite any strong evidence to support this assignment. In this work, we undertook a careful and detailed investigation into the genetics and biochemistry of TagE and have provided unambiguous proof that the enzyme is responsible for wall teichoic acid glycosylation in *B. subtilis* 168. This is the first biochemical study of a wall teichoic acid glycosyltransferase employing purified protein and well-defined substrates. More importantly, this work fills a critical gap in our understanding of wall teichoic acid biogenesis.

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

2.1 PREFACE

The work presented in this chapter has been submitted to the *Journal of Biological Chemistry* under the authorship of Sarah E. Allison, Michael A. D'Elia, Sharif Arar, Mario A. Monteiro and Eric D. Brown. All experiments, with the exception of those pertaining to NMR analysis of wall teichoic acid polymers, were conducted by Sarah E. Allison. The manuscript was written by Sarah E. Allison and edited by Michael A. D'Elia and Eric D. Brown.

2.2 INTRODUCTION

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* (3) and pathogenesis in *Staphylococcus aureus* (4-5). 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 (2). 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 (2,31-32). 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 (2). Classical genetic experiments in *B. subtilis* led to the isolation of the *tag* gene cluster (12,43) for wall teichoic acid synthesis 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 (6,14-17). In addition, the pathway responsible for teichoic acid D-alanylation has been characterized in *B. subtilis*

(24), as well as in other bacteria such as *S. aureus* (32). 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. Once synthesis is complete, the polymer is exported to the outside of the cell where it is attached to the 6-hydroxyl of *N*-acetylmuramic acid of peptidoglycan and modified with cationic D-alanyl esters (2,6). 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 (40,44-45). 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* (39). Disruption of this gene led to wall teichoic acid that completely lacked *N*-acetylglucosamine. The wall teichoic acid glycosyltransferase activity of TarM was subsequently confirmed by demonstration that crude extracts containing recombinant enzyme catalyzed the transfer of *N*-acetylglucosamine onto an uncharacterized membrane acceptor *in vitro* (39).

Burger and Glaser conducted the first and only *in vitro* study of poly(glycerol phosphate) polymer glycosylation in *B. subtilis* nearly fifty years ago (46). This was a traditional study of multi-step purification of glucosyltransferase 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 (44).

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 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.

2.3 MATERIALS AND METHODS

General Methods

Strains, plasmids and oligonucleotides used in this work are listed in Table 2.1. *Escherichia coli* and *B. subtilis* strains were grown in LB (Luria–Bertani) 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 (47). *B. subtilis* competent cells were prepared and transformed as previously described (48). 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-[^{14}C]-glucose and scintillation fluid were purchased from Perkin-Elmer Life Sciences (Boston, MA, U.S.A). CDP-glycerol was synthesized according to established methods (49). MnaA, TarA, TagB, TarD and TagF were purified as previously described (14,16,49-50). Chromatography was performed on a Waters HPLC system (Mississauga, ON, Canada).

Table 2.1 Strains, plasmids and oligonucleotides used in this study.

Strain, Plasmid or Oligonucleotide	Description	Source
Strains		
Novablue	General *E. coli* cloning strain (*endA1 hsdR17*(r_{K12}^{-} m_{K12}^{+})*supE44 thi-1 recA1 gyrA96 relA1 lac*F' [*proA*$^{+}$ *B*$^{+}$ *lacI*^{q}ZΔ*M15*::Tn*10*(Tc^{R})])	Novagen
EB863	*E. coli* strain used for protein over-expression (F^{-} *ompT hsdS*$_B$(r_B^{-} m_B^{-}) *gal dcm araB*::T7RNAP-*tetA*)	Invitrogen
EB6	Wild-type *B. subtilis* (*hisA1 argC2 metC3*)	(63)
EB2252	*tagE* deletion strain derived from EB6 (*hisA1 argC2 metC3 tagE::spec*)	This study
Plasmids		
pUS19	pUC19 derivative used as a source for a $spec^{R}$ cassette	(64)
pDEST17-*tagE*	Expression plasmid for N-terminal His_6-tagged TagE	This study
Oligonucleotides		
tagE-F	5' – ggggacaagtttctacaaaaaagcaggcttcttgtctttacatgcggtgagtgaatc – 3'	
tagE-R	5' – ggggaccactttgtacaagaaagctgggtcttaactctcttttatttccgtgaccctc – 3'	
tagE-a	5' – ggctatagtcgtttactctgatac – 3'	
tagE-b	5' – ctataaactatttaaataacagatttaaaaaattataaacagttaaaggcaatttctcttgg – 3'	
tagE-c	5' – attaatttgttcgtatgtattcaaatatatcctcctcacttttttttactccctttcggcatcta – 3'	
tagE-d	5' – gttaagttactgttaacataaggaata – 3'	
spec-F	5' – agtgaggaggatatatttgaatac – 3'	
spec-R	5' – ttataatttttttaatctgttat – 3'	

Construction of a Δ*tagE* Strain

To create a clean Δ*tagE* 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 Δ*tagE* strain was also examined for resistance to bacteriophage SPO1. A liquid culture of wild-type *B. subtilis* 168 and the Δ*tagE* 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 (51). 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 (52). 1D and 2D ^{1}H and ^{31}P 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 D_2O (99.9 %). TSP (δ_H 0.00, δ_C 0.0) in D_2O was used as reference for both ^{1}H and ^{13}C experiments. Orthophosphoric acid (δ_P 0.0) was used as the external reference for the ^{31}P 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 OD_{600} 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 Coommassie-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 φ.n Analogue Synthesis

Lipid α, β and φ.1 analogues were synthesized according to established methods (15,50). Reactions for the synthesis of the lipid φ.40 analogue contained 50 mM Tris, pH 8.0, 30 mM $MgCl_2$, 100 nM TagF, and 39 equivalents of CDP-glycerol per lipid φ.1 analogue. Lipid φ.n 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 (50). 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). A polymer containing only glycerol phosphate residues was synthesized as previously reported by incubating 4 mM of CDP-glycerol

with 100 nM TagF in a buffer containing 50 mM Tris, pH 8.0, and 30 mM $MgCl_2$ at room temperature overnight (53).

Wall Teichoic Acid Glycosyltransferase Assay

Reactions were conducted at room temperature 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-[^{14}C]-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:

$$v = \frac{V_{max}[S]}{(K_m + [S])}$$

Equation 2:

$$v = \frac{V_{max}[A][B]}{(K_{ia}K_{mb} + K_{mb}[A] + K_{ma}[B] + [A][B])}$$

Equation 3:

$$v = \frac{V_{max}[A][B]}{(K_{mb}[A] + K_{ma}[B] + [A][B])}$$

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 (54).

Poly(glycerol phosphate) Polymerization Assay

A [^{14}C]-lipid φ.5 analogue was synthesized by incubating a lipid φ.1 analogue (100 μM) with a mixture of CDP-glycerol (300 μM) and [^{14}C]-CDP-glycerol (100 μM at 0.01 μCi/μL) in a reaction buffer containing 50 mM Tris, pH 8.0, and 30 mM $MgCl_2$ and 100 nM TagF. Reactions were allowed to proceed to completion and the [^{14}C]-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 262nm. The non-

glycosylated and glycosylated [^{14}C]-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.

2.4 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 Δ*tagE* 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 Δ*tagE* strain contained wild-type levels of phosphate in its cell wall (Figure 2.1A), confirming that there were no polar effects on *tagF*. We then tested the Δ*tagE* and wild type parent strains for resistance to bacteriophage SPO1, which recognizes glycosylated teichoic acid as a receptor (55). *B. subtilis* 168 was susceptible to SPO1 phage while the Δ*tagE* strain was resistant (Figure 2.1B). 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 Δ*tagE* strain and analyzed the polymers by ^{1}H NMR. As shown in Figure 1C, the ^{1}H NMR spectrum revealed an anomeric proton signal at δ5.07 ($J_{1,2}$ 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 (46). By contrast, this signal was absent in the ^{1}H NMR spectrum of wall teichoic acid from the Δ*tagE* strain (Figure 2.1C). Taken together, these results demonstrate that *tagE* is involved in wall teichoic acid glycosylation in *B. subtilis* 168.

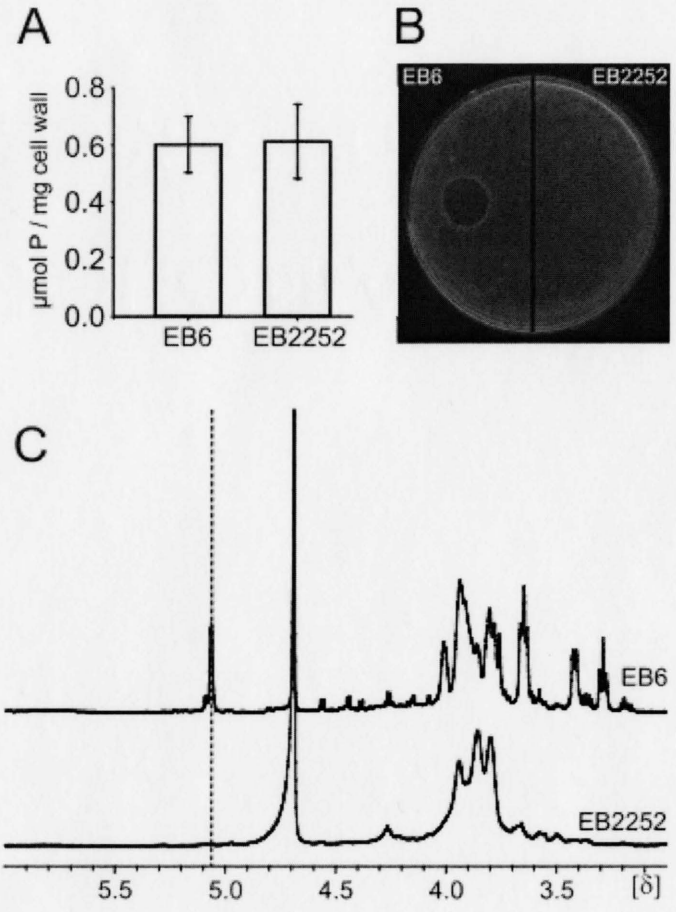

Figure 2.1 Deletion of *tagE* leads to the loss of α-glucose at the C-2 position of poly(glycerol phosphate) wall teichoic acid. (A) Phosphate analysis and (B) SPO1 phage susceptibility of the Δ*tagE* (EB2252) and the wild-type *B. subtilis* 168 parent strain (EB6). (C) ^{1}H NMR spectra of wall teichoic acid isolated from *B. subtilis* 168 (top) and the Δ*tagE* strain (bottom). The α-glucose anomeric resonance at δ5.07 is indicated by a dashed vertical line.

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 TagE *in vitro*. The reaction catalyzed by TagE in our *in vitro* assay is depicted in Figure 2.2. Purified recombinant TagE 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 2.2 (56). 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 (16). 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-[^{14}C]-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 2.3). 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.

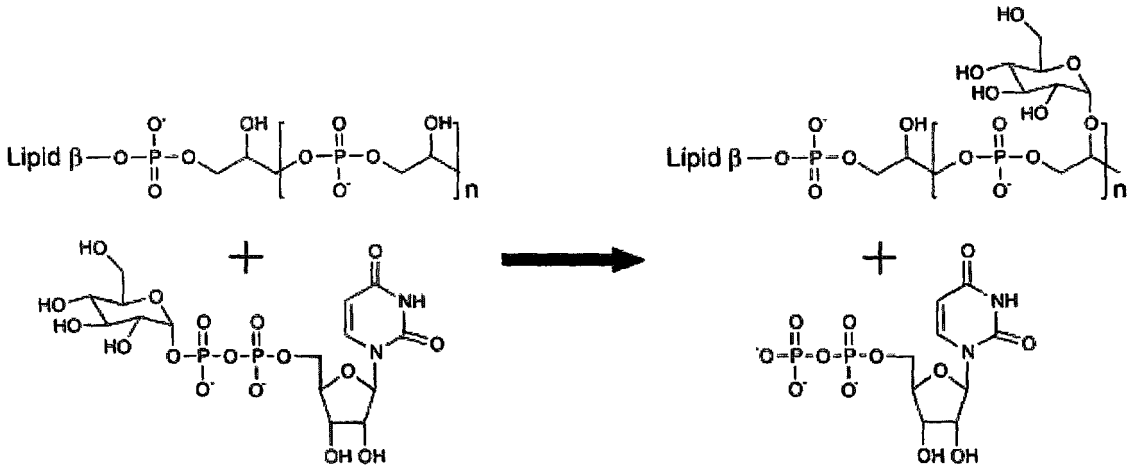

Figure 2.2 Reaction catalyzed by TagE *in vivo*. In the *in vitro* TagE activity assay, the lipid β portion of the poly(glycerol phosphate) polymer has been replaced by a soluble analogue of lipid β (ManNAc-*β*-(1-4)-GlcNAc-1-P-P-tridecane).

Table 2.2 Nomenclature for wall teichoic acid intermediates (29).

Enzyme	Substrate or Substrate Analogue	Chemical Composition[a]
TagA	Lipid α	GlcNAc-1-P-P-und
TagB	Lipid β	ManNAc-*β*-(1-4)-GlcNAc-1-P-P-und
TagF	Lipid φ.n	$(GroP)_n$-ManNAc-*β*-(1-4)-GlcNAc-1-P-P-und
TagA	Lipid α analogue	GlcNAc-1-P-P-tridecane
TagB	Lipid β analogue	ManNAc-*β*-(1-4)-GlcNAc-1-P-P-tridecane
TagF	Lipid φ.n analogue	$(GroP)_n$-ManNAc-*β*-(1-4)-GlcNAc-1-P-P-tridecane

[a]Lipid φ.1 is the product of the TagB reaction and contains a single glycerol phosphate unit. Lipid φ.1 serves as a substrate for TagF which catalyzes the addition of *n* glycerol phosphate units. Und, undecaprenol; P, phosphate; GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; GroP, glycerol phosphate.

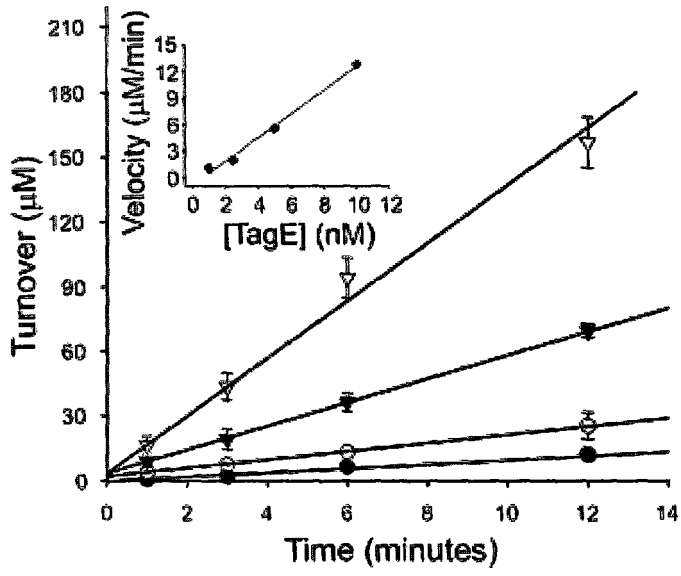

Figure 2.3 Dependence of TagE activity on time and enzyme concentration. Reactions contained 3 mM UDP-glucose, 15 μM lipid φ.40 analogue and 1 (●), 2.5 (○), 5 (■) or 10 nM (□) TagE. Reactions were quenched with urea to a final concentration of 6 M following 1, 3, 6 and 12 minute incubations. The conversion 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}).

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-glucuronic 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 k_{cat} of 17 ± 0.20 s^{-1} and with a K_m for UDP-glucose of 770 ± 52 μM (k_{cat} / K_m = 2.2 × 10^4 $M^{-1}s^{-1}$) at saturating concentrations of the lipid φ.40 analogue acceptor. The specificity constants for UDP-galactose, UDP-*N*-acetylglucosamine and UDP-glucuronic acid were at least 60-fold lower than the specificity constant for UDP-glucose (Table 2.3). 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 2.3). 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 φ.n analogues, ranging from 5 to 80 glycerol phosphate units. As shown in Table 2.3, the turnover numbers were comparable for all of the lipid φ.n 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 φ.n analogues are similar (approximately $1.0 \times 10^5\ M^{-1}s^{-1}$). For instance, if the K_m value of the lipid φ.40 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 φ.40 analogue decreases from 4.1×10^6 to $1.0 \times 10^5\ M^{-1}s^{-1}$ (Table 2.3). The similar specificity constants of the lipid φ.n 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 φ.n analogues (Table 2.3). 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.

Table 2.3 Kinetic parameters determined for sugar donor and acceptor substrates.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Sugar Donors[a]			
UDP-Glc	770 ± 52	17 ± 0.20	2.2×10^4
UDP-Gal	ND[c]	ND	2.9×10^2
UDP-GlcNAc	ND	ND	3.5×10^2
UDP-GalNAc	---[d]	---	---
UDP-GlcA	ND	ND	9.1×10^1
Acceptors[b]			
Gro	---	---	---
GroP	---	---	---
CDP-Gro	---	---	---
Lipid α analogue (TagA Susbstrate)	---	---	---
Lipid β analogue (TagB Substrate)	---	---	---
Lipid φ.1 analogue (TagF Substrate)	---	---	---
Lipid φ.5 analogue	23 ± 3.1 (120)[e]	14 ± 0.72	6.1×10^5 (1.2×10^5)
Lipid φ.10 analogue	24 ± 0.92 (240)	26 ± 0.45	1.1×10^6 (1.1×10^5)
Lipid φ.40 analogue	4.4 ± 0.19 (180)	18 ± 0.24	4.1×10^6 (1.0×10^5)
Lipid φ.80 analogue	1.2 ± 0.11 (96)	20 ± 0.35	1.7×10^7 (2.1×10^5)
Polymer (no linkage unit)	NA[f]	23 ± 1.6	NA

[a] The lipid φ.40 analogue acceptor concentration was fixed at 20μM

[b] The concentration of UDP-Glc was held constant at 3mM

[c] Specific kinetic constants were not determined as the reaction rate was not saturable at experimentally practical sugar donor concentrations

[d] Denotes that there was no detectable production of UDP

[e] Values in brackets represent kinetic parameters that have been adjusted to account for the number of glycerol phosphate units per polymer

[f] Kinetic parameters are not reported as polymer concentrations could not be determined

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 φ.40 analogue acceptor by monitoring the conversion of UDP-glucose to UDP. Double reciprocal plots of the initial rate data are shown in Figure 2.4. The data fit very well to a sequential (ternary) 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 2.4. The K_m values were 3.7 ± 0.61 mM for UDP-glucose and 0.72 ± 0.10 μM for the lipid φ.40 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 φ.40 analogue, respectively. These data are consistent with the kinetic parameters determined for the two substrates under saturating conditions (Table 2.3).

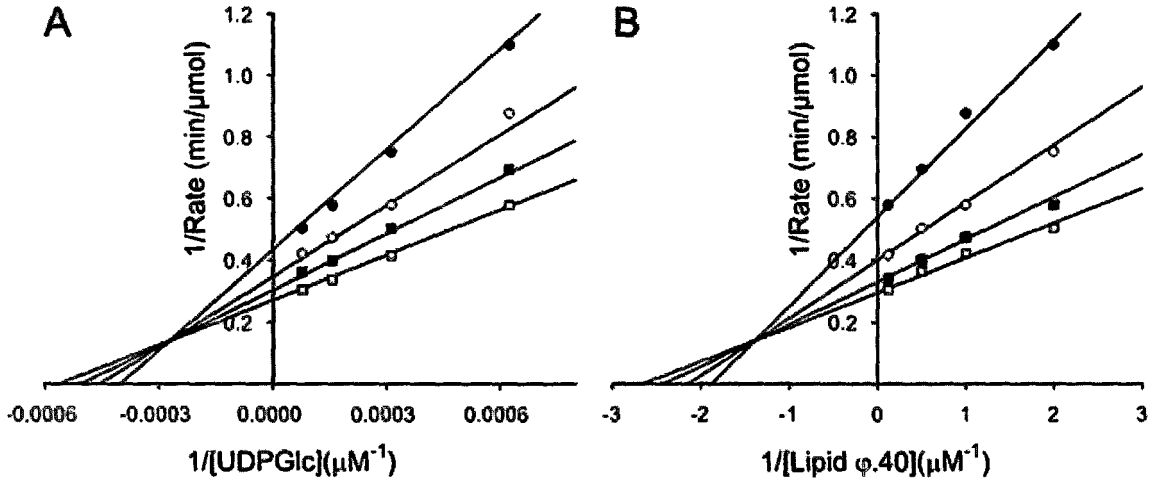

Figure 2.4 TagE utilizes a sequential mechanism. (A) Double reciprocal plot of 1/velocity versus 1/[UDP-glucose]. UDP-Glucose was varied from 1600-12800 μM while the lipid φ.40 concentration was fixed at 0.5 (●), 1 (○), 2 (■) and 8 μM (□). (B) Double reciprocal plot of initial rate data with varying 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).

Table 2.4 Summary of Kinetic Parameters for TagE.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	Dissociation Constant[a]
UDP-Glc	3700 ± 610[b]	25 ± 0.56	6.8×10^3	0.46 ± 0.098
Lipid φ.40	0.72 ± 0.10		3.5×10^7	

[a] Obtained from Equation (2)
[b] Standard error in the value is based on the fit of data to a sequential model

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 (50). 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 φ.5 analogue or a glycosylated [^{14}C]-lipid φ.5 analogue. The products of these reactions were subsequently separated by size exclusion chromatography. Under these conditions, all of the [^{14}C]-lipid φ.5 substrate analogue was converted to a higher molecular weight product as indicated by the shift in retention time (Figure 2.5). In the reaction containing the glycosylated [^{14}C]-lipid φ.5 substrate analogue, only a small percentage was converted to a larger glycerol phosphate containing product (Figure 2.5). 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.

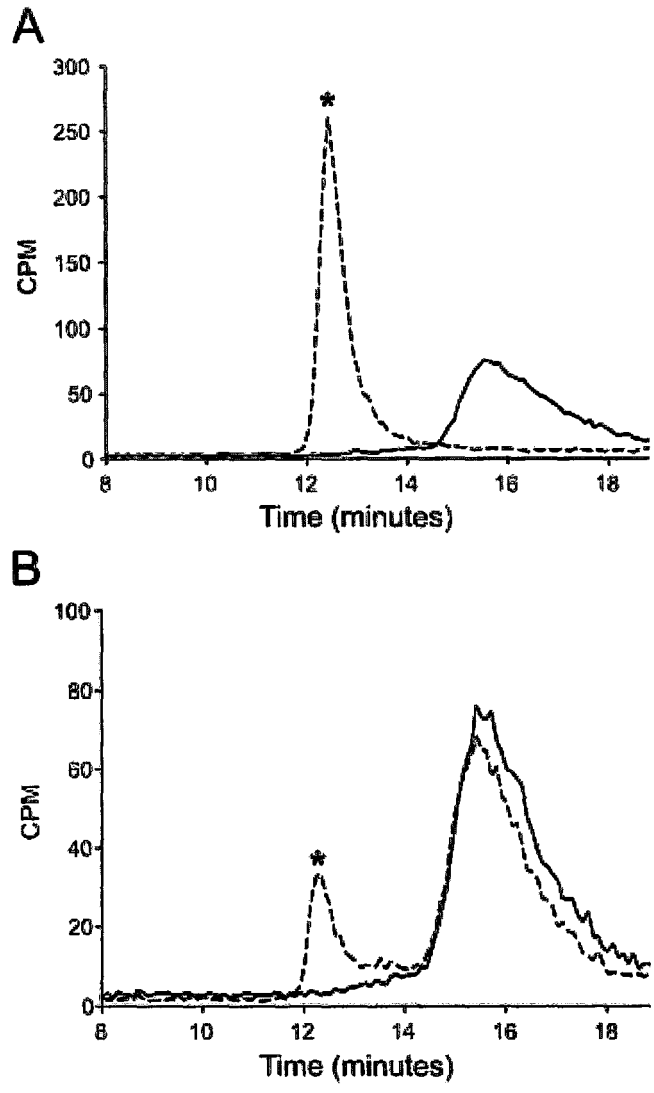

Figure 2.5 Glycosylation impairs poly(glycerol phosphate) polymerization by TagF. (A) The dotted black trace indicates elution for the [^{14}C]-lipid φ.5 analogue. The solid black trace indicates [^{14}C]-lipid φ.5 analogue elution for a reaction containing CDP-glycerol and TagF. Formation of a higher 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 (solid line) or without (dotted 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.

2.5 DISCUSSION

The biosynthesis of wall teichoic acids has been well-characterized and the roles of many of the enzymes have been established with *in vitro* biochemical assays using pure recombinant proteins and well defined substrates (14-17). Wall teichoic acid glycosylation, however, is less well characterized. The TagE protein of *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 biochemical proof that TagE is responsible for wall teichoic acid glycosylation in *B. subtilis* 168 and have characterized the substrate specificity and steady state kinetic mechanism of the enzyme.

We created the first precise *tagE* deletion strain reported to date and confirmed that there were no downstream effects on the polymerase *tagF* whose ribosome binding site lies at the 3' end of *tagE*. Our ability to create a *tagE* deletion strain in the absence of complementation confirms that this wall teichoic acid modification is dispensable for cell growth (40). 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 (40,44). 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 Burger and Glaser who used α and β-glucosidases to elucidate the stereochemistry of the glucose

substituent (46). 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 (39,46). 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 φ.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 ± 0.56 s^{-1}, K_m of 3.7 ± 0.61 mM for UDP-glucose and K_m of 0.72 ± 0.10 μM 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, the glycosylation reaction catalyzed by TagE 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 (50).

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.n 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 2.6A). We also showed that TagE can glycosylate a polymer that lacks a lipid φ.1 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 (ternary complex) mechanism. Many glycosyltransferases, including those that bind undecaprenol-linked acceptors, utilize a sequential mechanism in which the sugar donor binds before the acceptor substrate (13,57-58). 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 (59). 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 (59). 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 2.6B).

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 (50). Indeed, we have shown that the addition of glucose near the terminal end of a polymer impairs polymer extension by TagF. Despite this, previous work from our research group showed length-regulation by TagF on association with heat- and proteinase-treated *B. subtilis* membranes *in vitro* (53). Clearly further work is required to understand the functional significance of this wall teichoic acid modification.

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.

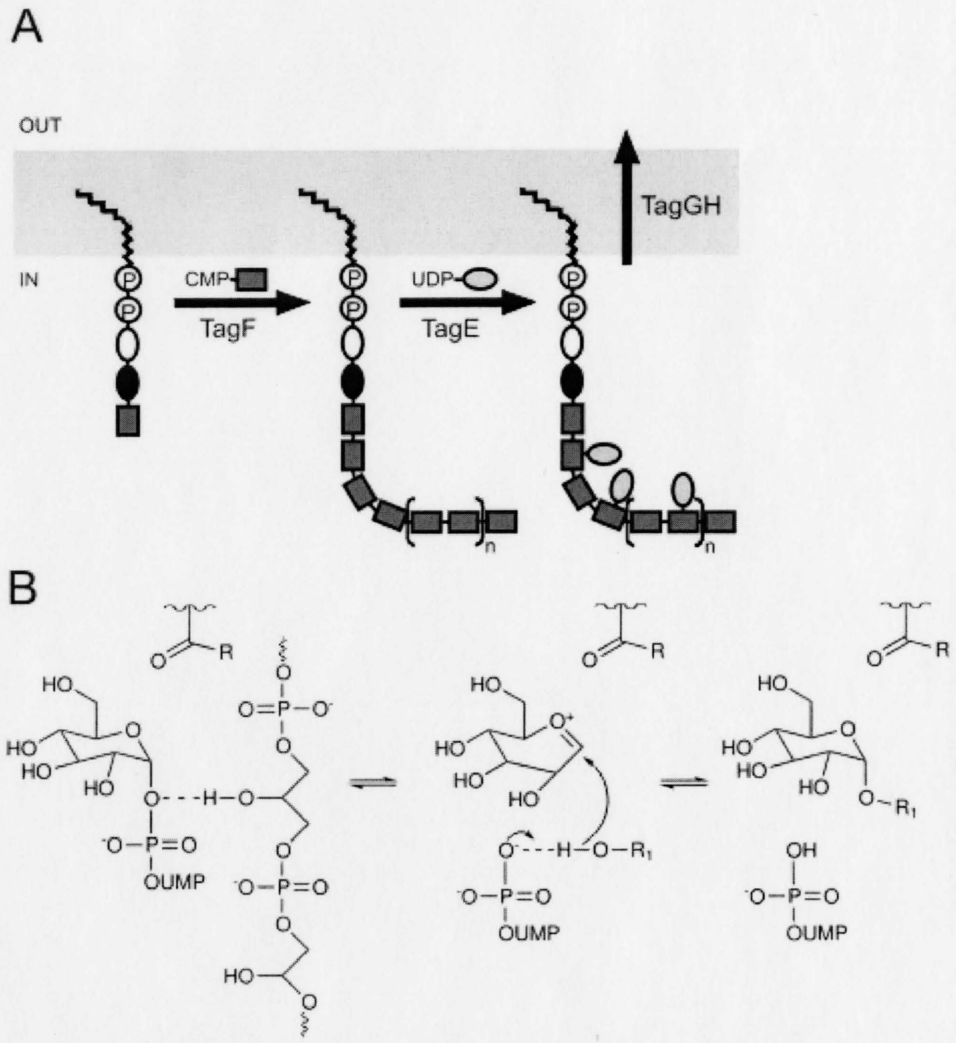

Figure 2.6 Mechanism of wall teichoic acid glycosylation in *B. subtilis 168 in vivo*. (A) TagF synthesizes a polymer of approximately 40 units of glycerol phosphate from CDP-glycerol onto lipid φ.1. Once polymer synthesis is complete, or near complete, TagE transfers glucose from UDP-glucose onto the 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. Symbols: wavy line with a phosphate unit, undecaprenyl-phosphate; white oval, *N*-acetylglucosamine; black oval, *N*-acetylmannosamine; square, glycerol phosphate; light grey oval, glucose. (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 NH_2 group on an amino acid in the active site of the enzyme; R_1, the poly(glycerol phosphate) acceptor substrate.

Chapter Three – Future Work and Conclusions

The aim of this thesis was to determine whether TagE is involved in wall teichoic acid glycosylation in *B. subtilis* 168. Although this work has unambiguously established that TagE is the wall teichoic acid glycosyltransferase in this organism, there are still some outstanding issues regarding this wall teichoic acid modification. These issues are discussed herein.

3.1 FUTURE WORK

Determining the Processivity of *B. subtilis* 168 TagE *in vitro*

To date, we have provided compelling evidence to suggest that TagE is a distributive enzyme. Despite this, future work will be required to definitively determine whether TagE catalyzes the glycosyltransfer reaction through a distributive or processive mechanism. A distributive glycosyltransferase catalyzes the addition of only a single sugar residue onto an acceptor before dissociation of the enzyme-acceptor complex while a processive glycosyltransferase transfers successive glycosyl residues without releasing the acceptor substrate (50). Elucidating the processivity of TagE will require examining patterns of product accumulation under initial rate conditions (60). For instance, in the TagE reaction system developed in this work, if TagE is a distributive polymerase one would expect that in a large excess of substrates the majority of lipid φ.40 substrate analogues would be glycosylated only once. The amount of UDP-glucose consumed would therefore be equal to the amount of glycosylated lipid φ.40 substrate analogues. By contrast, if TagE is a processive polymerase one would expect only a small portion of

lipid φ.40 substrate analogues to be glycosylated, and thus the ratio of UDP-glucose consumed to glycosylated lipid φ.40 analogues would be greater than 1. The challenge will lie in devising a means to separate non-glycosylated substrate analogues from glycosylated ones. Ultimately, determining the processivity of TagE will provide mechanistic insight into this prototypical wall teichoic acid glycosyltransferase.

Phenotypic Investigations into Teichoic Acid Glycosylation

The functional significance of teichoic acid glycosylation is unknown. We have long suspected that teichoic acid glycosylation, like the D-alanylation modification, may be important for virulence. To test this hypothesis, a *S. aureus* Δ*tarM* strain will need to be created and tested in an animal model of infection. This work will be instrumental in determining whether teichoic acid glycosyltransferases are viable targets for antimicrobial therapies.

Efforts to understand the physiological importance of teichoic acid glycosylation have been hindered by the lack of observable phenotypes for teichoic acid glycosyltransferase mutants. In preliminary work, we have created single deletion strains and a double deletion strain of the two predicted wall teichoic acid glycosyltransferases in *B. subtilis* W23. We have uncovered a growth defect, a minor biofilm defect and an altered antibiotic susceptibility pattern of these strains. Although more detailed investigations are required, the phenotypes of our *B. subtilis* W23 deletion strains provide an exciting basis for future studies that aim to determine the functional significance of teichoic acid glycosylation.

3.2 CONCLUSIONS AND PERSPECTIVES

Teichoic Acid Modification as a Novel Target for Antimicrobial Compounds

As multi-drug resistant bacteria become more prevalent, there is a pressing need to develop new antibiotics. The bacterial cell wall has been a celebrated target for antibiotics and holds great promise for the discovery of novel antimicrobial compounds. Cell wall active antibiotics have largely targeted peptidoglycan synthesis and have excluded other cell wall components. The lipoteichoic acid and wall teichoic acid biosynthetic pathways have been considered to be antibiotic targets for many years (6). The complex dispensability pattern of wall teichoic acids suggests that there may be two antimicrobial targets in the pathway: anti-virulence targets and antibiotic targets. Anti-virulence targets have garnered considerable attention as of late because it has been speculated that resistance to these targets will not develop as readily as it would to conventional antibiotic targets (61).

The D-alanylation and glycosylation modifications of teichoic acids would fall in the anti-virulence category. Mutants lacking D-alanyl esters on these cell surface polymers are sensitized to host-generated cationic peptides and have colonization and virulence defects in animal models of infection. Although glycoyslation mutants have not been tested in animal models of infection, it is intriguing to speculate that these mutants would exhibit similar defects. Given these profound phenotypes the modification of teichoic acid warrants consideration as a target for the development of new therapeutic strategies. Neuhaus and Baddiley have suggested targeting (i) the Dcl-catalyzed activation and ligation of D-alanine to Dcp and (ii) the incorporation of D-

alanine into lipoteichoic acid by D-alanyl-Dcp (2). In 2005, May *et al.* reported on the first inhibitor of the D-alanylation modification pathway. The compound, D-alanylacyl-sulfamoyl-adenosine, which inhibited the D-Ala adenylation reaction catalyzed by Dcl significantly reduced the growth of various *B. subtilis* strains when used in combination with vancomycin (62). This finding supports the idea that the modification reactions of teichoic acids are attractive antimicrobial targets. The recent studies that have focused on elucidating the genetics, biochemistry and physiological significance of these modifications should facilitate future work that aims to develop inhibitors of the D-alanylation and glycosylation aspects of the teichoic acid biosynthetic pathways.

Final Comments

Teichoic acids, together with peptidoglycan, define the Gram-positive cell wall. Extensive studies on teichoic acids have firmly established their importance to bacterial physiology. The D-alanylation and glycosylation modifications of these cell surface polymers have been a point of focus for a number of recent investigations. The mechanisms by which these substituents are incorporated into teichoic acid polymers and the functional significance of these modifications are now becoming clearer. However, critical gaps still remain in our understanding of teichoic acid D-alanylation and glycosylation.

With the availability of mutants lacking teichoic acid modifications and the *in vitro* assays that have been developed to monitor the activity of these tailoring enzymes, the stage is now set for comprehensive functional and biochemical studies. Addressing

these uncharacterized aspects of teichoic acid modification will provide insight into the importance of D-alanylation and glycosylation to bacterial physiology and may ultimately lead to the development of novel antimicrobial compounds.

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