INVESTIGATING GRAM-POSITIVE WALL TEICHOIC ACID

INVESTIGATING THE DISPENSABILITY OF WALL TEICHOIC ACID IN THE GRAM-POSITIVE BACTERIA *BACILLUS SUBTILIS* AND *STAPHYLOCOCCUS AUREUS*

By

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ABSTRACT

In recent decades, the emergence of multi-drug resistant pathogenic organisms has proven to be a significant threat to human health. Today, resistant organisms have been identified for every class of antibiotics introduced into clinical practice. Combining this with the knowledge that the introduction of new antimicrobial classes has been almost non-existent since the early 1960's, stresses the importance for new chemical scaffolds and novel therapeutic targets to combat this growing crisis. This thesis focuses on one potential target in Gram-positive bacteria, the biosynthesis of wall teichoic acid polymers. In particular, this study centers on investigating the dispensability of these polymers in both *Bacillus subtilis* and *Staphylococcus aureus*, highlighting the utility of these biosynthetic enzymes as novel therapeutic targets.

Despite significant examination of teichoic acid gene dispensability in the model organism, *B. subtilis*, the complexity surrounding gene dispensability was not appreciated until this study. Studying both *B. subtilis* and *S. aureus*, a peculiar gene dispensability pattern was uncovered in which early acting enzymes; those responsible for disaccharide synthesis, were dispensable for cell viability, yet the remaining late-acting enzymes remained essential. This paradox was reconciled by demonstrating that the lethality associated with the deletion of late-acting genes could be suppressed by deletion of the early genes. Notwithstanding the ability to survive devoid of wall teichoic acid, these polymers remain an absolute requirement for pathogen virulence.

The discoveries presented here have provided remarkable insight into understanding of wall teichoic acid gene dispensability and the complexity associated with this pathway. Furthermore, the ability to generate bacterial strains devoid of wall teichoic acid in both *B. subtilis* and *S. aureus* will provide tremendous utility for uncovering a function for this important cell wall component.

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ABBREVIATIONS

Amp – ampicillin
CA-MRSA – community acquired methicillin-resistant *Staphylococcus aureus*
CDPgro – cytidine diphosphate glycerol
Chl – chloramphenicol
CTP – cytidine triphosphate
EDTA – ethylenediaminetetraacetic acid
Erm – erythromycin
GlcNAc – *N*-acetylglucosamine
GroP – glycerol phosphate
IPTG – isopropyl-β-thiogalactopyranoside
Kan – kanamycin
LB – Luria-Bertani
Linc – lincomycin
LTA – lipoteichoic acid
ManNAc – *N*-acetylmannosamine
MHA – Mueller Hinton agar
MHB – Mueller Hinton broth
MIC – minimum inhibitory concentration
MRSA – methicillin-resistant *Staphylococcus aureus*
MurNAc – *N*-acetylmuramic acid
PBP – penicillin binding protein
RitP – ribitol phosphate
SDS-PAGE – sodium dodecyl sulphate polyacrylamide electrophoresis
Spec – spectinomycin
TRIS – trishydroxymethylaminomethane
VISA – vancomycin-intermediate *Staphylococcus aureus*
VRSA – vancomycin-resistant *Staphylococcus aureus*
WTA – wall teichoic acid

CHAPTER ONE

INTRODUCTION

ROLE OF ANTIBIOTICS AND THE PROBLEM THEY CAUSE

Utility of antibiotics

The discovery of penicillin by Alexander Fleming in 1929 (Fleming, 1929) revolutionized human health, immensely impacting modern medicine. Simple cuts and scrapes, which were often considered a death sentence stemming from uncontrolled bacterial infections, could now be routinely treated. During the early part of the 20th century, the mortality rate of patients with a *Staphylococcus aureus* infection was greater than 80 % (Skinner and Keefer, 1941). The clinical use of antibiotics in the 1940's contributed to a dramatic decrease in death rate throughout the century. Unfortunately, just 50 years later, we have approached a period where simple bacterial infections are again contributing to mortality, now from multi-drug resistant organisms, making the use of these once 'miracle drugs' ineffective.

The discovery of antibiotics in the first half of the last century figured prominently in the decline in mortality at the time. In the United States there was a steady decline of 2.5 % per year in the mortality rate until 1980. However, the 15-year period between 1938 and 1952 saw a remarkable 8.2 % per year decrease (Armstrong *et al.*, 1999). Although the modestly stable decrease in mortality can be attributed to a number of factors, the substantial drop seen between 1938 and 1952 coincides with the first clinical uses of antibiotics, including sulfonamide (1935), penicillin (1941) and streptomycin (1943). In addition to the overall mortality numbers, the spectrum of causes has changed dramatically. In the early 20th century, many deaths resulted from complications from uncontrolled infectious diseases. In today's era, the 'age of degenerative and man-made diseases', the spectrum represents more chronic diseases (Mackenbach, 1994; Omran, 1971). Although the nature of infectious diseases is volatile (eg. 1918 spanish flu), all in all, mortality caused by infectious diseases declined from 797, at the beginning of the century, to a low of only 36 deaths per 100 000 individuals in 1980 (Armstrong *et al.*, 1999). Despite this, alarming is the fact that since 1980, there has been a rise in mortality stemming from infectious diseases (Armstrong *et al.*, 1999; Hughes, 2001; Pinner *et al.*, 1996). In the US from 1980 to 1992, there was a staggering 58 % increase in deaths where infectious diseases were listed as the underlying cause (Pinner *et al.*, 1996). Although HIV infection is likely one aspect for the increase in mortality from infectious disease, this does not present itself significantly enough to account for the whole issue. A significant contributor to this problem is the rise of troublesome multi-drug resistant organisms. With the recurrence of familiar bacteria, *Staphylococcus aureus* and *Streptococcus pneumoniae*, as well as the emergence of less known species of *Clostridium difficile* and *Acinetobacter baumannii*, bacterial infections are becoming increasingly more difficult to control. Therefore, these compounds once heralded as 'wonder drugs', are losing their potency and bacteria are again beginning to win the battle. Despite being able to control lethal infectious diseases for the majority of the last century, of recent years, we have lost our stronghold over these microbes.

Antibiotic resistance

Since the time of the earliest antimicrobial drugs, shortly after their discovery resistant organisms have been isolated in the laboratory (Demerec, 1945). In all cases, clinical manifestation of resistant organisms followed closely behind the introduction into clinical use (Figure 1.1). This revelation, in combination with the extremely limited novel drug discovery since the early 1960's, has lead to a pandemic of untreatable microorganisms spreading through the population. This crisis raises such a great concern that even mainstream magazines have highlighted the significant problem that antibiotic resistance poses (Harrell, 2009).

In this day and age, multi-drug resistant organisms are becoming a significant burden on health care systems throughout the world (Grundmann *et al.*, 2006; Woodford and Livermore, 2009). There are a myriad of organisms where resistance is a formidable concern, including, but not limited to multi-drug resistant strains of *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and the archetypal hospital 'superbug' MRSA (methicillin-resistant *S. aureus*). Drug resistant *S. aureus* is an interesting, likely common, example of the adaptations an organism can make in response to human intervention, tracing back to the discovery and advancements of penicillin. Since the mid 1940's, infections caused by penicillin-resistant strains have increased in hospitals. With the advances in β-lactam modifications, specifically the development of methicillin, *S. aureus* concurrently adapted to develop into the currently familiar, MRSA. In 2003, 64.4 % of hospital *S. aureus* isolates were MRSA, a considerable increase from the 35.9 % in 1992 and only 2 % in 1974 (Klevens *et al.*, 2006). Furthermore, the drug used as a "last line" defence to treat MRSA infections soon fell victim with the increased use of vancomycin. VISA (vancomycin-intermediate *S. aureus*) and VRSA (vancomycin-resistant *S. aureus*) have recently been isolated (Weigel *et al.*, 2003). With the development of resistance to vancomycin, we are quickly exhausting treatment options for this dangerous pathogen. Even more alarming than the discovery of VRSA, is that these once believed obligate hospital acquired infectious are circulating through the community. Since the early 1990's (Coombs *et al.*, 2004; O'Brien *et al.*, 2004), community-associated MRSA (CA-MRSA) have been isolated and quickly spread. CA-MRSA strains have been reported in Canada, the United States, Asia, South America, Australia and throughout Europe, including Norway, the Netherlands, Denmark and Finland, which traditionally have a low incidence of MRSA (Weigel *et al.*, 2003). The adjustment made by *S. aureus* over the years in response to antibiotic intervention has demonstrated the power of adaptation in these organisms and demonstrates the current prevalent problem regarding drug treatment options. Currently, not only the adaptation of well-known pathogens, but also, the emergence of less common strains, *Clostridium difficile* (Coia, 2009) and *Acinetobacter baumannii* (Hanlon, 2005), as successful human pathogens, is causing infectious diseases to become a significant world-wide concern once again.

The "golden age" of antibiotic discovery ended nearly 50 years ago, yet one half of the drugs still in use today were discovered during that period (Davies, 2006). Since this time, there has been a significant lack of newly discovered drug classes. Before the

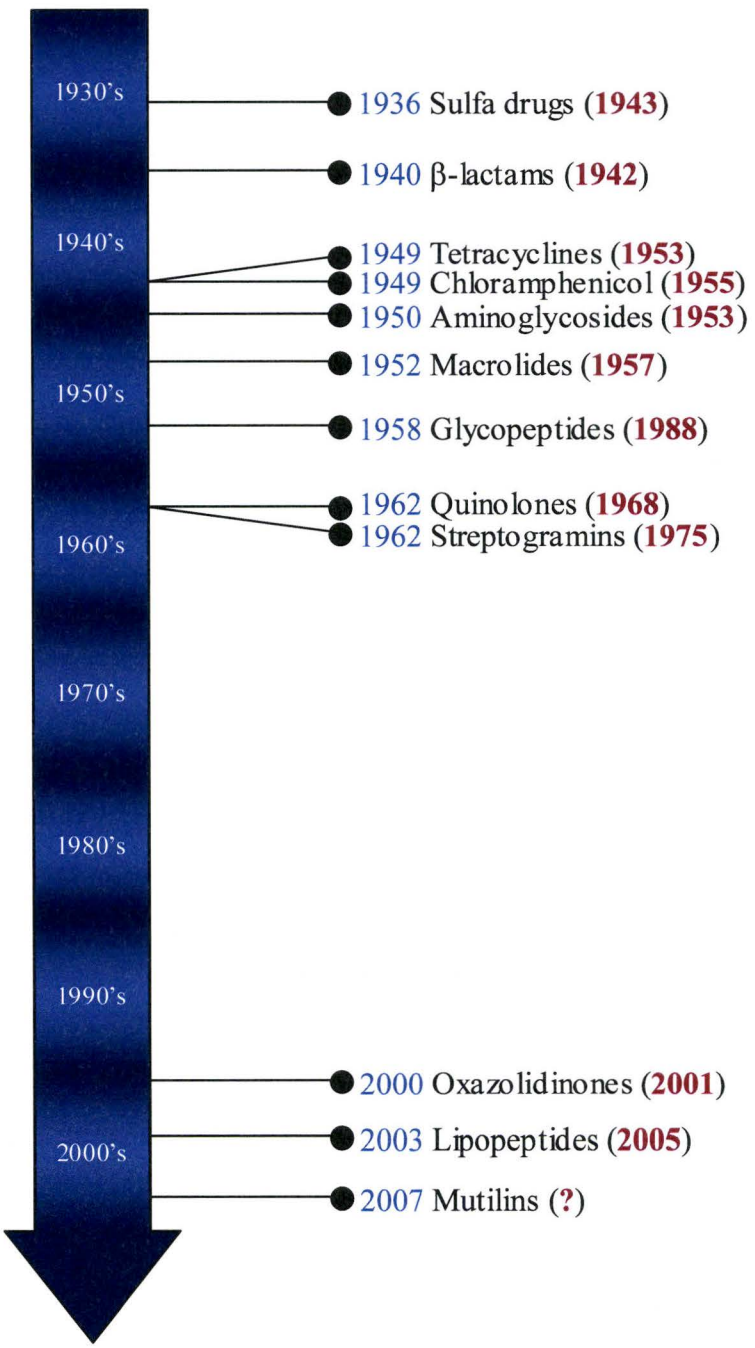

Figure 1.1 – Timeline of antibiotic usefulness. All antibiotics introduced into clinical practice have limited utility. The year of launch for different antibiotic classes are indicated in blue with the year that resistance emerged shown in red. Note the significant gap (termed the innovation gap) between 1962 and 2000.

introduction of linezolid in 2000, no new chemical class had been discovered since 1962 (Fischbach and Walsh, 2009), representing a significant innovation gap in drug discovery (Figure 1.1). During this time, a number of new antibiotics have been used clinically, but these new entities were modifications of drug classes already known. In fact, between 1981 and 2005, 73 % of all antibacterial new chemical entities were built upon only four scaffolds – cephalosporins, penicillins, quinolones, and macrolides (Newman and Cragg, 2007). With the lack of discovery towards at new antimicrobial targets, or at least new molecular scaffolds, drug discovery heavily relies on building upon existing scaffolds, likely accelerating resistance, as it may have with the emergence of MRSA (Chambers and Deleo, 2009; Hiramatsu *et al.*, 2001).

THE BACTERIAL CELL WALL /ENVELOPE

Traditional antibiotics target only a few cellular processes, including cell wall synthesis, DNA synthesis, RNA synthesis and a limited number of metabolic processes (Figure 1.2). The current arsenal of antimicrobials includes a large complement of drugs that target the cell wall as their site of action. The bacterial cell wall makes a particular valuable target given its essential presence, with very limited exception, in all bacteria and its absence in mammalian cells. As such, drugs targeting this area can be regarded as "the magic bullet", a term coined by Dr. Paul Ehrlich in the early 1900's (Ehrlich, 1908).

The Gram-Positive Cell Wall

The Gram-positive and Gram-negative cell envelopes are similar in many ways, yet have some significant differences. The Gram-negative cell wall contains a thin peptidoglycan layer, typically 1 strand thick (~6-7 nm (Labischinski *et al.*, 1991; Leduc *et al.*, 1989; Yao *et al.*, 1999)) and a characteristic outer membrane, containing lipopolysaccharides (LPS) in its outer leaflet. The Gram-positive envelope, on the other hand, in addition to the protein components, the cell wall contains peptidoglycan several layers thick (>30nm (Graham and Beveridge, 1994; Matias and Beveridge, 2005)) and anionic polymers often covalently attached to peptidoglycan.

Peptidoglycan

The peptidoglycan (murein) layer contains glycan stands composed of repeating β-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues. These strands are cross-linked by short peptides, attached to MurNAc, typically an L-Ala-γ–D-Glu-A_2pm (2,6-diaminopimelic acid) -D-ala-D-ala pentapeptide. The cross-link between glycan strands covalently bind the carboxyl group of the penultimate D-ala residue and the amino group of the diaminopimelic acid at position 3, either directly or though a short peptide bridge. Variations in both the peptide stem and cross-link occur (Schleifer and Kandler, 1972; Vollmer *et al.*, 2008a), but the glycan stand is uniform among bacteria. The peptidoglycan meshwork plays a vital role in the

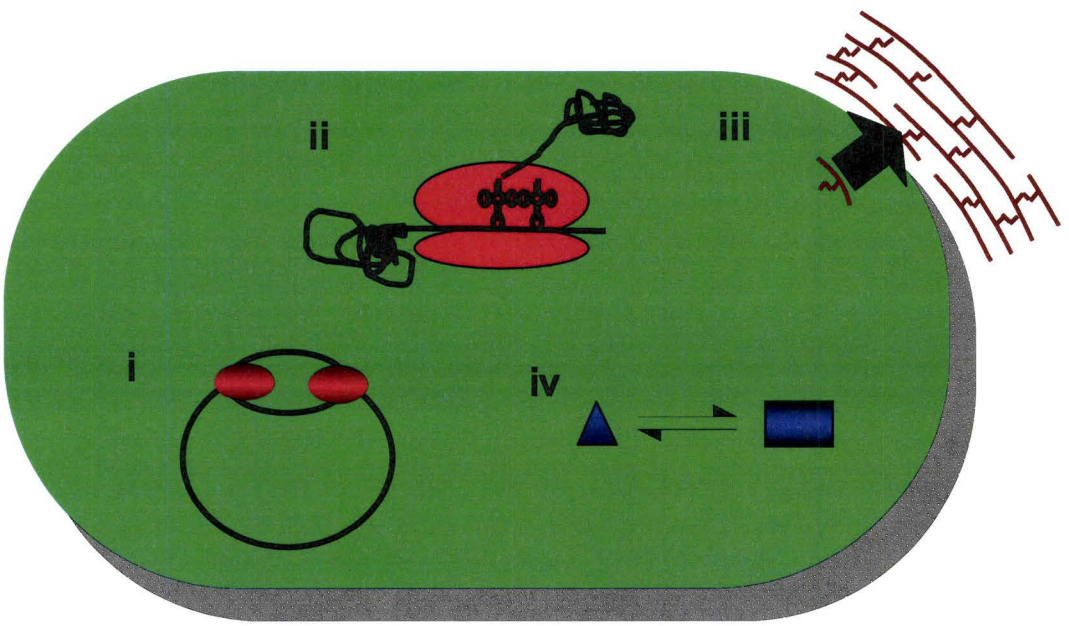

Figure 1.2 – Classical antimicrobial targets. Traditionally antibiotics target a limited number of cellular processes, including (i) DNA synthesis, (ii) protein synthesis, (iii) cell wall synthesis (exclusively peptidoglycan), and (iv) few metabolic processes.

cell, providing a scaffold for attachment of proteins and exopolysaccharides. It is an important structural support to the cell, so much so that degradation of the cell wall peptidoglycan by lysozyme results in cell lysis (Salton, 1958).

Since its discovery in 1956 (Strange and Dark, 1956), the structure and biosynthesis of peptidoglycan has been significantly studied. With the exception of a defined flippase, responsible for moving lipid II, the lipidated GlcNAc-MurNAc-pentapeptide unit, from the inside of the cell membrane to the outside, each enzyme responsible for peptidoglycan synthesis has been discovered (reviewed in (van Heijenoort, 2001a; van Heijenoort, 2001b)). Generally, the Mur enzymes are responsible for the generation of an isoprenoid-linked disaccharide pentapeptide on the inner-face of the cytoplasmic membrane, which is subsequently transferred outside of the cell and added to a growing glycan strand by the action of penicillin binding proteins (PBPs) (Figure 1.3). The current state of peptidoglycan research centers on the understanding of the physical architecture of this macromolecule and how this contributes to its stress-bearing, yet dynamic features. Huge advances are being made with advanced microscopy techniques to visualize peptidoglycan using both sacculi and intact cells (Hayhurst *et al.*, 2008).

Since the discovery of penicillin, peptidoglycan has been a celebrated target of antibiotics. Throughout the 1950's, penicillin played a central role in defining our understanding of peptidoglycan synthesis. Currently, peptidoglycan serves as the target for many antibiotics; penicillin, vancomycin, ramoplanin and nisin, to name a few, including many of the clinically important and routinely used classes. Therefore, the bacterial cell wall has proven to be an immense reservoir for antibiotic development.

Anionic Polymers

In addition to peptidoglycan, the Gram-positive cell envelope contains a number of modular extracellular anionic polymers. Polysaccharides can be found ubiquitously in nature, in both intracellular and extracellular forms. Extracellular polysaccharides, exopolysaccharides, can be released by the cell, or remain attached to outer cellular components (Wilkinson, 1958). Exopolysaccharides have found important roles in commercial applications. Due to their ability to thicken and stabilize aqueous solutions they have found utility in both food and industrial applications, such as use in dressings, sauces, syrups, detergents, paints, textiles, adhesives, amongst many others (Baird *et al.*, 1983; Sandford, 1979). Two of the most notable exopolysaccharides in Gram-positive bacteria are wall teichoic acid and lipoteichoic acid.

Wall teichoic acid polymers are abundant phosphate-rich polyols of varying chemical composition covalently linked to peptidoglycan. They have been shown to make up as much as 60 % of the cell wall weight (Boylen and Ensign, 1968). The two most commonly occurring polymers are repeating units of glycerol phosphate, as in *Bacillus subtilis* 168, and ribitol phosphate, as in *B. subtilis* W23 and *S. aureus*. These polymers are often decorated with alanine and glucose moieties (Figure 1.4). Since wall teichoic acid is the focus of this thesis, further discussion follows.

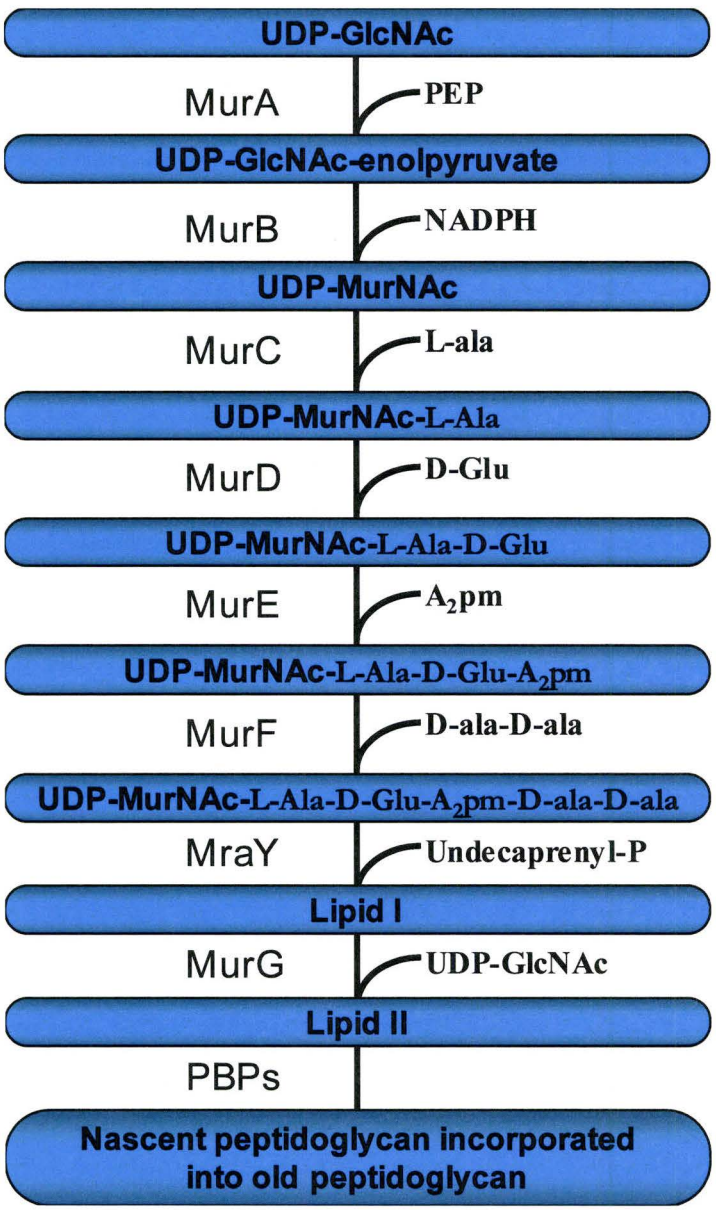

Figure 1.3. Stepwise assembly of peptidoglycan. Highlighted are the typical steps involved in the synthesis of peptidoglycan.

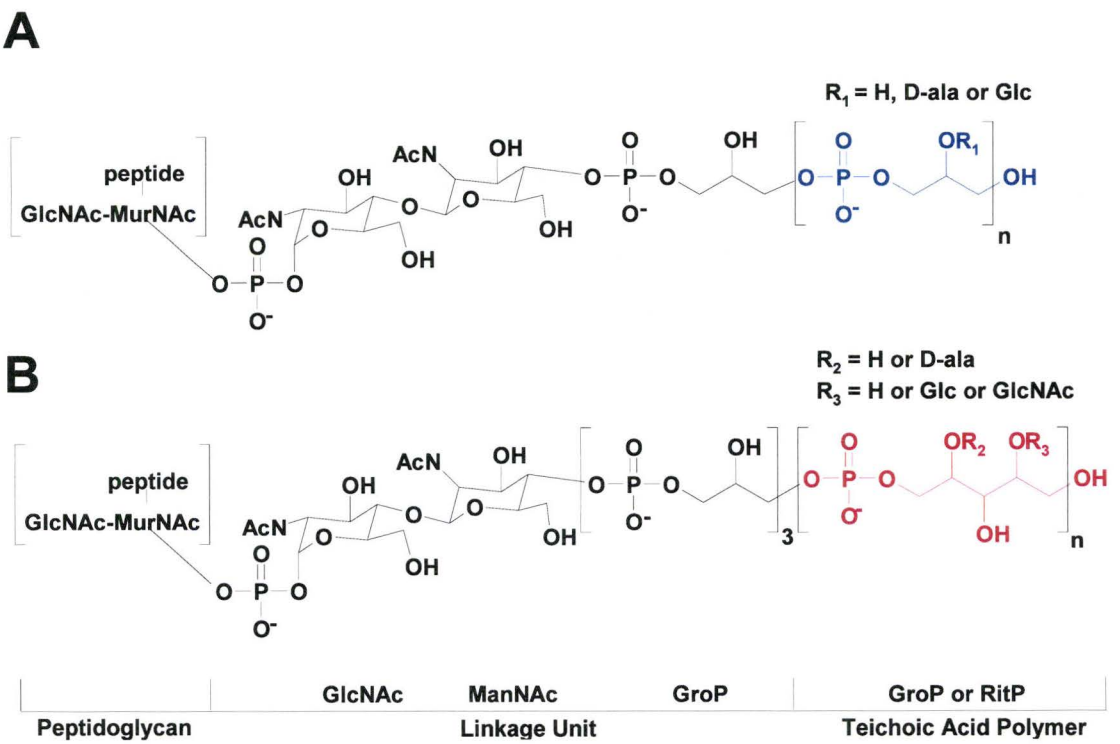

Figure 1.4 – Chemical structure of wall teichoic acid. The chemical structures of the glycerol phosphate containing teichoic acid polymers (A) and the ribitol phosphate containing polymer (B) are given. Stains such as *B. subtilis* 168 produce glycerol polymers, while ribitol polymers are found in strains like *B. subtilis* W23 and *S. aureus*. Although the polymer component in wall teichoic acid can vary, the linkage connecting the polymer and MurNAc of peptidoglycan is invariant, containing a GlcNAc, ManNAc and up to three glycerol phosphate units. The polymer component is often decorated with D-ala or sugar components including glucose and GlcNAc.

Irrespective of the species, lipoteichoic acid is invariant in composition, composed of a repeating glycerol phosphate polymer extending into the cell wall matrix. The polymer is covalently attached to a glycolipid moiety in the cell membrane (Fischer, 1988). Unlike wall teichoic acid, the origin of glycerol phosphate incorporated into lipoteichoic acid is generated from phosphatidylglycerol (Emdur and Chiu, 1975; Glaser and Lindsay, 1974). Not until recently has the lipoteichoic acid synthesis machinery been uncovered. Although the components generating the glycolipid anchor have been know for a several years (Kiriukhin *et al.*, 2001), only very recently were the enzymes responsible for glycolipid transport, LtaA (Grundling and Schneewind, 2007a), and polymerization, LtsS (Grundling and Schneewind, 2007b) identified, completing our understanding of the assembly pathway. The precise function of lipoteichoic acid in the membrane of cells remains elusive; however, it has been proposed to be involved in accurate septal development (Grundling and Schneewind, 2007b). With the discovery of LtaA and LtaS, there has been tremendous advancement in our understanding of lipoteichoic acid structure and function.

In phosphate rich media, a secondary polymer can be isolated from the cell wall peptidoglycan in *B. subtilis*; a minor teichoic acid polymer comprised of poly(glucosyl-*N*-acetylgalactose phosphate) (Duckworth *et al.*, 1972; Estrela *et al.*, 1991; Shibaev *et al.*, 1973). This polymer represents less than 20 % of the total cell wall hexosamines (Estrela *et al.*, 1991) and is considered dispensable for growth. The synthesis of this polymer has been known to involve the *ggaAB* operon. However, recently in *B. subtilis*, TagABDGH and TagO that are known to be required for wall teichoic acid synthesis have also been implicated in its assembly (Freymond *et al.*, 2006). The participation of TagABDGHO in minor teichoic acid synthesis is intriguing and it remains to be seen if the involvement of these wall teichoic acid enzymes holds true.

Conversely under phosphate limitation, another anionic polymer can be identified covalently attached to peptidoglycan, a teichuronic acid polymer. Under phosphate depletion, it has been shown that the concentration of the wall teichoic acid polymer decreases significantly and is replaced by teichuronic acid (Ellwood and Tempest, 1972). The enzymes, encoded in the *tua* operon, assemble this glucuronyl-*N*-acetylgalactosamine polymer (Soldo *et al.*, 1999; Ward, 1981). The advantage of remodelling the exopolysaccharide composition of the cell wall in this manner is that it allows the cell to conserve phosphate when limiting, while maintaining the anionic nature afforded by the uronic acid moiety. Interestingly, despite significant remodelling, there still remains approximately one third the amount of phosphate in the cell wall, indicating that synthesis of the teichuronic acid does not bypass entirely the synthesis of wall teichoic acid (Bhavsar *et al.*, 2004). The function of teichuronic acid polymers in the cell wall has not been established, it is possible that this polymer complements the phosphate rich wall teichoic acid under phosphate limiting conditions.

WALL TEICHOIC ACID

The commencement of the study of wall teichoic acid began with the discovery by Baddiley and Mathias in 1954 (Baddiley and Mathias, 1954). Over the last 50 years, significant strides have been made in understanding the chemical character of these polymers and their synthesis in the cell. Despite this, a precise function for these polymers remains elusive.

Structure of Wall Teichoic Acid

The first hints of the existence of wall teichoic acid came in 1954 with the discovery of two acid labile cytidine-containing molecules extracted from cystine-deficient *Lactobacillus arabinosus* (Baddiley and Mathias, 1954). Interest in these molecules quickly developed and within just four years these compounds were identified and a role for them in the cell was proposed. Chemical analysis identified these two molecules as CDP-glycerol (Baddiley *et al.*, 1956c; Baddiley *et al.*, 1956d) and CDP-ribitol (Baddiley *et al.*, 1956a; Baddiley *et al.*, 1956b). In addition, ribitol containing derivatives were being isolated from the cell walls of a number of bacterial species (Armstrong *et al.*, 1958a; Armstrong *et al.*, 1958b; Baddiley *et al.*, 1957). Given the lack of precedence for the utilization of ribitol in nature, in addition to the recently uncovered relationship between uridine derivatives and muramic acid-pentapeptide within the cell wall (Park and Strominger, 1957), these cytidine derivatives were believed to be involved in cell wall synthesis, likely as a polymer (Baddiley and Buchanan, 1959). The manner in which the polymer was attached to the cell wall was difficult to ascertain. An early hypothesis suggested that ionic interaction existed between the cell wall polymer and peptidoglycan. Huge advances in this area came from Ghuysen *et al.* and Strominger *et al.* with the isolation of a teichoic acid-glycopeptide conjugate demonstrating this interaction was through covalent binding of the teichoic acid polymer to peptidoglycan (Ghuysen *et al.*, 1965; Strominger and Ghuysen, 1963). Work continued to chemically dissect the linkage between the teichoic acid polymer and peptidoglycan to aid in our final understanding of the chemical composition of teichoic acid polymers and how they are attached to peptidoglycan (Button *et al.*, 1966; Coley *et al.*, 1976; Coley *et al.*, 1977; Coley *et al.*, 1978; Heckels *et al.*, 1975; Kojima *et al.*, 1983; Sasaki *et al.*, 1980). A compilation of this work resulted in the chemical composition highlighted in Figure 1.4. The polymer component of wall teichoic acid is typically composed of repeats of glycerol or ribitol phosphate, but other repeating units have been identified (Navarre and Schneewind, 1999). The polyol polymer is covalently attached to carbon 6 of MurNAc residues within peptidoglycan through an invariant 'linkage unit' (*N*-acetylglucosamine (GlcNAc), *N*-acetylmannosamine (ManNAc) and 1 – 3 glycerol phosphate residues).

Synthesis of Teichoic Acid

Biochemical characterizations of the products obtained from crude fractionation experiments played a key role in understanding the process of wall teichoic acid

assembly. Reports using both soluble and particulate factions, showed wall teichoic acid polymerization proceeding subsequent to the generation of CDP-glycerol and CDP-ribitol (Burger and Glaser, 1964; Glaser, 1964; Shaw, 1962). It was later revealed that the polymerization of ribitol into wall teichoic acid was dependent on linkage onto a glycerol residue (Bracha and Glaser, 1976a; Bracha and Glaser, 1976b; Hancock and Baddiley, 1976). In addition, a collection of reports using particulate fractions discovered that the wall teichoic acid polymer was assembled on a membrane bound fraction that could be readily extracted by alcohol (Douglas and Baddiley, 1968; Hussey and Baddiley, 1972; McArthur *et al.*, 1978; Wyke and Ward, 1977). This membrane component was found to be an isoprenoid moiety shared with peptidoglycan, undecaprenyl phosphate (Anderson *et al.*, 1972; Watkinson *et al.*, 1971). The nucleotide composition of precursor molecules and the membrane assembly of the polymer led to the speculation that polymer synthesis occurs on the inner face of the cytoplasmic membrane (Shockman and Barrett, 1983). Together these early experiments assembled a basis that provided the groundwork for our current understanding of wall teichoic acid assembly (explained below).

Genetics of wall teichoic acid

Akin to the role James Baddiley played in the deconstruction of the chemical composition of wall teichoic acid, decades later, Dimitri Karamata played an equally important role in the characterization of the genetic components required to synthesize wall teichoic acid polymers.

Genetic investigations into wall teichoic acid began with the generation of mutants by chemical means, which displayed either a temperature sensitive (*ts*) or osmotically sensitive rod to sphere transition (Boylan and Mendelson, 1969; Rogers *et al.*, 1968; Rogers *et al.*, 1970). In both mutant types, this rod to sphere transition was accompanied by gross morphological defects in shape, variable thickening in the cell wall, and erratic septal formation, but no specific cause for these defects was established. Follow up studies by Boylan *et al.* and Karamata *et al.* genetically linked 2 mutants to the *hisA1* locus. Consistent with the initial observations, these mutants displayed altered gross cell morphology defects and cell wall abnormality. In addition, they were found to contain decreased teichoic acid content and the inability to bind phage, implicating a defect in teichoic acid. Thereby this mutant was designated *tag-1* (Boylan *et al.*, 1972; Karamata *et al.*, 1972). These early studies stimulated Karamata's interest in this area and began a study into understanding the defects associated with this class of mutants.

To date, our current understanding of the genetic organization, gene identification and essentiality of wall teichoic acid, at least in *B. subtilis*, can be attributed to Karamata and his group. This group was the first to examine in detail teichoic acid gene dispensability (discussed below), in addition to identifying and annotating the gene cluster responsible for wall teichoic acid biosynthesis. A landmark experiment by Karamata *et al.* involved the transformation of chromosome from *B. subtilis* W23, which expresses a poly(ritP) teichoic acid polymer, into *B. subtilis* 168, which expresses a poly(groP) polymer, containing the *tag-1* temperature sensitive mutation. Selection for recombinants at the non-permissive temperature indicated a replacement of the *tag-1*

locus. Interestingly, chemical analysis and phage sensitivity indicated that in all cases isolated recombinants contained poly(ritP) replacing poly(groP) teichoic acid entirely. Furthermore, transformation mapping indicated that the exchange region was small; no more than 20 genes. These results indicated that genes controlling poly(ritP) and poly(groP) polymer synthesis occur in corresponding regions in the two strains and also that all of the genes responsible for polymer production reside in a limited area adjacent to *tag-1* (Karamata *et al.*, 1987). Sequence identification of this region by Mauel *et al.* identified two divergently transcribed operons, *tagAB* and *tagDEF*, responsible for teichoic acid synthesis in *B. subtilis* 168 (Figure 1.5A) (Mauel *et al.*, 1991). A similar study in *B. subtilis* W23, uncovered a related divergently transcribed operons, *tarABIJKL* and *tarDF*, in that organism (Figure 1.5B) (Lazarevic *et al.*, 1995). These studies, in addition to preliminary *in vitro* enzymatic analysis, (Pooley *et al.*, 1991; Pooley *et al.*, 1992) provided the basis for assigning predictive functions to each gene in both operons.

Through a series of bioinformatic and ever growing biochemical data, functional predictions have been assigned to every gene responsible for wall teichoic acid production (Figure 1.6). Gene nomenclature was designated as *tag* (***t***eichoic ***a***cid ***g***lycerol) to identify genes involved in the synthesis of poly(groP) polymers and *tar* (***t***eichoic ***a***cid ***r***ibitol) for those generating poly(ritP) polymers. The synthesis, export and attachment of the teichoic acid polymer are accomplished by as many as 13 different enzymes acting in succession. Polymer synthesis begins on the cytoplasmic face of the cell membrane onto undecaprenyl phosphate. Using UDP-GlcNAc as a substrate, TagO adds GlcNAc-1-P to the undecaprenol moiety releasing UMP. Undecaprenyl-diphosphate-disaccharide synthesis is completed with the addition of a β-1,4-linked ManNAc though the action of TagA. The substrate for the reaction, UDP-ManNAc is provided by MnaA, which epimerizes UDP-GlcNAc into UDP-ManNAc. A second important nucleotide precursor, which is one of the molecules isolated by Baddiley in 1954, is CDP-glycerol. CDP-glycerol is formed by the reaction of CTP and glycerol phosphate by the enzyme TagD and is used as a precursor for the addition of glycerol phosphate into the growing chain by the activities of both TagB and TagF. TagB has been shown to add only a few, one to three, glycerol phosphate units to the polymer, while in poly(groP) polymers, TagF adds the remaining units to complete polymer synthesis. This scheme represents the synthesis of poly(groP) polymers, like in *B. subtilis* 168. Poly(ritP) polymer synthesis occurs in a very similar manner, except that the homologous enzymes to TagB and TagF, TarB and TarF, only add a total of three glycerol phosphate units; TarB adding one and TarF adding two. The synthesis is completed by the activities of TarK, adding one ribitol phosphate, and TarL, adding the remaining units to complete synthesis. Ribitol phosphate is provided as nucleotide activated CDP-ribitol, generated from ribulose phosphate, NADPH, and CTP by the TarJ and TarI enzymes. Once complete, the polymer is exported out of the cell, through the ABC transporter TagGH, before it is transferred to peptidoglycan, by a still unknown transferase. Transfer of the polymer to peptidoglycan allows for the release of undecaprenyl phosphate, as done in peptidoglycan synthesis, allowing this limiting molecule to be recycled and used in another cycle of synthesis.

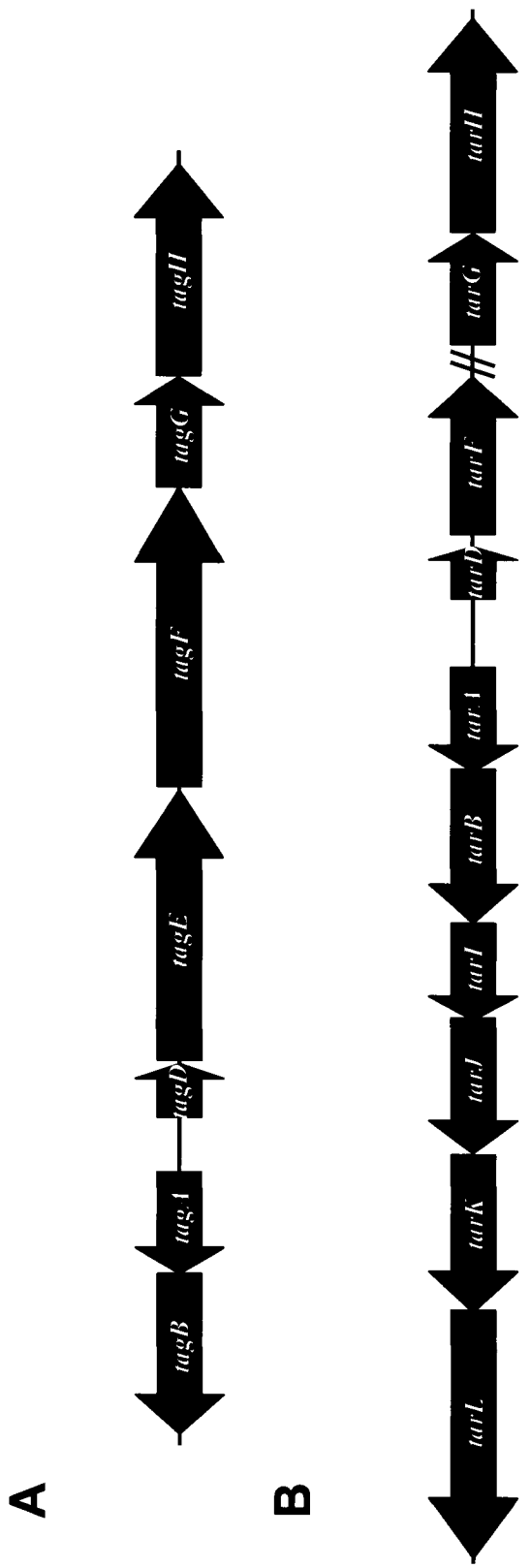

Figure 1.5 – Genetic organization of wall teichoic acid genes. The wall teichoic acid biosynthetic operons for *B. subtilis* 168 (A) and *B. subtilis* W23 (B) are displayed. A glycerol containing teichoic acid polymer is generated by the *tag* operon of strain 168, with the *tar* operon of strain W23 generating a ribitol containing polymer.

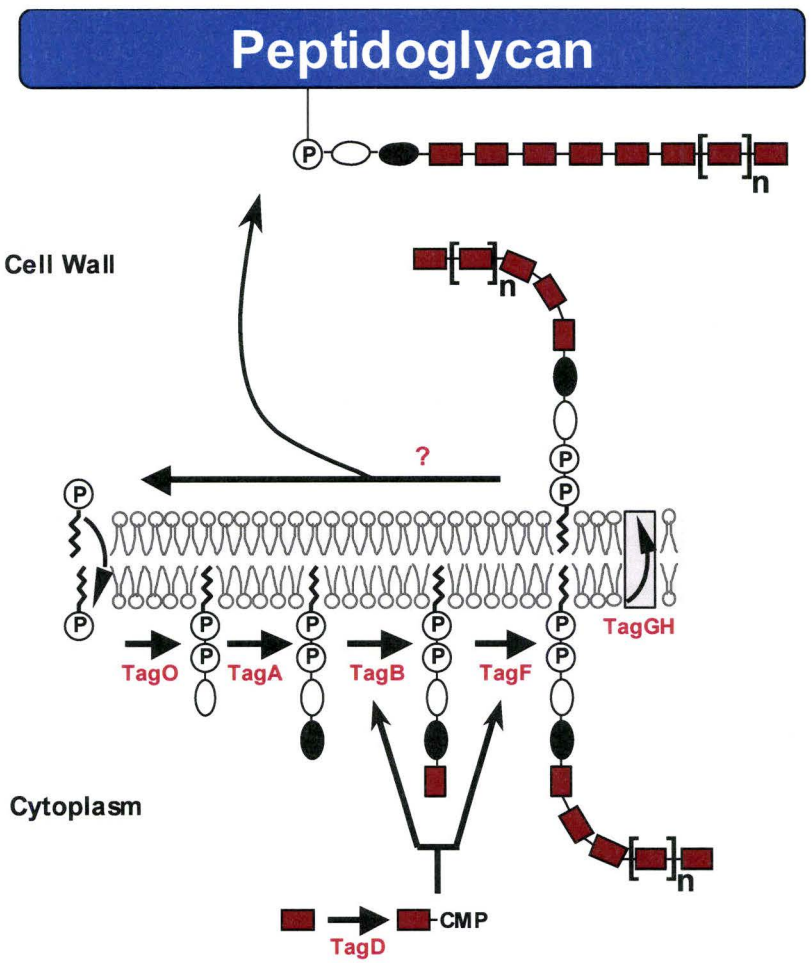

Figure 1.6 – Proposed pathway for glycerol teichoic acid polymer synthesis in *B. subtilis*. *B. subtilis* 168 teichoic acid comprises a polymer composed of a disaccharide containing *N*-acetylglucosamine-1-phosphate (open oval) and *N*-acetylmannosamine (closed oval) and ~40 repeating glycerol phosphate (square) units. These polymers are synthesized in a stepwise manner on the cytoplasmic face of the cell membrane onto undecaprenyl phosphate (wavy line). Following synthesis, the entire polymer is exported out of the cell and attached to *N*-acetylmuramic acid of peptidoglycan. A similar scheme is proposed for the synthesis of ribitol containing teichoic acid polymers, as in *S. aureus* and *B. subtilis* W23. In a ribitol pathway, this stepwise scheme continues. Only 3 glycerol phosphate units are present, while TarIJ form CDP activated CDP-ribitol. TarK then adds a single ribitol phosphate to the polymer and TarL adds the remaining (~30) units, before transfer.

Teichoic acid gene dispensability

In an attempt to understand the functional role for these poly anionic polymers, dispensability studies were undertaken. The start of this work stretches back nearly 40 years since the isolation of mutants displaying altered cell morphology. The belief that wall teichoic acid was an essential component in the cell wall was first presumed with the generation of the temperature sensitive mutants that were predicted to target teichoic acid synthesis (Boylan *et al.*, 1972; Karamata *et al.*, 1972). This was fortified with the isolation of several temperature sensitive mutations isolated to loci surrounding the teichoic acid operon, implicating essential phenotypes for these genes (Briehl *et al.*, 1989). These mutants were found to display a disrupted cell morphology and impairment in growth at the non-permissive temperature.

Even before sequence information for the teichoic acid locus had been obtained, the first targeted approach at gene disruption was attempted. Using integrative plasmids containing fragments of two genes, identified as *tagA* and *tagB*, attempts to disrupt these genes through single integration methods failed to yield mutants (Mauel *et al.*, 1989). Despite their crude nature, these experiments lent significant support to the indispensable nature of these genes and the polymer entirely.

Although these early studies were advanced for their time, they employed crude techniques, and often used non-physiological conditions to study dispensability. As the molecular tools progressed, so did the study of teichoic acid dispensability. Much of the direct evidence for the dispensability of teichoic acid biosynthesis genes came from our own lab. The study of essential genes is particularly difficult since deletions or non-functional mutations render the cell non-viable. The development of systems to allow for the induced / regulated expression of genes, especially in *B. subtilis* (Bhavsar *et al.*, 2001; Vagner *et al.*, 1998), has advanced the analysis of essential genes through protein depletion studies. One such system developed in our lab (Bhavsar *et al.*, 2001), places the tightly regulated target gene at a secondary loci, which is regulated by the addition of xylose into the media. Accordingly, in the absence of xylose no protein is expressed, but the addition of xylose permits protein expression to varied levels. Providing an inducible copy of the targeted gene at the secondary locus allows for the deletion of the gene at its native loci. Using this system our group performed studies on several wall teichoic acid biosynthetic genes, including *tagD* (Bhavsar *et al.*, 2001), *tagB* and *tagF* (Bhavsar *et al.*, 2004), with each gene shown to be indispensable for cell viability. Using a similar approach, Karamata's group believed *tagO* to be indispensable (Soldo *et al.*, 2002). The invention and implementation of these systems have been invaluable in assessing and characterizing phenotypes associated with the loss of function mutations of essential genes. The studies described using these systems further supported the early studies demonstrating the requirement for the teichoic acid biosynthetic machinery. It should be noted that at the time of this work, all studies investigating the dispensability of wall teichoic acid biosynthetic genes were limited to the model organism *B. subtilis* 168.

Although the study of the teichoic acid biosynthetic gene dispensability has progressed significantly over several decades, only recently were tools available to assess dispensability in the 'normal' state. These most recent studies appeared to solidify the

essential nature of teichoic acid biosynthesis. However, work presented in this thesis will demonstrate that wall teichoic acid gene dispensability is significantly more complex than originally believed.

Function of teichoic acid

Despite its discovery more than half a century ago and the considerable structural and genetic investigations over this time, a precise function for these polymers has not been identified. The chemical nature of this molecule and phenotypes associate with loss of this polymer have led to several postulated functions.

As highlighted above, teichoic acid polymers have been well known to be a site for phage binding, and this property was considerably exploited in the early isolation of mutant strains. Additionally, autolysin enzymes, responsible for the hydrolysis of the cell wall, have been shown to interact with teichoic acid polymers (Brown *et al.*, 1970; Heptinstall *et al.*, 1970; Holtje and Tomasz, 1975), likely through electrostatic interactions (Archibald *et al.*, 1973; Fan, 1970). Their highly anionic character of these polymers pointed to a role in cation binding for these polymers. The importance of teichoic acid in cation binding was demonstrated *in vivo*, where cells were found to possess large amounts of teichoic acid in Mg^{2+}-limiting conditions (Tempest *et al.*, 1968). Additionally, this ability to bind Mg^{2+} was found to be dependent upon the total charge of the polymer (Lambert *et al.*, 1975a; Lambert *et al.*, 1975b). The importance for teichoic acid mediated cation binding is rooted in the importance of cations in enzyme activity and the possibility for teichoic acid to act as a cation reservoir or scavenger. Hughes *et al.* demonstrated that membrane bound anionic polymer synthesis activity was highly dependent on the Mg^{2+} concentration. Furthermore, this dependence could be alleviated if the activity was tested in the presence of teichoic acid polymers, presumably due to the ability of the polymer to bind the limiting supply of Mg^{2+} in the solution (Hughes *et al.*, 1973).

In addition to charge, the presence of high amounts of phosphate, a functional group found ubiquitously in the bacterial cell, is a common feature to teichoic acid polymers. It has been well established that upon phosphate limitation, teichoic acid synthesis halts and is replaced by teichuronic acid; a polymer retaining charge, but devoid of phosphate. It was also found that concurrent to this conversion, growth is slowed, but not halted. In fact, immediately upon phosphate exhaustion bacterial growth is halted for a short time before resuming (Grant, 1979). Interestingly, addition of exogenous teichoic acid just after phosphate exhaustion resulted in increased growth rates, which are comparable to the addition of exogenous inorganic phosphate. This suggests that the phosphate supplied by the teichoic acid polymer provides a growth advantage to the cell (Grant, 1979). Finally, other roles, including a role in biofilm formation (Gross *et al.*, 2001) and protein translocation (Chambert and Petit-Glatron, 1999; Hancock, 1997) have been proposed for the polymer, but significantly fewer studies have been undertaken in these areas.

It is clear from the very earliest of discoveries that inhibition of teichoic acid assembly has a dramatic effect on cell morphology. It is highly likely that the true role of

teichoic acid polymers in the cell wall controls cell morphology, although the precise mechanism for this has not been suitably established.

ORGANIZATION OF THESIS

The experiments conducted throughout this thesis were aimed at understanding the dispensability of teichoic acid biosynthetic enzymes in both *B. subtilis* 168 and *S. aureus* with the ultimate objective of understanding the utility of this pathway as a novel therapeutic target. Please note that areas of this thesis were previously published so there may be some degree of repetition between chapters. Chapter two describes studies on the dispensability of enzymes in the biosynthesis of the ribitol teichoic acid polymer in *S. aureus*. Given the lack of genetic tools for use in *S. aureus*, a novel system to assess gene dispensability was established and used. The course of this study involved the investigation of a number of enzymes in the pathway, covering all steps, linkage unit synthesis, polymer synthesis, and export. Chapter three revisits teichoic acid gene dispensability in *B. subtilis* 168, focusing on the dispensability of *tagO*, the *N*-acetylgluosamine-1-phosphate transferase, coding for the first step in the biosynthetic pathway. Chapter four addresses the dispensability of a single teichoic acid biosynthetic enzyme not previously investigated with respect to dispensability, the *N*-acetylmannosamine transferase. Lying on the border between disaccharide and polymer synthesis, as it will be discussed, this step is unique in the pathway. The dispensability of the *B. subtilis* 168 and *S. aureus* counterparts, *tagA* and *tarA*, were investigated in this chapter. In addition, preliminary studies on the phenotype associated with deletion of teichoic acid were undertaken. Finally, chapter five highlights the progress made through this work and the outstanding issues in the area of wall teichoic acid biogenesis.

CHAPTER TWO

DISPENSABILITY OF WALL TEICHOIC ACID BIOSYNTHETIC GENES IN *STAPHYLOCOCCUS AUREUS*

CHAPTER TWO PREFACE

The work presented in this chapter was previously published in:

D'Elia, M.A., Pereira, M.P., Chung, Y.S., Zhao, W., Chau, A., Kenney, T.J., Sulavik, M.C., Black, T.A. and Brown, E.D. (2006) Lesions in teichoic acid biosynthesis in *Staphylococcus aureus* lead to a lethal gain of function in the otherwise dispensable pathway. J. Bacteriol. 188(12): 4183-4189.

Permission has been granted from the publisher to reproduce the material here.

I conducted all the experiments described in this chapter, with the exception of creation of pSAKO and the *S. aureus* parent strain SA178RI and the creation of the *tarO* / *tarIJ* double mutant.

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We would like to thank D. E. Heinrichs for providing bacteriophage 80α.

INTRODUCTION

The bacterial cell wall is composed of polymers of carbohydrate and amino acids, present as a rigid, cross-linked structure, termed peptidoglycan, which plays a critical role in bacterial functions including growth, division, maintenance of cell shape, and protection from osmotic lysis (Hancock, 1997). While the walls of Gram-negative bacteria are composed primarily of peptidoglycan, those of Gram-positives are more complex. They contain, in addition to peptidoglycan, large amounts of wall teichoic acid, an anionic polyol-phosphate polymer that is covalently attached to peptidoglycan. Despite making up a significant portion of the Gram-positive cell wall, the precise function of teichoic acid remains elusive. Indeed, a large body of evidence assembled for the model Gram-positive *Bacillus subtilis* has established that the biosynthetic genes for wall teichoic acid biosynthesis are indispensable in that organism. Conversely, Weidenmaier *et al.* recently published on a mutant in *Staphylococcus aureus* that was apparently devoid of wall teichoic acid (Weidenmaier *et al.*, 2004).

An analysis of the gene clusters for the synthesis of teichoic acid in *B. subtilis* strains 168 and W23 along with the benefit of many years of physiological, genetic, biochemical and analytical work on wall teichoic acids, some in *S. aureus*, provided a rational basis for proposing functional roles for most of the gene products (Bhavsar *et al.*, 2005; Navarre and Schneewind, 1999; Neuhaus and Baddiley, 2003; Schertzer and Brown, 2003). The major wall teichoic acids of the model Gram-positive bacteria *B. subtilis* strains 168 and W23 are linear 1,3- and 1,5-linked poly(glycerol phosphate) and poly(ribitol phosphate) polymers, respectively. The poly(ribitol phosphate) polymer of *B. subtilis* W23 is also common to *S. aureus*. Figure 2.1 summarizes our understanding with a model for the biogenesis of poly(ribitol phosphate) wall teichoic acid in *S. aureus*.

Teichoic acid biosynthesis begins with the formation of an undecaprenyl-pyrophosphoryl disaccharide on the cytoplasmic face of the cell membrane through the successive action of proteins TarO (*N*-acetylglucosamine-1-phosphate transferase) and TarA (*N*-acetylmannosamine transferase). Subsequently a primase (TarB) and oligomerase (TarF) is believed to add a trimer of 1-3-linked glycerol-3-phosphate units to the 4-hydroxyl of *N*-acetylmannosamine. Glycerol-3-phosphate is provided by the action of TarD in an activated form, CDP-glycerol. Analogously, TarK and TarL, have been proposed to prime and polymerize, respectively, a 1-5-linked polymer (n ~ 30) of ribitol-5-phosphate on the terminal hydroxyl of the trimer of glycerol phosphate. TarIJ provides activated ribitol-5-phosphate in the form of CDP-ribitol. Intracellular synthesis of the complete polymer is thought to be followed by extrusion by TarGH, an ABC transporter, before transfer to the 6-hydroxyl of *N*-acetylmuramic acid moiety of peptidoglycan by an unknown transferase.

The first evidence of an essential role for wall teichoic acid came from temperature sensitive mutants isolated in *B. subtilis* 168, created through chemical mutagenesis, that mapped to the poly(glycerol phosphate) teichoic acid gene cluster (*tag* genes) (Bhavsar *et al.*, 2004; Brandt and Karamata, 1987; Briehl *et al.*, 1989; Pooley *et al.*, 1991). More recently, a xylose-induced expression system was used to facilitate the construction of precise deletions of teichoic acid synthetic genes in *B. subtilis* with

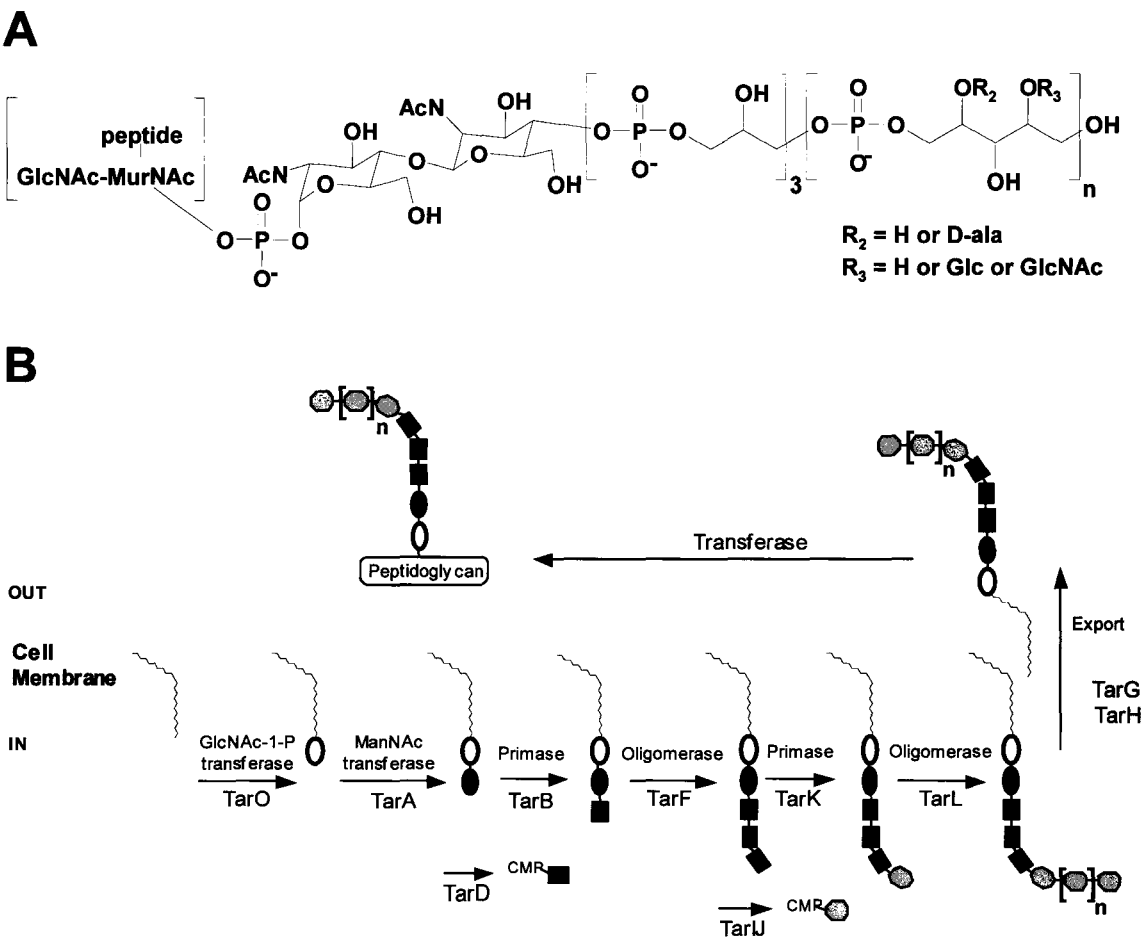

Figure 2.1 – Proposed scheme for teichoic acid synthesis in *S. aureus*. (A) The chemical structure for the completed polymer covalently bound to *N*-acetylmuramic acid is depicted. The repeating ribitol-5-phosphate polymer is often substituted with D-alanine or glucose (R_2 and R_3). (B) *S. aureus* teichoic acid comprises a polymer composed of a disaccharide containing *N*-acetylglucosamine-1-phosphate (closed oval) and *N*-acetylmannosamine (open oval), 3 units of glycerol-3-phosphate (square) and ~30 repeating ribitol-5-phosphate (octagon) units. These polymers are synthesized in a stepwise manner on the cytoplasmic face of the cell membrane onto undecaprenyl phosphate (wavy line). Following synthesis, the entire polymer is exported out of the cell and attached to *N*-acetylmuramic acid of peptidoglycan.

complementing copies of the target genes ectopically integrated into the chromosome and subject to tight control of the xylose regulon (Bhavsar *et al.*, 2001; Bhavsar *et al.*, 2004). The resulting strains were used to study the impact of controlled depletion of Tag gene products in a defined genetic background and at a physiologically relevant temperature. The mutants all showed lethal phenotypes that were fully rescued through induction of the complementing copy of the targeted gene. In all cases depletion of teichoic acid biosynthetic genes resulted in a transition from rod-shape to bloated spheres, followed by division defects typified by aberrant septal localization, partial septation and septal curvature; thickening of the cell wall and ultimately cell lysis. Thus with the exception of *tagA* (the *N*-acetylmannosamine transferase, orthologue of *S. aureus tarA*), all of the genes responsible for biosynthesis of the main chain of teichoic acid in *B. subtilis* 168 have been tested to date for dispensability. Each gene, including *tagO* (*N*-acetylglucosamine-1-phosphate transferase, orthologue of *S. aureus tarO*), has been shown to be indispensable for growth in *B. subtilis* 168 (Bhavsar *et al.*, 2001; Bhavsar *et al.*, 2004; Lazarevic and Karamata, 1995; Soldo *et al.*, 2002).

Here, we report a comprehensive investigation of the dispensability of teichoic acid biosynthetic genes in *S. aureus* using a novel allelic replacement methodology. Our findings challenge the emerging doctrine of teichoic acid essentiality in Gram-positive bacteria. We have confirmed that the gene encoding the first step of the pathway, *tarO*, is readily dispensable in *S. aureus* and that its deletion results in a strain lacking wall teichoic acid. Surprisingly, genes coding for subsequent biosynthetic steps in the pathway, *tarB, tarD, tarF, tarIJ* and *tarH*, resisted deletion and thus show an essential phenotype. This paradox of apparent indispensability of late-acting genes in an otherwise non-essential pathway was resolved with a systematic construction of double mutants. All of the late-acting genes became dispensable in a strain of *S. aureus* that lacked *tarO*. These surprising findings point to a mechanism for indispensability in late-acting teichoic acid biosynthetic genes where a lethal gain of function results from a lesion in late-acting steps of the teichoic acid biogenesis pathway. The work has broad implications for our understanding of essential gene sets, gleaned through single gene dispensability studies, of bacteria and higher organisms. These findings likewise point to a novel and exploitable drug development strategy targeting later steps in teichoic synthesis in *S. aureus*, an infectious pathogen of menacing renown in the clinic and in the community (Diekema *et al.*, 2001; Pooley and Karamata, 1988).

MATERIALS AND METHODS

Strains, plasmids and growth conditions – Cloning was performed in *E. coli* DH5α (Promega) or Novablue (Novagen) strains grown in Luria-Burtani broth at 37 °C. All *S. aureus* strains, described in Table 2.1, were grown in Mueller Hinton Broth (MHB) at 37 °C. For selection in *E. coli*, ampicillin (50 μg/ml) and kanamycin (50 μg/ml) were used. In *S. aureus*, kanamycin (20 μg/ml), erythromycin (10 μg/ml), spectinomycin (300 μg/ml), chloramphenicol (60 μg/ml), sucrose (5 % wt/vol) and IPTG (0.4 mM) were used.

S. aureus teichoic acid biosynthesis sequences were identified using BLAST analysis and comparing the protein sequences of *B. subtilis* 168 and W23 taken from the NCBI database (www.ncbi.nih.gov) against the *S. aureus* COL genome obtained from The Institute for Genomic Research database (www.tigr.org). *S. aureus* COL gene identifications can be found in Table 2.1 next to the corresponding *tar* designations for each gene studied.

Chromosomal manipulations of *S. aureus* – DNA manipulations were performed using both transformation and transduction. Transformation was accomplished through standard electroporation procedures (Lee *et al.*, 1991). Transductions were performed using bacteriophage 80α and standard protocols (Novick, 1991).

Generation of *S. aureus* SA178RI – The *S. aureus* expression strain SA178RI was constructed via integrative transformation into the strain CYL316, a derivative of RN4220. The T7 polymerase gene from *λDE3* was placed under the control of the Gram-positive promoter P_{spac} and cloned upstream of the *lacI* repressor driven by the constitutive promoter P_{pen} to generate the expression cassette. Two *lac* operator sequences were introduced into the P_{spac} promoter upstream of the ribosome binding site as well as a *trpA* terminator upstream of the promoter region. The *trpA* term-P_{spac}/T7-P_{pen}/*lacI* cassette was cloned into the integration vector pCL84 and transformed into CYL316. Tetracycline resistant and lipase-negative colonies were the result of successful integration of the cassette into the *geh* locus.

Construction of pSAKO, a *S. aureus* gene deletion plasmid – pSAKO is a *S. aureus* suicide vector utilized to disrupt chromosomal copies of *S. aureus* genes. pSAKO has a Gram-negative p15A origin of replication subcloned from pACYC184, making it non-replicative in *S. aureus*. It has a multiple-cloning site (MCS) and a *trpA* transcriptional terminator cloned from pQF50. It also has the gene encoding the bifunctional enzyme AAC(6')-APH(2"), conferring aminoglycoside resistance for selection in both *E. coli* and *S. aureus*, which was cloned as a PCR product from *S. aureus* genomic DNA (Freitas *et al.*, 1999). pSAKO also has a copy of the mutated levansucrase gene, *sacB*[*BamP*]W29 (Bramucci and Nagarajan, 1996), which confers sucrose-induced lethality in *S. aureus*, allowing for counterselection.

Construction of pG164, a *S. aureus* complementation vector – pG164 is an *E. coli* / *S. aureus* shuttle vector constructed by fusing pUC19 and pSK265. The vector has both a pUC and a pC194 origin for replication in Gram-negative and positive bacteria respectively. The expression cassette in pG164 is comprised of a T7 promoter into which a *lac* operator has been introduced to enhance regulation in the absence of inducer, as well as a Gram-positive optimal ribosome binding site, a multiple cloning site, and a *C*-terminal hexa-histidine tag. pG164 also has a copy of the *lacI* repressor regulated by the constitutive promoter P_{pen} to reduce background expression in the absence of induction.

Creation of gene specific deletion plasmids – Primer sequences are found in Table A.1 (see appendix). All PCR was performed using Vent DNA polymerase (New England Biolabs) or Roche High Fidelity PCR System (Roche Applied Science). The spectinomycin and erythromycin cassettes were PCR amplified using SpecF-SpecR and ErmF-ErmR respectively. For each gene, the left and right flanks were independently amplified from the SA178RI chromosome using gene specific primers sets A-B, and C-D respectively. Primers B and C contain regions complementary to the resistance cassette to allow binding. A final PCR using gene specific primers A-D was performed using the resistance cassette and both left and right flanks as template. The resulting product was purified and ligated into the XhoI site of pSAKO.

Creation of integrants in *tarO, tarB, tarD, tarF, tarIJ* and *tarH* – SA178RI was independently transformed with gene specific pSAKO integration plasmids (Table 2.1). Integrants were selected on Mueller Hinton agar (MHA) supplemented with kanamycin, and spectinomycin or erythromycin. Strains generated were confirmed by PCR.

Testing for gene essentiality – Integrants (EBII1, EBII2, EBII3, EBII4, EBII29 and EBII43) were plated on MHA containing sucrose. 100 colonies from each strain were selected and passaged twice more onto MHA with sucrose. Each colony was then patched onto MHA containing kanamycin, MHA containing erythromycin or spectinomycin and MHA alone to determine phenotype. For strains in which mutants could not be generated, the same procedure was performed on complemented integrants (EBII9, EBII10, EBII15, EBII27, EBII50). In addition to the above antibiotics, this media was supplemented with chloramphenicol and IPTG. Finally, the dispensability of *tarB, tarD, tarF, tarIJ* and *tarH* in a Δ*tarO* background was determined using integrants EBII35, EBII36, EBII37, EBII46 and EBII38 as described above. The growth media contained spectinomycin through all steps in the procedure. The phenotype of each colony was determined and the data summarized in Table 2.2.

Creation of Δ*tarO* and Δ*tarO* pG164-*tarO* – EBII29 was passaged three times on MHA containing sucrose and spectinomycin to generate a *tarO* deletion (EBII44). EBII44 was transformed with pG164-*tarO* to generate a complemented Δ*tarO* strain (EBII53).

Creation of *tarB, tarD, tarF, tarIJ, tarH* integrants in a Δ*tarO* background – For the generation of the *tarIJ* integrant in the Δ*tarO* background, pSAKO-Δ*tarIJ* was

transformed into EBII44 and selected for on MHA containing erythromycin and kanamycin to generate EBII46. For all other clones, EBII53 was used to generate bacteriophage 80α lysate. This lysate was used to transduce each integrant (EBII1, EBII2, EBII3, EBII4, and EBII29) and selected on MHA containing spectinomycin and kanamycin. This allowed the movement of the *tarO* deletion, marked with spectinomycin, into each single integrant.

Creation of double gene deletions – Strains EBII35, EBII36, EBII37, EBII38 and EBII46 were plated for three successive rounds on MHA containing spectinomycin, erythromycin and sucrose to generate double mutants.

PCR verification of single integrant and deletion strains – All single integrant and deletion strains were verified by PCR analysis. For deletion strains, analysis was performed using a drug cassette specific primer and primers designed to anneal to sequences of the flanking regions. For single integrant strains, analysis was performed using a drug cassette specific primer and a primer designed to anneal to the flanking regions or to sequences in pSAKO. In all cases, PCR analysis was performed for both the upstream and downstream sequences flanking the insertion.

Cell wall phosphate content determination – Strains were grown overnight in 5 ml of MHB and used to inoculate 100 ml of fresh MHB and grown at 37 °C overnight to saturation. Cell wall isolation and phosphate content determination were carried out as described previously (Bhavsar *et al.*, 2004). Briefly, cell wall was extracted by boiling in sodium dodecyl sulphate (MacDonald and Beveridge, 2002), DNase, RNase and trypsin treated (Kruyssen *et al.*, 1980), and mineralized (Ames, 1966). Wall phosphate content was assayed by absorbance using KH_2PO_4 as a standard (Chen *et al.*, 1956).

Table 2.1 – *S. aureus* strains and plasmids used in this study.

Strain	Genotype / Description	Source
S. aureus		
RN4220	Restriction-deficient host	(Kreiswirth *et al.*, 1983)
CYL316	RN4220 derivative, recipient strain for plasmid pCL84	(Lee *et al.*, 1991)
SA178RI	CYL316 containing T7 polymerase, (tet^r)	This study
***tar* gene integrants**[a]		
EBII1	SA178RI *tarF*$_{(SACOL0239)}$::pSAKO-Δ*tarF* (erm^r, kan^r)	This study
EBII2	SA178RI *tarB*$_{(SACOL0696)}$::pSAKO-Δ*tarB* (erm^r, kan^r)	This study
EBII3	SA178RI *tarD*$_{(SACOL0698)}$::pSAKO-Δ*tarD* (erm^r, kan^r)	This study
EBII4	SA178RI *tarH*$_{(SACOL0694)}$::pSAKO-Δ*tarH* (erm^r, kan^r)	This study
EBII29	SA178RI *tarO*$_{(SACOL0810)}$::pSAKO-Δ*tarO* ($spec^r$, kan^r)	This study
EBII43	SA178RI *tarIJ*$_{(SACOL0240/0241)}$::pSAKO-Δ*tarIJ* (erm^r, kan^r)	This study
Uncomplemented deletion		
EBII44	SA178RI *tarO*::spec ($spec^r$, kan^s)	This study
Complemented deletions		
EBII5	*tarF*::erm pG164-*tarF*	This study
EBII6	*tarB*::erm pG164-*tarB*	This study
EBII7	*tarD*::erm pG164-*tarD*	This study
EBII8	*tarH*::erm pG164-*tarH*	This study
EBII53	*tarO*::spec pG164-*tarO*	This study
EBII56	*tarIJ*::erm pG164-*tarIJ*	This study
Complemented *tar* gene integrants		
EBII9	EBII2 pG164-*tarB*	This study
EBII10	EBII4 pG164-*tarH*	This study
EBII15	EBII3 pG164-*tarD*	This study
EBII27	EBII1 pG164-*tarF*	This study
EBII50	EBII43 pG164-*tarIJ*	This study
***tar* gene integrants in Δ*tarO* background**		
EBII35	EBII2 *tarO*::spec	This study
EBII36	EBII3 *tarO*::spec	This study
EBII37	EBII1 *tarO*::spec	This study
EBII38	EBII4 *tarO*::spec	This study
EBII46	EBII43 *tarO*::spec	This study
Double gene deletions		
EBII47	SA178RI *tarO*::spec, *tarIJ*::erm	This study
EBII67	SA178RI *tarO*::spec, *tarB*::erm	This study
EBII68	SA178RI *tarO*::spec, *tarD*::erm	This study
EBII70	SA178RI *tarO*::spec, *tarF*::erm	This study
EBII71	SA178RI *tarO*::spec, *tarH*::erm	This study

Plasmid		
pCL84	*S. aureus* integration vector (tet^r)	(Lee *et al.*, 1991)
pACYC184	*E. coli* plasmid containing p15A origin of replication	NEB
pQF50	source of MCS and *trpA* terminator for pSAKO	(Farinha and Kropinski, 1990)
pUS19	pUC19 derivative containing spec^r cassette	(Benson and Haldenwang, 1993)
pDG1664	source for erm^r cassette	(Guerout-Fleury *et al.*, 1996)
pSAKO	*E. coli* replicating vector containing *sacB*[*BamP*]W29 and kan^r cassette	This study
pUC19	source for pUC origin and *bla* gene for selection and replication in *E. coli* (amp^r)	NEB
pSK265	source of pC194 origin and CAT gene for selection and replication in *S. aureus* (chl^r)	(Jones and Khan, 1986)
pG164	*E. coli* / *S. aureus* shuttle vector for T7 based protein expression (amp^r, chl^r)	This study
pSAKO-Δ*tarO*	single integration plasmid: *tarO* flank (spec^r, kan^r)	This study
pSAKO-Δ*tarB*	single integration plasmid: *tarB* flank (erm^r, kan^r)	This study
pSAKO-Δ*tarD*	single integration plasmid: *tarD* flank (erm^r, kan^r)	This study
pSAKO-Δ*tarF*	single integration plasmid: *tarF* flank (erm^r, kan^r)	This study
pSAKO-Δ*tarIJ*	single integration plasmid: *tarIJ* flank (erm^r, kan^r)	This study
pSAKO-Δ*tarH*	single integration plasmid: *tarH* flank (erm^r, kan^r)	This study
pG164-*tarO*	pG164 containing wild-type *tarO* from *S. aureus*	This study
pG164-*tarB*	pG164 containing wild-type *tarB* from *S. aureus*	This study
pG164-*tarD*	pG164 containing wild-type *tarD* from *S. aureus*	This study
pG164-*tarF*	pG164 containing wild-type *tarF* from *S. aureus*	This study
pG164-*tarIJ*	pG164 containing wild-type *tarIJ* from *S. aureus*	This study
pG164-*tarH*	pG164 containing wild-type *tarH* from *S. aureus*	This study

[a]*S. aureus* COL gene identification given in brackets.

RESULTS

Testing gene dispensability in *S. aureus* with pSAKO

While the genetic tools for studies of gene dispensability in *B. subtilis* are relatively robust, those for *S. aureus* are considerably less definitive. In the work reported here we developed a novel plasmid (pSAKO) for allelic replacement in *S. aureus* that facilitates testing gene essentiality (outlined in Figure 2.2). Plasmid pSAKO encodes a kanamycin resistance marker (AAC(6')-APH(2")), allowing for selection in both *Escherichia coli* and *S. aureus*. A key feature of this plasmid is a counter selectable marker, the *B. subtilis* SacB variant (*sacB*[*BamP*]W29) that is impaired for secretion and produces a product toxic to *S. aureus* in the presence of sucrose (Bramucci and Nagarajan, 1996). The flanks (~1000 bp) surrounding the gene targeted for deletion are cloned into the polylinker of pSAKO along with an intervening drug marker (erm). Transformation and selection results in single recombinants containing a tandem duplication of the targeted locus consisting of wild-type and mutant copies. Subsequent plating on sucrose selects for loss of the plasmid sequences through two possible excision events. If the gene is dispensable, both mutant (erm resistant, kan sensitive) and wild-type (erm sensitive, kan sensitive) clones will be generated. In the case of an essential gene, only the wild-type allele will be isolated among the resulting clones. The strength of this methodology is in a passive approach to the isolation of excisants and subsequent evaluation of resistance profiles of the resulting clones to reveal frequencies at which the organism can dispense with the targeted gene.

Investigating teichoic acid biosynthetic gene dispensability in *S. aureus*

Plasmid pSAKO was used to test the dispensability of each step of *S. aureus* teichoic acid biosynthesis, including disaccharide formation (*tarO*), glycerol phosphate oligomerization (*tarD, tarB, tarF*), ribitol phosphate polymerization (*tarIJ*), and teichoic acid export (*tarH*). A sample of the data for 36 clones obtained for *tarD* is shown in Figure 2.3A. Here all integrants excised under counterselection to produce kan sensitive colonies, of which none were erm resistant, indicating no propensity to generate viable deletions in *tarD*. As a positive control, selection for excisants was also done with a plasmid-encoded copy of *tarD* provided *in trans* (Figure 2.3B). Here 28 of 36 integrants excised to produce a deletion of *tarD* (erm resistant) while 8 excisants restored the wild-type locus. The ability to produce a deletion in *tarD* in the presence of a complementing copy confirmed that our inability to generate mutants in the absence of complementation was due to the indispensable nature of the target gene and not a systematic inability to generate a mutant excisant. Thus these data were consistent with an essential phenotype for *tarD* demonstrating that our deletion strategy could be used to distinguish between dispensable and indispensable genes.

Table 2.2 details the outcome of counterselection on sucrose for 100 integrants in each targeted gene. Each integrant was examined for excision of the plasmid and replacement of the targeted gene with a resistance cassette. With a low frequency, some

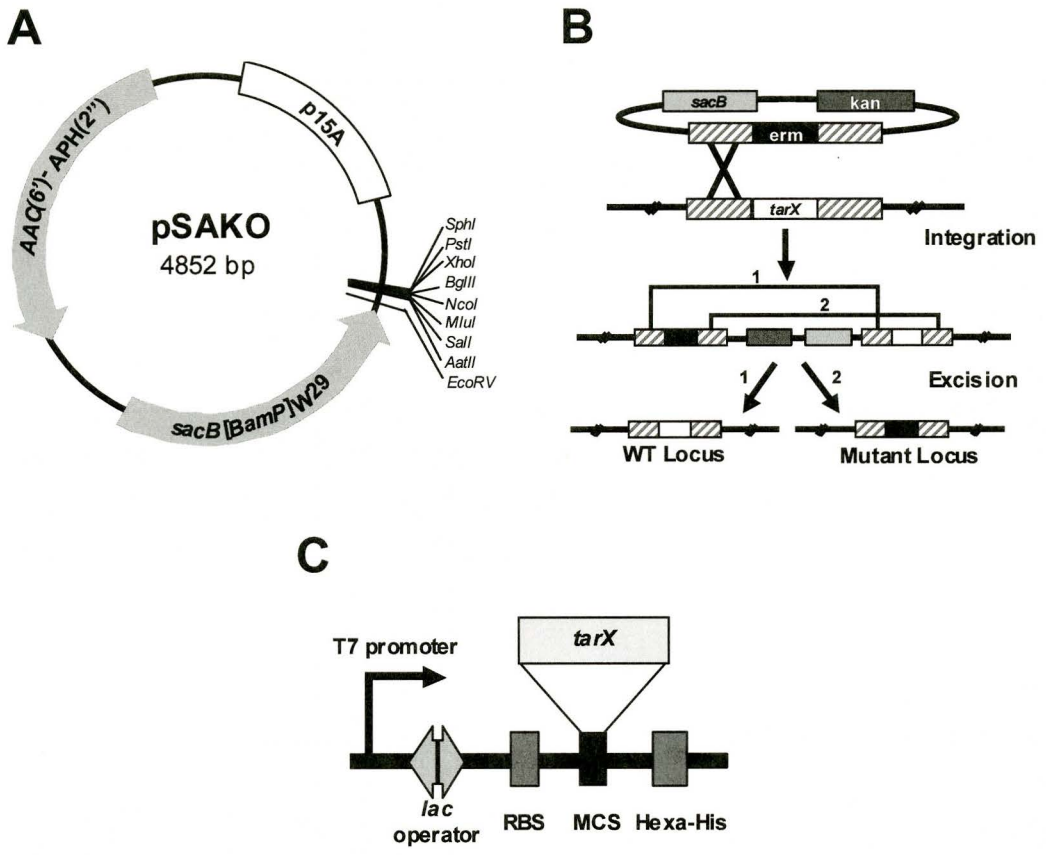

Figure 2.2 – A novel genetic strategy for testing dispensability in *S. aureus*. (A) pSAKO contains a Gram-negative p15A replication origin that allows replication in *E. coli*, but not in *S. aureus*. Selection in both *E. coli* and *S. aureus* is accomplished using the kanamycin resistance cassette, AAC(2')-APH(6"), while a mutant form of SacB (*sacB*[*BamP*]W29) permits counterselection. Unique restriction sites found within the multiple cloning site (MCS) are highlighted on the outside of the plasmid. (B) Integration of pSAKO encoding kanamycin (kan) resistance and containing an erythromycin resistance cassette (erm), the latter flanked by ~1000 bp of chromosomal sequences upstream and downstream of the targeted gene (*tarX*), occurs through single recombination. Selection for excision of plasmid sequence is accomplished using *sacB*[*BamP*]W29 (*sacB*) on media containing sucrose. Excision results in restoration of the wild-type (WT) locus or generation of a mutant locus. (C) The expression cassette located on pG164 was used to express the complementing copy of the gene of interest (*tarX*) cloned into the MCS. Protein expression was driven by a T7 promoter controlled by a chromosomally integrated T7 polymerase induced by IPTG.

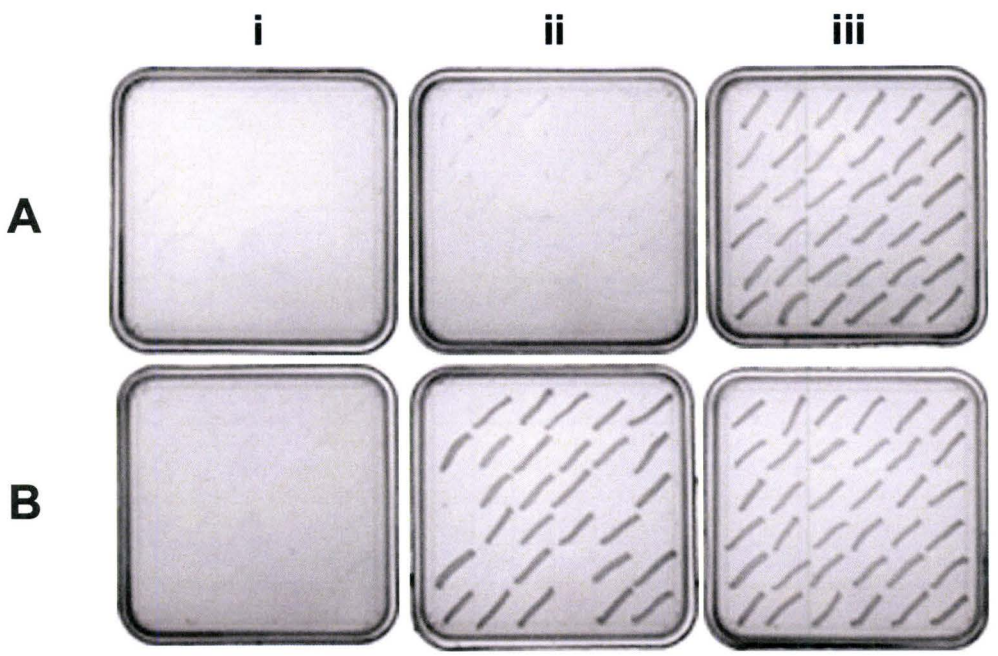

Figure 2.3 – Dispensability analysis of *S. aureus tarD*. Integrants targeting *tarD* (EBII3, EBII15) were subject to selection for excisants on (A) Mueller Hinton agar (MHA) containing sucrose (EBII3) and (B) MHA containing chloramphenicol (chl), IPTG and sucrose (EBII15). The phenotypic outcome of selection was revealed in the resistance profile of three test plates containing (i) kan, (ii) erm and (iii) no antibiotic.

colonies failed to excise the plasmid, remaining single integrants, and were identified as non-excisants. Gene *tarO* showed roughly equal propensity to generate either mutant or wild-type on excision in the absence of complementation, indicating that *tarO* was dispensable in a wild-type background, which is consistent with the previous study (Weidenmaier *et al.*, 2004). Also consistent with the previous study, the growth of the *tarO* mutant was not significantly impaired compared to wild-type (Figure 2.4). Surprisingly, in contrast to *tarO*, integrants targeted to *tarB*, *tarD*, *tarF*, *tarIJ* and *tarH* consistently failed to produce a mutant phenotype. All excised to leave the wild-type locus in the absence of complementation. When a complementing copy of each gene was supplied *in trans*, integrants resolved to produce both wild-type and mutant alleles with roughly equal propensity. Thus, the data are consistent with an essential phenotype for these late-acting genes, all of which carry out biosynthetic reactions after the formation of undecaprenyl-pyrophosphoryl-disaccharide.

The requirement for TarO cannot be bypassed

One explanation for this paradox is that cells maybe making teichoic acid in the absence of *tarO*, perhaps through the use of a redundant enzyme to transfer *N*-acetylglucosamine-1-phosphate to undecaprenyl phosphate or by remodelling the teichoic acid polymer to exclude the need for the *N*-acetylglucosamine-1-phosphate moiety. This would be consistent with the conventional understanding in *B. subtilis* that teichoic acid polymers are an essential component of the cell wall in *S. aureus*. To address this we endeavoured to test the dispensability of the late-acting genes (*tarB*, *tarD*, *tarF*, *tarIJ*, *tarH*) in the Δ*tarO* genetic background. Again, 100 integrants targeting the late-acting genes in the Δ*tarO* strain were characterized following counterselection (Table 2.2). In all cases, excision generated both wild-type and mutant alleles with approximately equal propensity. Thus, the late acting genes (*tarB*, *tarD*, *tarF*, *tarIJ*, *tarH*) became non-essential in the absence of *tarO,* a finding inconsistent with the possibility that TarO function could be bypassed in any fashion. Indeed, we confirmed that the cell wall phosphate content was vastly reduced in the Δ*tarO* mutant relative to the parent strain (0.0069 ± 0.0003 and 0.48 ± 0.05 μmol phosphate/mg cell wall respectively), and that provision of plasmid-encoded *tarO in trans* could correct the defect in this mutant (0.37 ± 0.02 μmol phosphate/mg cell wall). Accordingly, our characterization of the teichoic acid content of the *tarO* mutant was in accord with the previous study indicating that the viable *tarO* deletion mutant lacked teichoic acid (Weidenmaier *et al.*, 2004).

DISCUSSION

In the work reported here we have embarked on a systematic examination of the dispensability of genes coding for poly(ribitol phosphate) teichoic acid in *S. aureus* with a novel allelic replacement vector pSAKO. Paradoxically, while wall teichoic acid polymers appeared to be dispensable for viability *in vitro*, several late-acting genes in the pathway for teichoic acid synthesis were indispensable. This apparent contradiction was

Table 2.2: Allelic replacement for testing gene dispensability.

	Phenotype		
	Wild-type	Non-excisant	Mutant
No complementation			
tarO	57	1	42
tarB	91	9	0
tarD	94	6	0
tarF	98	2	0
tarIJ	96	4	0
tarH	98	2	0
Complemented			
tarB	75	0	25
tarD	44	0	56
tarF	36	0	64
tarIJ	76	1	23
tarH	58	0	42
Δ*tarO*			
tarB	64	2	34
tarD	36	0	64
tarF	29	1	70
tarIJ	68	0	32
tarH	52	0	48

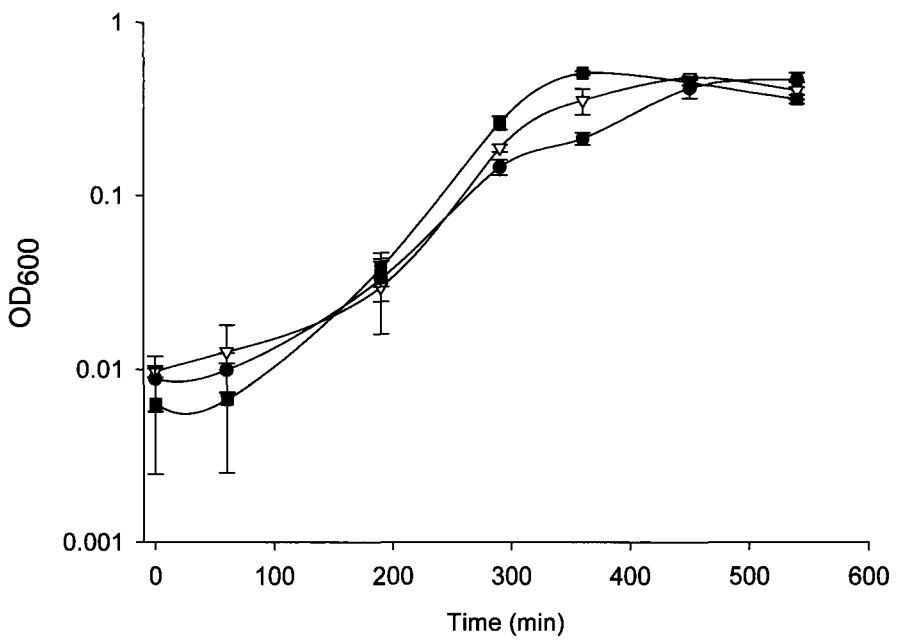

Figure 2.4 – Growth analysis for *S. aureus* wild-type and mutant. Growth analysis was performed in Mueller Hinton broth for the wild-type *S. aureus* (SA178RI, ▼) and the *tarO* deletion strain (EBII44) grown in the presence (○) and absence (●) of inducer (0.4 mM IPTG). Cultures were inoculated at a starting OD_{600} of 0.001 and absorbance measurements taken every 1 – 2 hours.

resolved with the demonstration that late-acting genes became dispensable in a strain that lacked *tarO*, coding for the first step in the pathway. The suppression of an essential phenotype associated with the late-acting genes in the pathway (*tarB, tarD, tarF, tarIJ* and *tarH*) by a deletion in the first step (*tarO*) is a remarkable and intriguing finding. This ability to suppress the lethal effects of gene deletion by a secondary site deletion is not an exceptional case. The best-characterized and most simplistic examples are with the toxin-antitoxin gene pairs of bacteria (Aizenman *et al.*, 1996). Here deletion of the antitoxin gene leads to cell death, unless there is a compensating deletion of the cognate toxin gene. Perhaps most interestingly, our observations are also echoed by some curious reports in systems involving bacterial exopolysaccharide synthesis. Studies of succinoglycan biosynthesis in *Rhizobium meliloti* revealed that a mutation in *exoR*, resulting in upregulation of the exo gene cluster, caused late acting genes to become essential, while double mutants in *exoR* and early steps of this pathway were viable (Reuber and Walker, 1993). Recently, Cuthbertson *et al.* showed that in *E. coli* O9a, the *wzm-wzt* ABC transporter for polymannan O-antegenic polysaccharide export was only dispensable in a strain containing a secondary mutation preventing polymannan production (Cuthbertson *et al.*, 2005). In the work reported here, we have discovered analogous genetic interactions that pervade an entire metabolic pathway.

Our observations regarding suppression of lethality within the teichoic acid biosynthetic pathway suggest a mechanism where a single mutation in late-acting steps leads to a lethal gain of function for the abbreviated pathway. The most parsimonious interpretation of our findings is that once teichoic acid production has commenced, it must be completed or else lethal intermediates accumulate. For example, the incomplete production of teichoic acid might cause an accumulation of toxic precursors, such as activated sugars or partially complete polymer, leading to cell death. Alternatively, a lesion in teichoic acid synthesis may interfere with the synthesis of some truly essential component of the cell, for example, by sequestering a shared building block. Apart from UDP-*N*-acetylglucosamine and undecaprenyl phosphate, which are shared with peptidoglycan biosynthesis, teichoic acid synthesis uses building blocks that are exclusive to its own synthesis. Interestingly, work using isolated *B. subtilis* membranes demonstrated that inhibition of peptidoglycan synthesis could occur by providing a soluble teichoic acid precursor, CDP-glycerol (Anderson *et al.*, 1972), suggesting a functional link between the assembly of these two major cell wall components. Along these lines, a failure to complete teichoic acid production could conceivably sequester the otherwise recycled undecaprenyl phosphate molecule (van Heijenoort, 2001b) upon which peptidoglycan is also built (Higashi *et al.*, 1967). Such a mechanism for lethality could surely be corrected by preventing the initiation of teichoic acid production with the loss of *tarO*. This explanation is further supported circumstantially by the phenotypes of conditional mutants in these metabolic pathways. Lesions in teichoic acid synthesis (Bhavsar *et al.*, 2001), peptidoglycan biogenesis (Wei *et al.*, 2003), and isoprenoid biosynthesis (Campbell and Brown, 2002), the latter pathway being responsible for undecaprenol production, all result in remarkably similar and profoundly altered cell morphology when examined by electron microscopy.

The essential phenotypes of the five late-acting loci studied in this work *tarB, tarD, tarF, tarIJ* and *tarH* suggest that all late-acting genes, at least from *tarB* onwards will be essential. The prediction therefore is that four additional genes (*tarK*, *tarL*, *tarG*, and the unknown transferase), and possibly *tarA*, will show an essential phenotype in *S. aureus*. The spectre of ten apparently indispensable genes coding for the eleven step synthesis of a non-essential polymer in *S. aureus* challenges the conventional understanding of essential gene sets garnered through single gene deletion experiments, at least in the laboratory setting.

The discovery of a lethal gain of function associated with lesions in the late steps of teichoic acid biosynthetic genes points to a novel therapeutic route to target the pathogen *S. aureus*. *S. aureus* is a major cause of hospital acquired infection and has become increasingly difficult to treat due to resistance to multiple antibiotics including methicillin (Salgado *et al.*, 2003) and vancomycin (Weigel *et al.*, 2003). The work outlined here suggests that inhibition of late-acting enzymes in teichoic acid biosynthesis leads to cell death, possibly through a corrupting impact on cell wall peptidoglycan biosynthesis. Indeed, while a mutation of the first-acting gene, *tarO*, would be capable of suppressing the lethal inhibition of late-acting steps, Weidenmaier *et al.* have shown wall teichoic acid in *S. aureus* to be a key virulence determinant for infection (Weidenmaier *et al.*, 2004). Thus wall teichoic acid biosynthesis appears to be critical for growth *in vivo* and represents a pathway that is vulnerable to an extraordinary mechanism for lethality upon inhibition.

CHAPTER THREE

DISPENSABILITY OF WALL TEICHOIC ACID BIOSYNTHETIC GENES IN *BACILLUS SUBTILIS*

CHAPTER THREE PREFACE

The work presented in this chapter was previously published in:

D'Elia, M.A., Millar, K.E., Beveridge, T.J. and Brown, E.D. (2006) Wall teichoic acid polymers are dispensable for cell viability in *Bacillus subtilis*. J. Bacteriol. 188(23): 8313-8316.

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I conducted all the experiments described in this chapter, with the exception of the transmission electron microscopy and the creation of the xylose induced *tagO* diploid strain.

Acknowledgements for this work:
We would like to thank Bob Harris of the University of Guelph for his technical assistance in preparing samples for electron microscopy.

INTRODUCTION

The cell wall of bacteria is a complex meshwork of carbohydrates and amino acids linked as a rigid structure termed peptidoglycan, which is responsible for a variety of cellular functions, including growth, division, maintenance of shape and protection from osmotic stress (Hancock, 1997). In Gram-positive organisms, in addition to this dense layer of peptidoglycan, the cell wall contains and equal amount of a highly charged anionic polymer of polyol phosphate, called wall teichoic acid. Although variability exists among the polymers from various organisms, these polymers have been found in all Gram-positive bacteria studied. Remarkably and despite its discovery nearly 50 years ago, the cellular function of wall teichoic acid remains speculative. Nevertheless, a significant body of literature using the model organism *Bacillus subtilis*, has identified a requirement for teichoic acid polymers in cell viability (Bhavsar and Brown, 2006).

Beginning with temperature sensitive mutants and more recently with the creation of deletion strains that were conditionally complemented using a tightly regulated promoter, nearly every gene responsible for wall teichoic acid biosynthesis has been shown to be required for viability in *B. subtilis* (Bhavsar *et al.*, 2001; Bhavsar *et al.*, 2004; Brandt and Karamata, 1987; Briehl *et al.*, 1989; Pooley *et al.*, 1991). In contrast, we recently demonstrated that wall teichoic acid was dispensable in *Staphylococcus aureus* (D'Elia *et al.*, 2006b). Paradoxically, that work indicated that the first step in polymer synthesis was dispensable, while the later steps were not (D'Elia *et al.*, 2006b). This apparent contradiction was resolved with the finding that a lesion in the first step of the biochemical pathway (TarO) suppressed the lethal phenotype associated with mutations in the later steps. Here, we have re-evaluated the dispensability of teichoic acid biosynthesis genes in *B. subtilis*, with particular attention to the dispensability of the first biosynthetic step encoded in *tagO* (orthologue of *tarO*).

MATERIALS AND METHODS

Strains, plasmids and growth conditions – Cloning was performed using *E. coli* Novablue strain (Novagen) grown in Luria-Burtani broth at 37 °C. All *B. subtilis* strains, described in Table 3.1, were grown in LB at 30 °C. For selection in *E. coli*, ampicillin (50 μg/ml) was used. In *B. subtilis*, erythromycin (0.5 μg/ml) and lincomycin (12.5μg/mL), spectinomycin (150 μg/ml), chloramphenicol (10 μg/ml), kanamycin (15 μg/ml), and xylose (2% wt/vol) were used.

Transformation and Congression – All transformations and congressions were performed using the system of Cutting and Youngman (Cutting and Vander Horn, 1990).

Creation of *tagO* deletion strain – Using Expand High Fidelity PCR system (Roche) or Vent DNA polymerase (NEB), 1kb sequences upstream and downstream of *tagO* was amplified from the chromosome of EB6 and an erythromycin resistance cassette was amplified from pMUTIN4 (see appendix Table A.2 for oligos used). The PCR product

was transformed into EB6 to generate strain EB1451. Replacement of *tagO* with the erythromycin cassette was confirmed by PCR.

Characterization of the *tagO* deletion strain – EB1451 along with the wild-type parental strain, EB6, were grown in liquid LB over a period of 24 hours. The starter cultures were obtained by plating each strain onto LB agar overnight. Following overnight growth, the cells were suspended in saline and washed once before diluting into 100 mL of liquid LB to a final OD_{600} of 0.001. For EB1451 cultures were grown in the presence and absence of 20 mM $MgCl_2$.

Microscopy – Phase contrast light microscopy was performed using an Olympus CX41 microscope (Carsen Group, Markham, Ontario, Canada) with an oil immersion x100 objective. Images were obtained with an Olympus Q-Color3 camera, and processed using the MediaCybernetics Image-Pro Express software (Carsen Group). Transmission electron microscopy was prepared and performed by conventionally embedding in thin sections as previously described (Matias and Beveridge, 2005). *B. subtilis* samples were harvested at late log phase of growth for preparation.

Creation of *tagO* deletion in complemented *tagB*, *tagD*, *tagF* deletion strains – Stains EB633, EB240 and EB669 were congressed with chromosome from EB1451 to generate strains EB1554, EB1559, EB1560. The resulting strains were confirmed by PCR.

Testing dispensability of *tagB*, *tagD*, and *tagF* – The chromosomes of strains EB1554, EB1559, EB1560 were congressed into the wild-type strain EB6 and selected for on LB containing spectinomycin and xylose. 100 colonies from each congression were selected and patched onto LB containing spectinomycin and xylose. Each clone was subsequently patched onto three test plates of LB containing erythromycin and xylose, chloramphenicol and xylose, spectinomycin and xylose. Assessing antibiotic resistance profiles allowed for the assignment of chromosomal markers and therefore the genotype for each clone. As a control, chromosome from EB1554 was congressed into EB859. Here 25 colonies were tested in the same manner as above, except kanamycin was added to each plate to ensure maintenance of the plasmid.

Cell wall phosphate content determination – Strains were grown overnight on LB agar washed once with saline and used to inoculate 200 ml of fresh LB, then grown at 30 °C to $OD_{600} \sim 0.7$. Cell wall isolation and phosphate content determination were carried out as described previously (Bhavsar *et al.*, 2004). Briefly, cell wall was extracted by boiling in sodium dodecyl sulfate (MacDonald and Beveridge, 2002), DNase, RNase and trypsin treated (Kruyssen *et al.*, 1980), and mineralized (Ames, 1966). Wall phosphate content was assayed by absorbance using KH_2PO_4 as a standard (Chen *et al.*, 1956).

Table 3.1 – *B. subtilis* strains and plasmids used in this study.

Strain	Genotype / Description	Source
B. subtilis		
EB6	wild-type *B. subtilis* 168, *hisA1 argC4 metC3* (L5087)	(Briehl *et al.*, 1989)
EB240	*hisA1 argC4 metC3 amyE*::*xylR* P_{xylA} *tagD cat86 tagD*::spec^r	(Bhavsar *et al.*, 2001)
EB633	*hisA1 argC4 metC3 amyE*::*xylR* P_{xylA} *tagB cat86 tagB*::spec^r	(Bhavsar *et al.*, 2004)
EB669	*hisA1 argC4 metC3 amyE*::*xylR* P_{xylA} *tagF cat86 tagF*::spec^r	(Bhavsar *et al.*, 2004)
EB892	EB6 transformed with pRB*tagBgfp*	(Bhavsar *et al.*, 2005)
EB1451	*hisA1 argC4 metC3 tagO*::erm^r	This study
EB1453	*hisA1 argC4 metC3 amyE*::*xylR* P_{xylA} *tagB cat86 tagB*::spec^r *tagO*::erm^r	This study
EB1559	*hisA1 argC4 metC3 amyE*::*xylR* P_{xylA} *tagD cat86 tagD*::spec^R *tagO*::erm^R	This study
EB1560	*hisA1 argC4 metC3 amyE*::*xylR* P_{xylA} *tagF cat86 tagF*::spec^R *tagO*::erm^R	This study
Plasmid		
pMUTIN4	IPTG inducible integration vector, source of erm^r cassette	(Vagner *et al.*, 1998)
pUS19	pUC19 derivative containing spec^r cassette	(Benson and Haldenwang, 1993)

RESULTS AND DISCUSSION

Dispensability of *B. subtilis tagO*

Gene *tagO* was the subject of a relatively recent dispensability study in *B. subtilis,* where failure to create insertional mutants, lead to the conclusion that disruption of *tagO* was lethal to the cell (Soldo *et al.*, 2002). In the work reported here, we employed a precise deletion strategy using double recombination of PCR product targeting *tagO*. The PCR producted contained a central erythromycin cassette flanked by 1000 bp regions 5' and 3' of *tagO*. To our surprise, we were able to successfully create a strain with a deletion in *tagO* (EB1451) that was viable but slow growing. The failure in the previous study (Soldo *et al.*, 2002) to isolate mutants in *tagO* by insertional inactivation may stem from the slow growth and altered colony morphology of this mutant. These colonies were significantly smaller and smoother compared to wild-type *B. subtilis* but could be repeatedly sub-cultured onto fresh media. Additionally, transformation (Cutting and Vander Horn, 1990) of chromosomal DNA from the deletion strain back into the wild-type background (EB6) occurred at a frequency of three-fold for that obtained by an unlinked, dispensable marker (283 ± 1 versus 93 ± 7 transformants / pg DNA of the unlinked marker), giving rise to colonies of identical morphology to the donor strain, arguing against the existence of a secondary site mutation leading to viability.

Cell wall phosphate content

Because a deletion in *tagO* is expected to disrupt the first step of wall teichoic acid biosynthesis we reasoned that the deletion strain should be devoid of any wall teichoic acid. Using previously established protocols, the cell wall from both wild-type and the deletion strain were isolated and the phosphate content was analysed (Bhavsar *et al.*, 2004). Compared to wild-type, the cell wall phosphate content was decreased by nearly 95% in the *tagO* null (EB1451) (2.01 ± 0.04 μg phosphate / mg cell wall versus 0.14 ± 0.02 μg phosphate / mg cell wall). These data support the absence of teichoic acid in the cell wall and indicate that the activity of TagO was not bypassed by an alternative biosynthetic mechanism.

B. subtilis Δ*tagO* deletion is significantly impaired for growth

Further characterization of the *tagO* deletion strain was performed through the investigation of the growth kinetics compared to wild-type in Luria Bertani broth (Figure 3.1A). It is clear that the failure to synthesize teichoic acid had a drastic effect on the growth of *B. subtilis*. Compared to the wild-type strain, the mutant strain had a considerably longer lag phase coupled with a decreased growth rate. The growth kinetics were also examined with the addition of 20 mM $MgCl_2$ in the medium. Previous reports have demonstrated that Mg^{2+} supplemented in the medium has a positive effect on the growth of certain morphology mutants (Formstone *et al.*, 2008; Leaver and Errington, 2005). The most dramatic effect was observed with an *mreB* mutant whose viability was

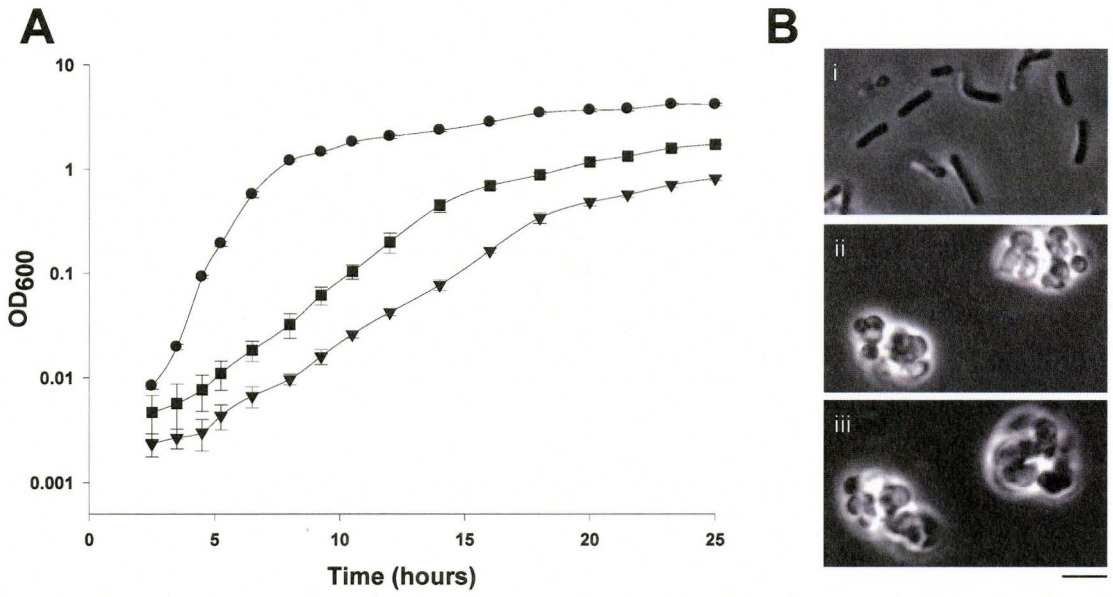

Figure 3.1 – Growth of *tagO* deletion mutant. (A) Growth analysis was performed in LB for the wild-type *B. subtilis* (EB6, ●) and the *tagO* deletion strain (EB1451) grown in the presence (■) and absence (▼) of $MgCl_2$. Cultures were inoculated at a starting OD_{600} of 0.001 and absorbance measurements taken every 1-2 hours. (B) Phase contrast microscopy was performed on stationary phase cultures of the (i) wild-type strain, and the *tagO* deletion strain grown in the (ii) presence and (iii) absence of $MgCl_2$. The bar represents 5 μm.

dependent on the addition of Mg^{2+}. Although the addition of $MgCl_2$ does not restore growth of the *tagO* deletion mutant to wild-type levels, supplementation resulted in a shorter lag phase and increased growth rate (doubling time of 1.4 ± 0.1 hours for the supplemented culture versus 2.1 ± 0.1 hours for the non-supplemented culture). Though, the effect of Mg^{2+} on the enhancement of growth is not well understood, several explanations have been suggested. Most proposals have implied some impact on peptidoglycan structure or the stabilization of cell wall-enzyme complexes that are relevant to cell wall remodelling or synthesis (Formstone *et al.*, 2008). Furthermore, given the potential role for teichoic acid polymers in binding Mg^{2+} ions (Lambert *et al.*, 1975b), supplementation of this ion might compensate for the loss of teichoic acid polymers in the cell wall.

Morphological defects associated with *tagO* deletion

Light microscopy and transmission electron microscopy in the presence and absence of $MgCl_2$ are shown in Figure 3.1B and 3.2, respectively. Light microscopy of the *tagO* mutant revealed a loss of rod shape and swelling of the cell volume in addition to cell aggregation. These phenotypes were not alleviated by the addition of $MgCl_2$. Interestingly, these characteristics were previously evident in micrographs of a TagO-depleted strain that were published by Soldo *et al.* (Soldo *et al.*, 2002). Transmission electron microscopy in the work reported here revealed aberrant septation and non-uniform thickening of the peptidoglycan layer, hallmarks associated with a loss of teichoic acid in *B. subtilis* (Bhavsar *et al.*, 2001). From these findings it is clear that the loss of teichoic acid polymers has a dramatic effect on the cellular morphology of *B. subtilis*.

Δ*tagO* suppresses lethality associated with deletion of late acting enzymes

Given the surprising dispensability pattern associated with teichoic acid biosynthesis genes in *S. aureus*, where the first step was dispensable and remaining steps had an essential phenotype (D'Elia *et al.*, 2006b), we were interested, in this work, to re-evaluate the dispensability of several late-acting teichoic acid genes (*tagB*, *tagD* and *tagF*) in *B. subtilis*. The low transformability of *B. subtilis* makes it difficult to differentiate between a failed transformation and a lethal event; therefore, we endeavoured to examine the dispensability of these late-acting genes by congression analysis (transformation of chromosomal DNA into the recipient strain and analysis of resistance markers transferred). Strains were generated that contained a deletion in *tagO* (marked with erm^r [*ermAB*]), a deletion in the late-acting gene (marked with $spec^r$ [AAD(9)]) and a complementing copy of the late-acting gene at *amyE* (marked with chl^r [*cat86*]). Each strain was produced by transforming chromosomal DNA from the *tagO* deletion strain (EB1451) into the complemented deletion strains of *tagB* (EB633), *tagD* (EB240), and *tagF* (EB669) giving rise to EB1453, EB1559 and EB1560.

Chromosomal DNA from each of the strains constructed (EB1453, EB1559 and EB1560) was transformed into a wild-type background (EB6) and growth selected on LB

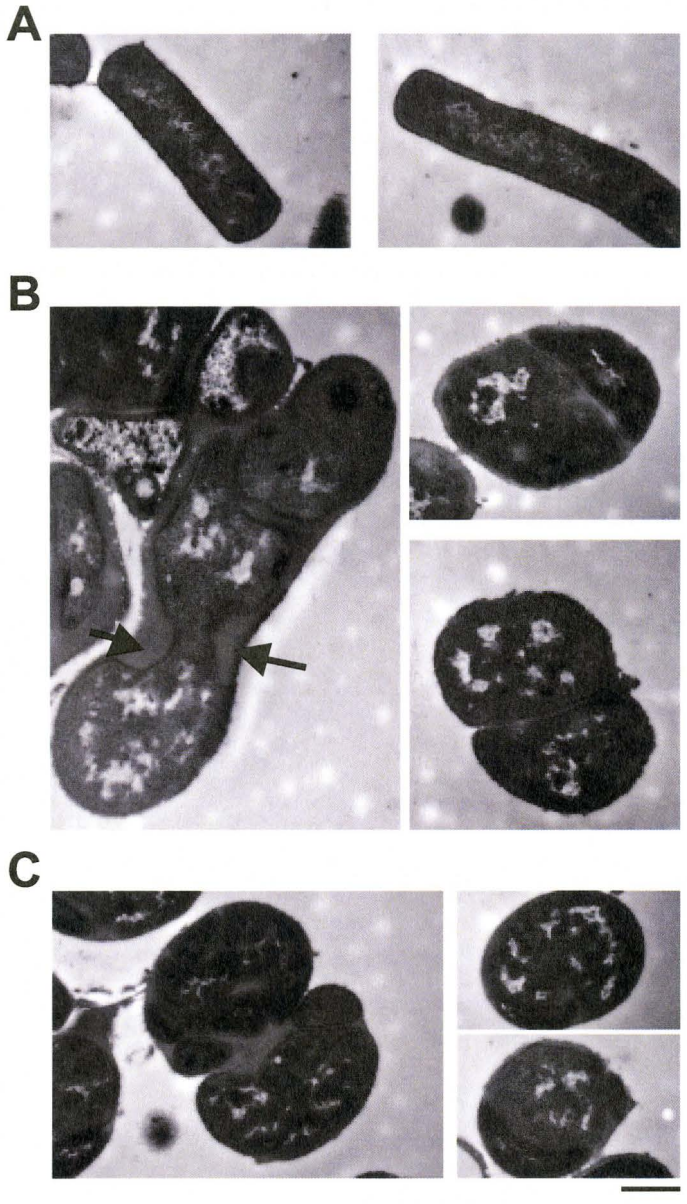

Figure 3.2 – Ultrastructure of *B. subtilis* lacking wall teichoic acid. Strains of *B. subtilis* 168 were harvested at late log phase of growth and conventionally embedded in thin sections for examination with transmission electron microscopy as in (Matias and Beveridge, 2005). The (A) wild-type strain (EB6) along with the *tagO* deletion mutant (EB1451) in the (B) absence and (C) presence of $MgCl_2$ are depicted. The bar represents 500 nm.

media containing spec (150 μg/mL) and xylose (2 %). After two days 100 colonies from each transformation were examined for erm and / or chl resistance. Figure 3.3A provides a schematic of the experimental methodology and possible outcomes expected. Figure 3.3B shows the outcome of a typical experiment where 36 clones were chosen from the transformation of chromosomal DNA of EB1559 into EB6. Here, 31 clones were $spec^r$ erm^r, 3 clones were $spec^r$ chl^r and 2 clones were $spec^r$ erm^r chl^r. Notably, we were unable to generate any clones that were solely $spec^r$ suggesting that *tagD* is indeed essential and that it is only possible to obtain a deletion in *tagD* if it is accompanied by a complementing copy or by a deletion in *tagO*. These results were echoed in larger scale screens performed for *tagB, tagD* and *tagF* outlined in Table 3.2. In each case, the majority of clones (80 – 90%) were $spec^r$ erm^r. Under no circumstances were clones generated that were exclusively $spec^r$. To confirm that $spec^r$ could be unlinked from erm^r and/or chl^r a similar congression sought to transform chromosomal DNA from EB1453 into EB892 (a strain containing a plasmid borne copy of *tagB*). Here 24 of the 25 clones selected were $spec^r$ erm^s chl^s suggesting that the $spec^r$ marker could be unlinked from the other two. This indicates that the *tagO* locus can be unlinked from the *tagB* locus and therefore the entire *tag* operon. Taken together, these data support the conclusion that the first enzyme of the teichoic acid biosynthesis pathway is dispensable, yet the remaining enzymes, at least *tagB* and beyond, are indispensable for viability. Furthermore, the ability to isolate clones that were $spec^r$ and erm^r, yet chl^s indicates that the essential nature of *tagB*, *tagD* and *tagF* can be suppressed by a deletion in *tagO*. These data parallel those obtained using *S. aureus* as a model and thus the peculiar dispensability pattern seen in these organisms may be a mechanistic feature associated with teichoic acid biosynthesis genes in Gram-positive bacteria.

Here we show that, despite a significant body of literature to the contrary, teichoic acid polymers are not essential to the viability of *B. subtilis*, but nevertheless appear to play a crucial role in maintaining the shape of this organism. Through the replacement of *tagO* with an erythromycin cassette in the absence of complementation, we have circumvented the ability of the organism to produce cell wall containing teichoic acid polymers, as shown by the drastic reduction in phosphate content. The creation of this mutant is in contradiction to the work by Soldo *et al.*, who reported the inability to generate viable mutants in *tagO* through insertional inactivation (Soldo *et al.*, 2002). We attribute this discrepancy to the slow growth and altered morphology of this mutant that may have mistakenly led those authors to conclude that these mutants were not viable. Perhaps most remarkable is that, despite the dispensability of *tagO*, late-acting gene products are required for viability. This is in agreement with the peculiar dispensability pattern seen in *S. aureus* teichoic acid genes. Indeed it may reflect a mechanistic feature that is paradigmatic of the dispensability patterns of these genes in all Gram-positive bacteria. As speculated in our previous work, we believe that the essentiality of the late-acting gene products may arise from the build up of toxic intermediates or from the sequestration of a crucial metabolite, such as undecaprenol phosphate, which is also required for the production of peptidoglycan.

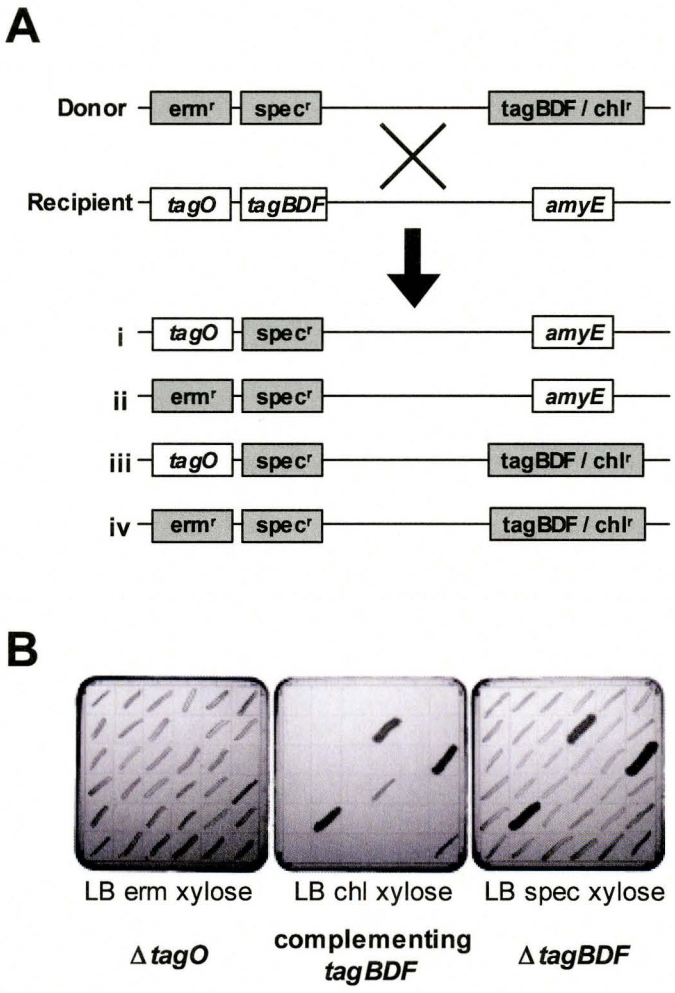

Figure 3.3 – Testing *tag* gene dispensability in *B. subtilis*. (A) To address the dispensability of *tagB, tagD and tagF* donor strains were created containing deletions of *tagO* (marked with erm^r) and one of *tagB, tagD or tagF* (marked with spec^r) (*tagBDF*) that contained a complementing copy of *tagBDF* at *amyE* (marked with chl^r). Transformation into a recipient (wild-type) strain and selection on spec (150 μg/mL) and xylose (2 %) could allow for 4 possible outcomes (i-iv). (B) The outcome of this selection procedure performed to test the dispensability of *tagD* is depicted. In addition to showing spec^r, all of the clones selected were also erm^r and/or chl^r.

Table 3.2 – Testing the dispensability of late acting gene products.

Gene Tested	Donor Strain	Recipient Strain	$spec^r$ erm^r chl^s	$spec^r$ erm^s chl^r	$spec^r$ erm^r chl^r	$spec^r$ erm^s chl^s
tagB	EB1453	EB6	95	4	1	0
tagD	EB1559	EB6	92	6	2	0
tagF	EB1560	EB6	83	17	0	0
tagB	EB1453	EB892	1	0	0	24

Total of 100 colonies for each experiment tested (except EB1453 into EB892 – only 25 colonies tested)

CHAPTER FOUR

THE *N*-ACETYLMANNOSAMINE TRANSFERASE CATALYZES THE FIRST COMMITTED STEP OF TEICHOIC ACID ASSEMBLY IN *BACILLUS SUBTILIS* AND *STAPHYLOCOCCUS AUREUS*

CHAPTER FOUR PREFACE

The work presented in this chapter was previously published in:

D'Elia, M.A., Henderson, J.A., Beveridge, T.J., Heinrichs, D.E. and Brown, E.D. (2009) The *N*-acetylmannosamine transferase catalyzes the first committed step of teichoic acid assembly in *Bacillus subtilis* and *Staphylococcus aureus*. J. Bacteriol. 191(12): 4030-4034.

Permission has been granted from the publisher to reproduce the material here.

I conducted all the experiments in this chapter, with the exception of the transmission electron microscopy and the inoculation of the mice for the *in vivo* infection model used.

Acknowledgements for this work:
We would like to thank Bob Harris of the University of Guelph for his technical assistance in preparing samples for electron microscopy.

INTRODUCTION

The cell wall of Gram-positive bacteria is composed not only of peptidoglycan, but contains a significant proportion of the polyol phosphate polymer known as teichoic acid. Wall teichoic acid has long been held as an essential component of the cell wall architecture (Baddiley *et al.*, 1956b; Bhavsar *et al.*, 2001; Bhavsar *et al.*, 2004; Brandt and Karamata, 1987; Briehl *et al.*, 1989; Pooley *et al.*, 1991). However, recently our group has demonstrated a complex pattern of dispensability for wall teichoic acid biosynthetic genes in both *Bacillus subtilis* and *Staphylococcus aureus* (D'Elia *et al.*, 2006a; D'Elia *et al.*, 2006b).

The synthesis of wall teichoic acid polymers occurs through the sequential action of several enzymes (Lazarevic *et al.*, 2002; Neuhaus and Baddiley, 2003). The action of no less than 7 enzymes is thought to synthesize the completed polymer on the cytoplasmic face of the cell membrane for export to the outside of the cell. Once outside, the completed polymer is covalently attached to the C-6 of *N*-acetylmuramic acid of peptidoglycan though the action of an uncharacterized transferase. The best characterized wall teichoic acid biosynthetic machinery is that for polymers composed of glycerol phosphate and ribitol phosphate. In the last several years, biochemical experiments have characterized the activities of nearly all of the enzymes responsible for the synthesis of both glycerol phosphate and ribitol phosphate polymers (Brown *et al.*, 2008; Ginsberg *et al.*, 2006; Pereira *et al.*, 2008).

Work on the essential nature of wall teichoic acid dates back many years to the discovery and characterization of temperature-sensitive mutants in the *B. subtilis tag* genes for poly(glycerol phosphate) synthesis by Karamata's lab (Brandt and Karamata, 1987; Briehl *et al.*, 1989; Pooley *et al.*, 1991). That work and follow-up studies by our research group (Bhavsar *et al.*, 2001; Bhavsar *et al.*, 2004; Schertzer and Brown, 2003) showed convincingly that genetic lesions in several wall teichoic acid biosynthetic steps led to cell death *in vitro*. Recently, however, we uncovered some remarkable complexity in the dispensability pattern of wall teichoic acid synthetic genes. Working in both *B. subtilis* and *S. aureus*, we showed that viable deletions could be generated in the first gene of the pathway, encoding the *N*-acetylglucosamine-1-phosphate transferase (*tagO* in *B. subtilis*; *tarO* in *S. aureus*) while deletions could not be made in late-acting genes, including those encoding the glycerol phosphate primase (*tagB* in *B. subtilis*; *tarB* in *S. aureus*) and downstream enzymes. This apparent paradox was resolved when it was discovered that all of the indispensable genes became dispensable in a *tagO* (or *tarO*) – null genetic background and suggested that lesions in late steps in wall teichoic acid synthesis lead to a gain of lethal function for the pathway, presumably with the formation of toxic intermediates (Figure 4.1A).

Prior to the action of TagB and after that of the TagO protein, the *N*-acetylmannosamine transferase encoded in *B. subtilis tagA* (*tarA* in *S. aureus*) completes the synthesis of undecaprenyl pyrophosphate-linked disaccharide, a core component of the 'linkage unit' of wall teichoic acid (Ginsberg *et al.*, 2006; Zhang *et al.*, 2006). Here we show that the *N*-acetylmannosamine transferases of both *B. subtilis* and *S. aureus* (*tagA* and *tarA*) are dispensable for growth *in vitro*. This finding makes *tagA* (*tarA*) the

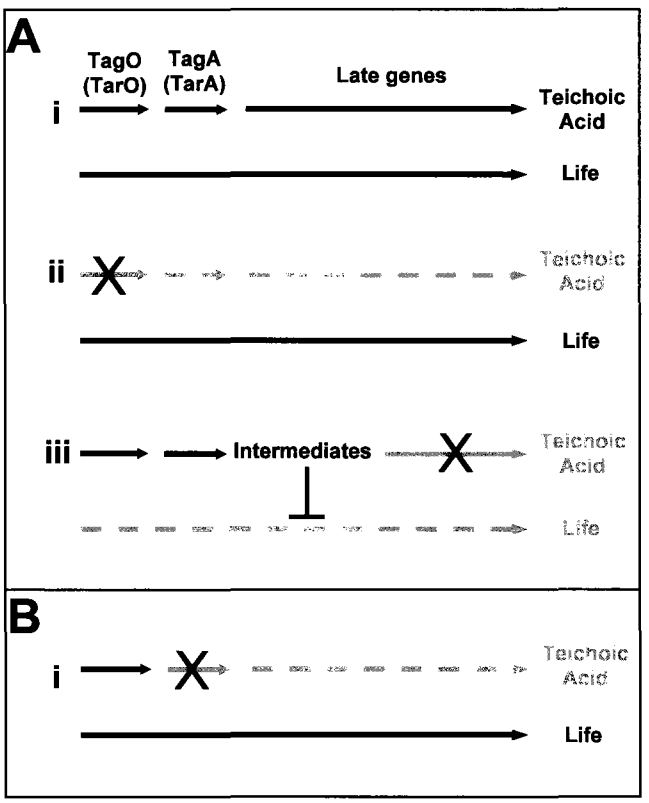

Figure 4.1 – Model for wall teichoic acid dispensability and lethal consequences of *B. subtilis tag* gene or *S. aureus tar* gene deletion. Panel (A) summarizes the previous studies, identified in chapters two and three, for the dispensability of late-acting *tag* (*tar*) genes and their interactions with *tagO* (*tarO*). Part (i) graphically depicts our understanding that the teichoic acid synthesis pathway is not a life-limiting process but that it parallels such processes. Part (ii) illustrates the findings that a deletion in *tagO* (*tarO*) blocks teichoic acid synthesis but does not impact on cell viability *in vitro*. Part (iii) summarizes our hypothesis for the lethal impact of deletions in the late genes of wall teichoic acid synthesis. We posit that a lesion in the late steps of teichoic acid synthesis results in the accumulation of intermediates that have toxic implications for life-giving function(s) in the cell. Panel (B) summarizes the findings reported herein. Deletion of gene *tagA* (*tarA*) has a phenotype like that of *tagO* (*tarO*). This makes *tagA* (*tarA*) the last dispensable gene in the pathway where the action of *tagA* (*tarA*) commits the cell to teichoic acid polymer production.

last dispensable gene in the pathway and suggests that the action of the TagA (TarA) enzyme commits the cell to synthesizing wall teichoic acid. Once committed to wall teichoic acid synthesis the cell must complete polymer assembly to avoid the lethal consequences that have been documented for partial synthesis.

MATERIALS AND METHODS

Strains, plasmids and growth conditions – Cloning was performed in an *E. coli* Novablue (Novagen) strain grown in Luria-Burtani broth at 37 °C. All *B. subtilis* and *S. aureus* strains used are described in Table 4.1. *B. subtilis* strains were grown in LB at 30°C and *S. aureus* strains grown in Mueller Hinton Broth (MHB) or Trypiticase Soy Broth (TSB) at 37 °C. For selection in *E. coli*, ampicillin (50 μg/ml) and kanamycin (50 μg/ml) were used. In *B. subtlis*, chloramphenicol (10 μg/ml), spectinomycin (100 μg/ml), and erythromycin (0.5 μg/ml) was used with lincomycin (12.5 μg/ml). In *S. aureus*, kanamycin (20 μg/ml), erythromycin (10 μg/ml), spectinomycin (300 μg/ml), chloramphenicol (60 μg/ml), sucrose (5 % wt/vol) and IPTG (0.4 mM) were used.

S. aureus teichoic acid biosynthesis sequences were identified using BLAST analysis and comparing the protein sequences of *B. subtilis* 168 taken from the SubtiList database (http://genolist.pasteur.fr/SubtiList) against the *S. aureus* COL genome obtained from The Institute for Genomic Research database (www.tigr.org).

Chromosomal manipulation of *B. subtilis* and *S. aureus* – Transformation of DNA into *B. subtilis* transformation was performed as described by Harwood *et al.* (Cutting and Vander Horn, 1990). DNA manipulations in *S. aureus* were performed using transformation and / or transduction. Transformation was accomplished through standard electroporation procedures (Lee, 1995). Transductions were performed using bacteriophage 80α and standard protocols (Novick, 1991).

PCR amplification – Primer sequences are found in Table A.3 (see appendix). All PCR was performed using Vent DNA polymerase (New England Biolabs) or Roche High Fidelity PCR System (Roche Applied Science). The spectinomycin and erythromycin cassettes were PCR amplified using SpecF-SpecR and ErmF-ErmR respectively. For each gene, the left and right flanks were independently amplified from the SA178RI chromosome using gene specific primers sets A-B, and C-D respectively.

Construction of *tagA* null mutant in *B. subtilis* 168 – The null mutant was generated by transforming wild-type *B. subtilis* (EB6) with a PCR product permitting double recombination and replacing *tagA* with a spectinomycin resistance cassette. This PCR product contains 1000 bp regions upstream and downstream of *tagA* flanking a central spectinomycin resistance cassette.

Construction of *tarA* single integrant and null mutant in *S. aureus* – The null mutant was created as previously described (D'Elia *et al.*, 2006b). Briefly, a PCR product containing 1127 bp upstream and downstream of *tarA* was cloned with a central spectinomycin or erythromycin cassette into pSAKO (D'Elia *et al.*, 2006b). This plasmid (pSAKO-Δ*tarA*) was transformed into EBII13 (SA178RI) to generate a single integrant (EBII57 and EBII85). The single integrant was then plated on MHA containing sucrose and spectinomycin or erythromycin for 3 successive generations, selecting for excision replacing *tarA* with the resistance cassette (EBII58 and EBII86 respectively).

Construction of a *tarO* and *tarA* null mutant in *S. aureus* Newman – EBII44 and EBII58 were independently transduced into *S. aureus* Newman (EBII61) to generate EBII65 and EBII82 respectively.

Testing *tarA* dispensability and suppressor activity in *S. aureus* – The dispensability of *tarA* was determined as previously described (D'Elia *et al.*, 2006b). Briefly, EBII57 were plated on MHA containing sucrose. 100 colonies were selected and passaged twice more onto MHA with sucrose. Each colony was then patched onto MHA containing kanamycin, MHA containing spectinomycin and MHA alone to determine phenotype. To determine if the dispensability of *tarA* could suppress the indispensability associated with teichoic acid enzymes previously demonstrated to be essential (D'Elia *et al.*, 2006b), the dispensability of *tarB, tarF* and *tarIJ* in a Δ*tarA* background was determined using integrants EBII90, EBII91 and EBII92 as described above. These strains were created by transduction as described previously (D'Elia *et al.*, 2006b). The growth media contained spectinomycin through all steps in the procedure. The phenotype of each colony was determined and the data summarized in Table 4.2.

Cell wall phosphate content determination – Strains were grown overnight in 100 ml of MHB at 37 °C. Cell wall isolation and phosphate content determination were carried out as described previously (Bhavsar *et al.*, 2004). Briefly, cell wall was extracted by boiling in sodium dodecyl sulfate (MacDonald and Beveridge, 2002), DNase, RNase and trypsin treated (Kruyssen *et al.*, 1980), and mineralized (Ames, 1966). Wall phosphate content was assayed by absorbance using KH_2PO_4 as a standard (Chen *et al.*, 1956).

Growth kinetic analysis – Wild-type (EB6) and the *tagA* null mutant (EB1494) were grown on LB agar overnight. The cells were resuspended in saline and diluted in 100 mL of LB to an OD_{600} of 0.005. The cells were grown at 37 °C with shaking (275 RPM) with OD measurements taken every 1-2 hours. *S. aureus* growth kinetics were measured by diluting an overnight of each strain to an OD_{600} of 0.005 in 200 µL of media in a sterile Costar 96 well microtitre plate (VWR). Strain EBII13, EBII44 and EBII58 were grown in MHB, while EBII53 and EBII77 were grown in MHB containing chloramphenicol and IPTG. Cultures were grown at 37 °C with shaking (200 RPM). OD measurements were taken every 1-2 hours. Each strain was performed in triplicate with the average of the replicates plotted as $\log_{10}$ versus time.

Ultrastruture of teichoic acid null mutants of *B. subtilis* and *S. aureus* – 50 mL cultures of *B. subtilis* EB6 and EB1494 and *S. aureus* EBII13, EBII44 and EBII58 were grown O/N. The cultures were pelleted and washed with saline. Cells were resuspended in 2 % glutaraldehyde and fixed O/N at 4 °C. The samples were prepared and imaged using standard techniques (Matias and Beveridge, 2006).

Testing the *in vivo* effects of teichoic acid null mutants using a murine kidney abscess model – Female Swiss-Webster mice (25 g each) were purchased from Charles River Laboratories Canada Inc., and housed in microisolator cages. *S. aureus* Newman (EBII61), EBII65 and EBII82, were grown overnight in TSB, washed three times with sterile saline and suspensions of 1 x 10^8 cfu / mL were made in sterile saline. One hundred microliters of the cell suspensions were administered intravenously via the tail vein. The number of viable bacteria injected was confirmed by plating serial dilutions of the inocula on TSA. In a blinded fashion, University of Western Ontario Animal Care and Veterinary Services (ACVS) personnel scored mice throughout the duration of the experiment for alertness, activity and coat condition. In each of the three categories, a score of 0 was normal, a score of 1 was slightly abnormal and a score of 2 was very abnormal. On day 5, the mice were euthanized and the kidneys were aseptically removed. Kidneys were then homogenized in sterile PBS + 0.1% Triton X-100 using a PowerGen 700 Homogenizer (Fisher). Homogenates were serially diluted and plated on TSB-agar to enumerate recovered bacteria. Data presented are the $\log_{10}$ CFU recovered per mouse.

Computer analyses – Microsoft Excel and SigmaPlot 2000 (SPSS Inc.) were used for data analysis and graphing applications.

MIC determination – MIC determination was preformed using a Costar 96 well microtitre plate (VWR). Cultures of EBII61, EBII65 and EBII82 were grown O/N. Cell cultures were first diluted 1 in 100 and grown to mid log at 37 °C with shaking. These cultures were subsequently diluted to a starting OD_{600} of 0.0002 in 150 µL MHB and grown at 37 °C in the absence of shaking before examining the OD_{600} at 24 and 48 hours. The MIC for each strain against a variety of drugs (ampicillin, bacitracin, chloamphenicol, cycloserine, daptomycin, erythromycin, fusidic acid, lincomycin, linezolid, phosphomycin, neomycin, novobiocin, spectinomycin, synercid, teichoplanin, tetracycline, triclosan, trimethoprim, tunicamycin and vancomycin) was determined by evaluating the minimal drug concentration preventing growth (OD_{600} lower than 0.005).

Table 4.1 – Strains and plasmids used in this study.

Strain	Genotype / Description	Source
S. aureus		
SA178RI	CYL316 containing T7 polymerase, (tet^r)	(D'Elia *et al.*, 2006a)
Newman	wild-type strain	(Duthie and Lorenz, 1952)
***tar* gene integrants**		
EBII1	SA178RI *tarF*::pSAKO-Δ*tarF* (erm^r, kan^r)	(D'Elia *et al.*, 2006a)
EBII2	SA178RI *tarB*::pSAKO-Δ*tarB* ($spec^r$, kan^r)	(D'Elia *et al.*, 2006a)
EBII43	SA178RI *tarIJ*::pSAKO-Δ*tarIJ* ($spec^r$, kan^r)	(D'Elia *et al.*, 2006a)
EBII57	SA178RI *tarA*::pSAKO-Δ*tarA* (erm^r, kan^r)	This study
EBII85	SA178RI *tarA*::pSAKO-Δ*tarA* ($spec^r$, kan^r)	This study
Uncomplemented deletion		
EBII44	SA178RI *tarO*::spec ($spec^r$, kan^s)	(D'Elia *et al.*, 2006a)
EBII58	SA178RI *tarA*::erm (erm^r, kan^s)	This study
EBII65	Newman *tarO*::spec ($spec^r$, kan^s)	This study
EBII82	Newman *tarA*::erm (erm^r, kan^s)	This study
EBII86	SA178RI *tarA*::spec ($spec^r$, kan^s)	This study
Complemented deletions		
EBII53	*tarO*::spec pG164-*tarO* (SA178RI)	This study
EBII77	*tarA*::erm pG164-*tarA* (SA178RI)	This study
EBII88	*tarA*::spec pLI50-*tarA* (SA178RI)	This study
Complemented *tar* gene integrants		
EBII40	EBII57 pG164-*tarA*	This study
***Tar* gene integrants in Δ*tarA* background**		
EBII90	EBII2 *tarA*::spec	This study
EBII91	EBII1 *tarA*::spec	This study
EBII92	EBII43 *tarA*::spec	This study
B. subtilis		
EB6	wild-type *B. subtilis* 168, *hisA1 argC4 metC3* (L5087)	(Briehl *et al.*, 1989)
EB1451	EB6 *tagO*::spec	(D'Elia *et al.*, 2006a)
EB1494	EB6 *tagA*::spec	This study

Plasmid		
pUS19	pUC19 derivative containing spec^r cassette	(Benson and Haldenwang, 1993)
pMUTIN4	source for erm^r cassette	(Vagner *et al.*, 1998)
pSAKO	*E. coli* replicating vector containing *sacB*[*BamP*]W29 and kan^r cassette	(D'Elia *et al.*, 2006a)
pG164	*E. coli* / *S. aureus* shuttle vector for T7 based protein expression (amp^r, chl^r)	(D'Elia *et al.*, 2006a)
pLI50	5.2-kb *E. coli/S. aureus* shuttle vector (amp^r chl^r)	(Lee *et al.*, 1991)
pSAKO-Δ*tarA*$_e$	plasmid for single integration into *tarA* flank containing central erm^r cassette (erm^r, kan^r)	This study
pSAKO-Δ*tarA*$_s$	plasmid for single integration into *tarA* flank containing central spec^r cassette (spec^r, kan^r)	This study
pG164-*tarA*	pG164 containing wild-type *tarA* from *S. aureus*	This study
pLI50-*tarA*	pLI50 containing wild-type *tarA* from *S. aureus*	This study

RESULTS

Dispensability of the *N*-acetylmannosamine transferase

Gene *tarA* from *S. aureus* COL was identified using BLAST analysis as SACOL0693. Dispensability testing of *tarA* was done in *S. aureus* strain SA178RI using an allelic replacement system developed by us (pSAKO) and described previously (D'Elia *et al.*, 2006b). This approach relies on chromosomal integration of the plasmid pSAKO, carrying upstream and downstream flanks of the target gene surrounding a drug resistance cassette. Integration results in a tandem duplication of the flanks surrounding the target gene with the plasmid sequence between the two copies. The plasmid contains a selectable marker for plasmid integration (kan^r) and a sucrose inducible counter-selectable marker (*B. subtilis sacB*[BamP]W29). Upon counterselection with sucrose, a recombination event excising the plasmid backbone is selected for. Excision can occur either through the upstream or downstream gene flank, resulting in chromosomal genotype of either a wild-type locus, or replacement of the targeted allele with a selectable marker. Gene dispensability is assayed by the frequency of recombination to either revert to wild-type or replace the locus with a resistance cassette. Using this methodology we demonstrated that *S. aureus tarA* could be readily replaced with an erythromycin resistance cassette indicating that this locus is dispensable for growth *in vitro* (Table 4.2).

In *B. subtilis*, we were likewise able to replace the *tagA* gene with a spectinomycin resistance cassette. The resulting colonies were small, smooth and very similar in morphology to the *tagO* mutant that we have described previously (D'Elia *et al.*, 2006a).

Table 4.3 shows our analysis of the cell wall phosphate contents for *B. subtilis* (*S. aureus*) wild-type, *tagO* (*tarO*) and *tagA* (*tarA*) null stains. These results reveal that the cell wall phosphate content of *tagA* and *tarA* null strains are approximately 10 % of the wild-type and are comparable to phosphate found in the *tagO* and *tarO* null strains. Together these results are consistent with the conclusion that *B. subtilis tagA* and *S. aureus tarA* mutants were devoid of wall teichoic acid.

Suppression of late gene essentiality by *tarA* deletion

Having succeeded in making strains of *B. subtilis* and *S. aureus* that lacked the *N*-acetylmannosamine transferase gene and wall teichoic acid, we were interested to test for genetic interactions with the late genes in the pathway. Previously we were able to leverage the capacity of allelic replacement with pSAKO to test the dispensability of late-acting teichoic genes in the presence and absence of a *tarO* deletion (D'Elia *et al.*, 2006b). We reasoned that the dispensable phenotype of *tarA* should provide for a dispensable phenotype of the downstream genes *tarB*, *tarF* and *tarIJ*, just as we have seen for *tarO*. From Table 4.2, it is clear that in the absence of *tarA*, the otherwise essential genes, *tarB*, *tarF* and *tarIJ,* become dispensable. Further demonstrating that *tarA* has the same peculiar genetic interactions previously observed with *tarO*.

Table 4.2 – Allelic replacement for testing gene dispensability in *S. aureus*.

	Phenotype		
	Wild-type	Non-excisant	Mutant
No complementation			
tarA	65	1	34
Δ*tarA* background			
tarB	75	0	15
tarF	50	2	48
tarIJ	88	0	12

Table 4.3 – Phosphate content of cell wall isolated from *B. subtilis* and *S. aureus*.

	Cell Wall (μmol phosphate / mg cell wall)
Bacillus subtilis	
Wild-type	1.6 ± 0.4
Δ*tagO*	0.09 ± 0.02
Δ*tagA*	0.10 ± 0.03
Staphylococcus aureus	
Wild-type	1.2 ± 0.1
Δ*tarO*	0.140 ± 0.003
Δ*tarA*	0.140 ± 0.005

Characterization of *B. subtilis tagA* and *S. aureus tarA* mutants

To further evaluate the phenotype of the deletion of the *N*-acetylmannosamine transferase in both *B. subtilis* and *S. aureus*, growth analysis and transmission electron microscopy was performed. The growth characteristics of the *B. subtilis tagA* and *S. aureus tarA* deletions with respect to the wild-type strains are very different (Figure 4.2). Figure 4.2A shows the growth kinetics of the *B. subtilis tagA* null strain (EB1494) compared to wild-type (EB6) and the *tagO* deletion (EB1451). The data reveal that the mutant is significantly impaired for growth compared to the wild-type strain, with a growth rate comparable to the *tagO* mutant previously described (D'Elia *et al.*, 2006a). In *S. aureus* the *tarA* deletion strain grew similarly to both wild-type and the *tarO* deletion (Figure 4.2B).

The differences shown in the growth curves were paralleled in the transmission electron micrographs shown in Figure 4.3. While the *S. aureus tarA* mutant did not have any significant morphological defects, the *B. subtilis tagA* deletion showed abnormalities that were very similar to those seen previously for the *tagO* deletion (D'Elia *et al.*, 2006a). These gross morphological defects included loss of rod shape, aberrant septation, and asymmetrical peptidoglycan architecture. Thus the loss of wall teichoic acid had a much more profound effect on *B. subtilis* than *S. aureus*.

Colonization of the *S. aureus tarA* mutant

Previous work has shown that *tarO* is a virulence determinant in *S. aureus* (Weidenmaier *et al.*, 2004; Weidenmaier *et al.*, 2005). The *tarO* deletion has been shown to lead to impaired colonization in a cotton rat nasopharaengeal, and rabbit endocarditis infection models. Here, we tested the hypothesis that the *tarA* deletion would similarly impair the colonization of *S. aureus* in a mouse kidney abscess model. Figure 4.4 charts the colony counts recovered from mouse kidneys five days after infection with a wild-type *S. aureus* Newman strain (EBII61) as well as the *tarO* and *tarA* null mutants in the Newman background (EBII65 and EBII82, respectively). At sacrifice, mice infected with the wild-type Newman strain had high bacterial cell numbers in their kidneys (average of $10^{6.5} \pm 10^{1.3}$ CFU). In stark contrast, we were unable to recover viable bacteria from most of the mice infected with either the *tarO* or *tarA* mutant strains, while some mice had low but detectable bacterial loads. The average cells recovered from mice infected with the *tarO* and *tarA* null strains was $10^{1.7} \pm 10^{2.0}$ and $10^{2.2} \pm 10^{2.7}$ CFU, respectively. Generally, mice infected with the mutant strains were significantly more healthy than those infected with wild-type bacteria. Clinical scoring through examination of the overall fitness of the mice showed that Δ*tarO* (EBII65) had an average score of 0.66 ± 1, Δ*tarA* (EBII82) had an average score of 0 ± 0, while the wild-type strain (EBII61) had a significantly higher score of 3.1 ± 1.2. As a further measure of health, we observed that mice infected with mutant strains lost, on average, significantly less weight than mice infected with wild-type Newman strain; 21 ± 6 % (wild-type Newman), 0.6 ± 3.4 % (*tarO* null), 1.4 ± 4.3 % (*tarA* null).

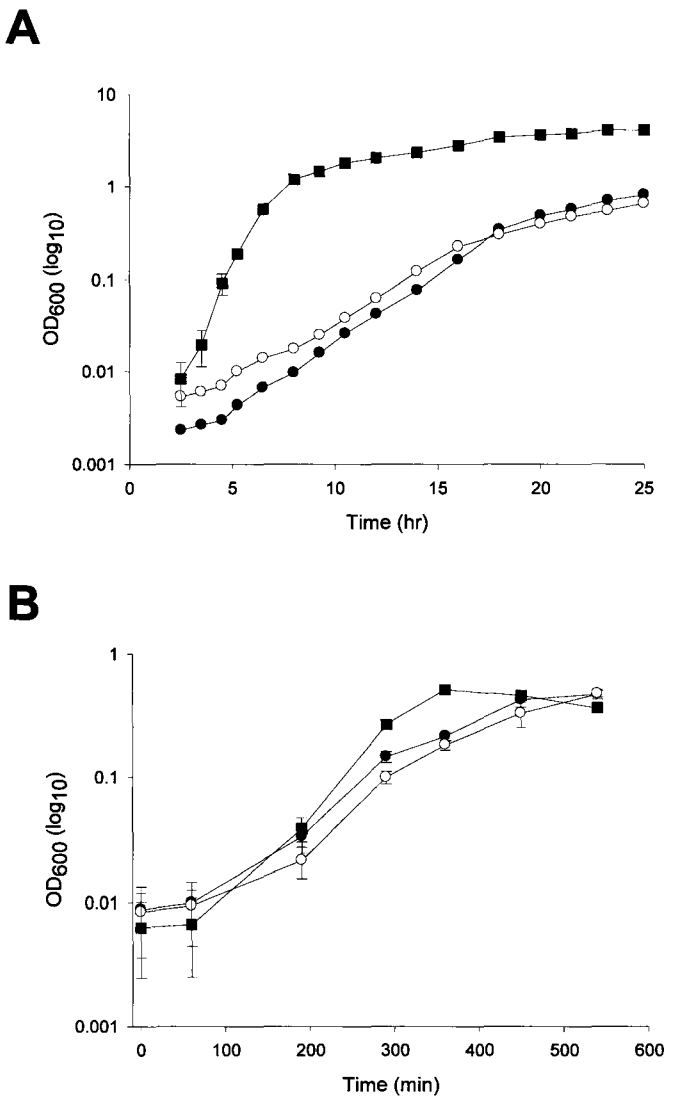

Figure 4.2 – Growth kinetics of *B. subtilis* and *S. aureus* deletion mutants. (A) Growth curves are depicted for the *B. subtilis tagA* deletion strain (EB1494, ○). Growth data for the wild-type (EB6, ■) and *tagO* deletion (EB1451, ●) strains are shown for comparison. (B) Growth curves are shown for the *S. aureus* (SA178RI) wild-type (■), *tarO* null (EBII44, ●) and *tarA* null (EBII58, ○) strains. All cultures were inoculated to a starting OD600 of 0.005 and absorbance measurements were taken every 1-2 hours.

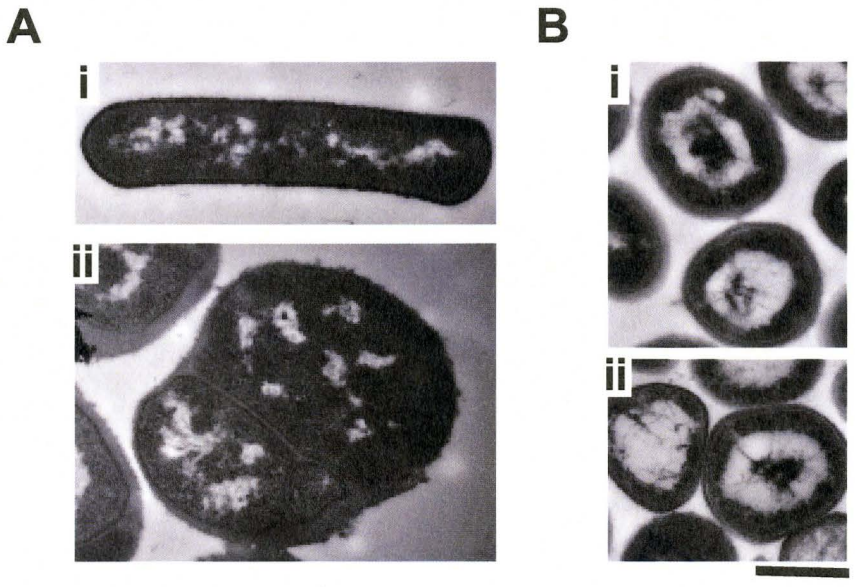

Figure 4.3 – Ultrastructure of *B. subtilis tagA* and *S. aureus tarA* null mutants. Bacteria were harvested at late log phase of growth and embedded in thin sections for examination with transmission electron microscopy as described in the Materials and Methods section. Panel (A) shows micrographs of (i) *B. subtilis* wild-type (EB6), and (ii) the *tagA* null (EB1494) strains. Panel (B) depicts micrographs of (i) *S. aureus* wild-type (SA178RI), and (ii) the *S. aureus tarA* null (EBII58) strains. The bar represents 500 nm.

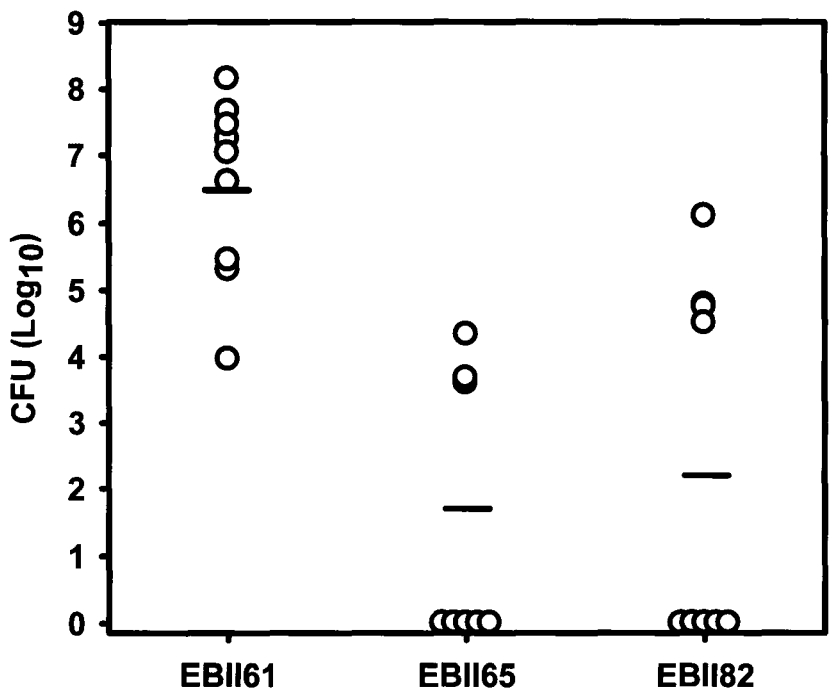

Figure 4.4 – Teichoic acid mutants are impaired for growth *in vivo*. The graph shows the colony forming units (CFU) recovered from the homogenized kidneys of mice infected with the *S. aureus* wild-type Newman strain (EBII61) and corresponding deletions in *tarO* (EBII65) and *tarA* (EBII81). In these experiments mice were injected with 10^7 bacteria and CFU was determined 5 days post-infection.

Antibiotic susceptibility of *S. aureus tarO* and *tarA* mutants to known antibiotics

Antibiotic susceptibility determinations were performed using the *S. aureus* Newman wild-type strain as well as derivatives containing *tarO* and *tarA* deletions. Some 20 antibiotics of varying chemical class and mechanism were tested and only fusidic acid and phosphomycin showed a change in minimal inhibitory concentration (MIC) greater than 2 fold with respect to the wild-type strain (Figure 4.5). These two antibiotics displayed a similar response in both mutants, showing 8-fold and 16-fold reduction in the MIC for fusidic acid and phosphomycin, respectively.

DISCUSSION

Recently our group demonstrated that wall teichoic acid biosynthetic genes show a complex dispensability pattern in both *B. subtilis* and *S. aureus* (D'Elia *et al.*, 2006a; D'Elia *et al.*, 2006b). That work revealed that the first step in the teichoic acid biosynthetic pathway encoded in *tagO* and *tarO*, were dispensable in *B. subtilis* and *S. aureus*, respectively. Paradoxically, late-acting wall teichoic acid biosynthetic genes were indispensable in both of these organisms. This remarkable inconsistency was resolved with the determination that the essential phenotype of the late acting genes could be suppressed by a deletion in the first enzyme in *B. subtilis* (*tagO*) and *S. aureus* (*tarO*). The simplest interpretation of these findings is that premature termination of teichoic acid biosynthesis causes a build up of toxic intermediates or the sequestration of a common precursor molecule.

While extensive investigations have charted the complex genetics of wall teichoic acid synthesis in both *B. subtilis* 168 (Bhavsar *et al.*, 2001; Bhavsar *et al.*, 2004; Brandt and Karamata, 1987; Briehl *et al.*, 1989; D'Elia *et al.*, 2006a; Lazarevic and Karamata, 1995; Mauel *et al.*, 1989; Pooley *et al.*, 1991; Soldo *et al.*, 2002) and *S. aureus* (D'Elia *et al.*, 2006b; Weidenmaier *et al.*, 2004), no experiments have so far been reported to characterize the dispensability phenotype of the *N*-acetylmannosamine transferase encoded in *tagA* (*B. subtilis*) and *tarA* (*S. aureus*). Indeed, *tagA* from *B. subtilis* was recently shown to catalyze the addition of *N*-acetylmannosamine to complete the synthesis of undecaprenyl pyrophosphate-linked disaccharide, a core component of the 'linkage unit' of wall teichoic acid (Brown *et al.*, 2008; Ginsberg *et al.*, 2006; Zhang *et al.*, 2006). This places TagA (TarA) as an enzyme catalyzing the second step in wall teichoic biosynthesis after TagO (TarO), the *N*-acetylglucosamine-1-phosphate transferase. Given the dispensable phenotype of *tagO* (*tarO*) and the capacity of this deletion for suppression of downstream essential late-acting genes, we were motivated to explore the dispensability phenotype of this yet unexplored step of wall teichoic acid synthesis.

In the work reported here we have established that *tagA* and *tarA* are dispensable for *in vitro* growth in both *B. subtilis* and *S. aureus*, respectively. Phenotypic characterization of these mutants indicated that the strains were devoid of wall teichoic acid. Furthermore we have shown that deletion of *tarA* in *S. aureus* is able to suppress

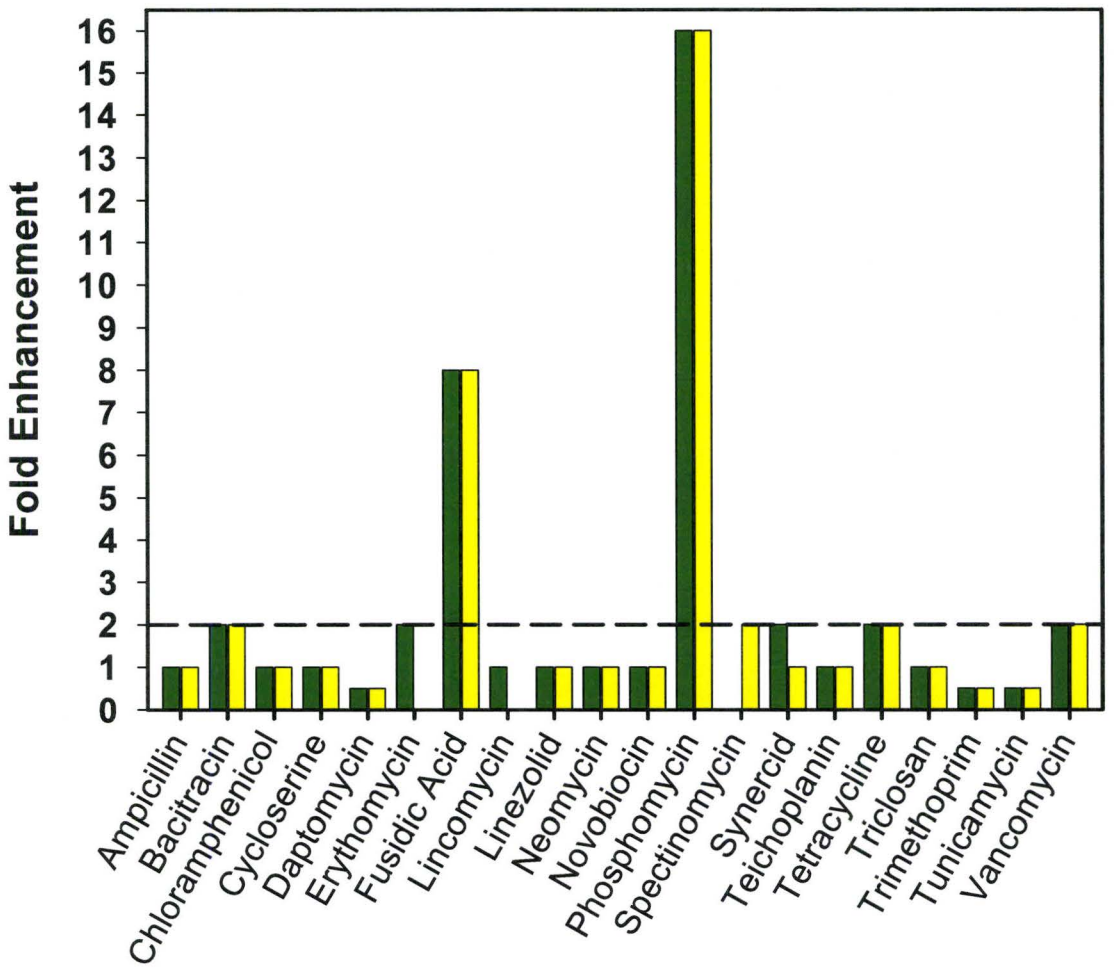

Figure 4.5 – Antibiotic sensitivity of *S. aureus tarO* and *tarA* deletions. The MIC for *S. aureus* wild-type and mutant strains against a panel of antibiotics was determined. The fold enhancement was calculated for the *tarO* (green) and *tarA* (yellow) mutants. Fold enhancement equals the MIC for the wild-type / MIC for the mutant; therefore a high fold enhancement represents a significant decrease in MIC for the drug against the wild-type strain. The dashed line represents a 2-fold enhancement. Missing bars for erythromycin and spectinomycin indicates a resistance cassette is present in the mutant strain.

the essential phenotypes of several late-acting wall teichoic acid synthesis genes. These findings reveal that *tagA* and *tarA* are the last dispensable genes in their respective biosynthetic pathways and suggests that the *N*-acetylmannosamine transferase commits the cell to synthesizing wall teichoic acid. Having committed to wall teichoic acid synthesis the cell must complete polymer assembly to avoid the lethality associated with blocks in the late steps of this pathway (Figure 4.1B).

Further characterization of the *tagA* and *tarA* mutants revealed that *N*-acetylmannosamine transferase deficiency had dramatically different impacts on the growth and morphology of *B. subtilis* and *S. aureus*. Deletion of *tagA* in *B. subtilis* resulted in a remarkable impact on ultrastructure including complete loss of rod shape, abberant septation and cell wall asymmetry. These observations were reminiscent of that seen for the *B. subtilis tagO* deletion described previously (D'Elia *et al.*, 2006a). In contrast, the *tarA* deletion in *S. aureus* had growth and ultrastructural characteristics that were not unlike wild-type.

Given the similar phenotypes of the *tagO* (*tarO*) and *tagA* (*tarA*) deletion strains, we broadened our search for phenotypes in this work to include antibiotic susceptibility. We restricted our investigations to *S. aureus* for these studies because of the robust growth of the *tarO* and *tarA* deletion strains in this organism. MIC determinations to a variety of antimicrobials are largely unchanged relative to wild-type. Among 20 antibiotics of varying chemical class and mechanism, *tarO* and *tarA* deletion strains showed increased susceptibility only to fusidic acid and phosphomycin. Interestingly, these two compounds are negatively charged, as are teichoic acid polymers. We posit that the increased susceptibility was due to improved delivery of these compounds to their intracellular targets. Thus our susceptibility data indicate that loss of teichoic acid polymers does not have an impact on drug susceptibility unless the antibiotic is negatively charged. This result predicts that drug-like molecules with a capacity to prevent teichoic synthesis ought to have potential in augmenting the antibacterial effect of negatively charged antibiotics such as fusidic acid and phosphomycin.

Having shown that *B. subtilis tagA* and *S. aureus tarA* deletions were viable and analogous in many respects to the *tagO* and *tarO* mutants characterized previously, we were interested to compare the *in vivo* phenotypes of the *tarO* and *tarA* mutants. Weidenmaier *et al.*, previous showed that the *tarO* null mutant was compromised for colonization in rat nasopharangeal and rabbit endocarditis models (Weidenmaier *et al.*, 2004; Weidenmaier *et al.*, 2005). Here, we found that the *tarO* and *tarA* mutants were similarly compromised relative to wild-type in a mouse kidney abscess model of infection. It has been well established that teichoic acid polymers play a significant role in the adherence of bacteria, likely the result of the charge associated with the polymer (Gross *et al.*, 2001; Weidenmaier *et al.*, 2005). The failure of the *tarA* null mutant to colonize and persist in the mouse model here provides additional support for the importance of wall teichoic acid to infection and draws further parallels with *tarO* in terms of phenotype.

In conclusion, our findings reveal that *B. subtilis tagA* and *S. aureus tarA* are the ultimate dispensable genes in their respective biosynthetic pathways. Indeed, the encoded *N*-acetylmannosamine transferases should be considered the committed steps in wall

teichoic acid polymer production. In this particular pathway, commitment to wall teichoic acid synthesis marks an obligation to complete polymer assembly and export. The consequence of failing to do so in these organisms is cell death. Thus despite the dispensability of the polymer for *in vitro* growth, wall teichoic acid biosynthesis represents an exploitable target for new antibiotic development. Interestingly, experiments shown here and elsewhere (D'Elia *et al.*, 2006a; D'Elia *et al.*, 2006b) predict that suppression of lethal phenotypes realized by targeting late steps in wall teichoic acid synthesis could be achieved with mutations in the first steps, namely *tagO* (*tarO*) and *tagA* (*tarA*). The apparent requirement of wall teichoic acid for colonization in various animal models nevertheless predicts that such suppressors would not be infectious. We maintain therefore that wall teichoic acid synthesis may well be an ideal target for new antibacterial drug discovery.

CHAPTER FIVE

CONCLUSIONS AND ONGOING STUDIES

Throughout the course of this thesis, two principal themes were emphasized: First the understanding and utility of teichoic acid biosynthetic enzymes as potential therapeutic targets, and second, the biological role for teichoic acid polymers in the cell wall. This chapter will highlight some of the outstanding issues surrounding the potential for therapeutic development and dissecting biological roles for teichoic acid polymers and their enzymes.

THERAPEUTIC POTENTIAL FOR TEICHOIC ACID BIOSYNTHESTIC ENZYMES

Over the last few decades, using a myriad of approaches, several reports have tried to address the question of the dispensability of teichoic acid biosynthetic genes (Bhavsar *et al.*, 2001; Bhavsar *et al.*, 2004; Lazarevic and Karamata, 1995; Mauel *et al.*, 1989; Pooley *et al.*, 1991). From the earliest of these observations, demonstrating that temperature sensitive phenotypes could be isolated in the teichoic acid operon, these biosynthetic genes were suggested to have therapeutic relevance (Pooley and Karamata, 1988). Although teichoic acid gene dispensability and therefore therapeutic importance was understood for sometime, not until this work has its complexity been fully appreciated.

During the course of this work, a complex relationship between dispensable and indispensable biosynthetic genes has been elucidated, in which early genes were found to be dispensable, yet late-acting genes essential; thereby demonstrating that despite significant belief to the contrary, teichoic acid polymers are not a strict requirement of the Gram-positive cell wall. Although this discovery significantly alters the long-standing view for their necessity, the underlying therapeutic potential for these biosynthesis genes remains intact. Work presented in chapter four and elsewhere (Weidenmaier *et al.*, 2004) has shown that despite the ability to produce strains devoid of wall polymers, these polymers remain an absolute requirement for pathogenicity, suggesting that targeting these enzymes continues to be a valid therapeutic opportunity.

Very recently, taking advantage of the discoveries highlighted in chapter two, whereby in a wild-type background late-acting enzymes are essential, but in a *tarO* null background late-acting enzymes are dispensable, Susan Walker's group identified an inhibitor of the wall teichoic acid export protein, TarG (Swoboda *et al.*, 2009). Interestingly, this molecule is specific only for *S. aureus* TarG and does not appear to be active against homologous proteins. As of yet, there is no understanding surrounding the specificity for this inhibitor, due in large part to a complete lack of any biochemical understanding of the mechanism of TarG activity. This ideally demonstrates the importance of biochemical comprehension for antimicrobial drug discovery. Despite this, the report by Swoboda *et al.* provides promise that inhibitors, synthetic or naturally occurring, can be identified to target the wall teichoic acid pathway.

BIOCHEMICAL INVESTIGATION OF TEICHOIC ACID BIOSYNTHETIC ENZYMES

While genetic dispensability studies have their own importance in the antimicrobial drug discovery field, biochemical characterization plays a significant role as well. The biochemical characterization of teichoic acid biosynthetic enzymes is significantly limited; it was almost exclusive restricted to work on the glycerol phosphate cytidylylransferase (GCT) from *B. subtilis*. In recent years, in large part due to the synthesis of a key soluble substrate, an undecaprenyl phosphate analogue (Ginsberg *et al.*, 2006; Zhang *et al.*, 2006), biochemical investigations in this field has exploded, specifically surrounding the 'TagF-like' family of enzymes. This class of proteins represent the polyol phosphate glycosyltransferases; those enzymes that add nucleotide activated glycerol or ribitol to the growing polymer. These are particularly interesting enzymes because they share significant sequence homology within their family, but no homology with outside proteins (Pereira, 2008), thus likely representing a novel protein structure. The membrane localization of the undecaprenyl-linked substrate has made detailed mechanistic studies impossible. With the availability of synthetic analogues these studies are now possible and underway using the prototypical *B. subtilis* TagF (Pereira *et al.*, 2008; Sewell *et al.*, 2009). Also, very recent progress has been made in solving the crystal structure for TagF from *Staphylococcus epidermidis* (personal communication E. Brown). Therefore, detailed biochemical studies on this and other 'TagF-like' family members will add significant value for future inhibitor discovery and design.

BIOLOGICAL SIGNIFICANCE OF TEICHOIC ACID POLYMERS

Chapters 2 through 4 establish that strains devoid of teichoic acid polymers in their cell wall can be generated in two different Gram-positive organisms, *B. subtilis* and *S. aureus*. Until now, a major hurdle in studying a biological role for teichoic acid polymers was the inability to completely remove the polymer from the wall. With the advantage of studying deletion, not just depletion of wall teichoic acid, it appears this has prompted a renewed vigour in understanding the biological significance of these polymers. Since the publication by Weidenmaier *et al.* (Weidenmaier *et al.*, 2004) and our own (D'Elia *et al.*, 2006b), concerning the dispensability of *tarO* in *S. aureus*, several groups have studied various biological implications with this mutant (Bera *et al.*, 2007; Kaito and Sekimizu, 2007; Kohler *et al.*, 2009; Koprivnjak *et al.*, 2008; Vergara-Irigaray *et al.*, 2008; Yamamoto *et al.*, 2008).

The most constant and unifying theme in all biological studies concerning wall teichoic acid has been this profound rod to sphere transition noted upon depletion in rod shaped bacteria, like *B. subtilis*. Additional complexity is noted in comparing observations made in chapter two and chapter three. In chapter three, deletion of teichoic acid in *B. subtilis* reveals the characteristic rod to sphere transition. This transition was accompanied by a dramatic reduction in growth rate (see Figure 3.1). However, similar

results were not observed upon investigating the corresponding *S. aureus* deletion. Obviously, given the coccoid character of *S. aureus*, no rod to sphere transition was seen. In fact, there was little morphological or growth defect observed (see Figure 4.2B and 4.3B). Surprisingly, it appears morphologically that coccoid organisms can tolerate the absence of wall teichoic acid, but rod shape bacteria may require this polymer for proper shape determination and/or cell wall maintenance.

Despite huge advances in our understanding of the cell wall, large holes, particularly in the insertion and maturation of the cell wall still exists. Autolysin enzymes cleave peptidoglycan and are thought to play a significant role in the maturation and remodelling of the cell wall (Vollmer *et al.*, 2008b). Additionally, teichoic acid polymers are believed comprise the binding site for autolysins in the wall (Brown *et al.*, 1970; Heptinstall *et al.*, 1970; Holtje and Tomasz, 1975). Therefore, one possible scenario is that these enzymes may mediate the observed rod to sphere transition. Also, recently Daniel *et al.* made significant strides in the understanding of nascent peptidoglycan insertion into the cell wall by revealing that peptidoglycan insertion into the cell wall occurs in a helical pattern co-localizing with the MreB structural protein (Daniel and Errington, 2003). Furthermore, Formstone *et al.* demonstrated that several teichoic acid proteins interact with Mre proteins, suggesting that teichoic acid and peptidoglycan insertion are linked through the Mre scaffold (Formstone *et al.*, 2008). It is interesting to speculate that the morphological defects may be caused by a role for teichoic acid polymers on the proper insertion of peptidoglycan. This idea can be further refined by suggesting teichoic acid polymers may control peptidoglycan insertion on the cell cylinder (cell cylinder growth). This notion is strengthened by the observed growth differences, compared to wild-type, between the *S. aureus* and *B. subtilis* mutants in chapters two and three respectively. This difference may be caused by the differential mechanisms of growth between the two organisms (Daniel and Errington, 2003). Being coccoid, therefore not containing an MreB cytoskeleton, *S. aureus* only undergoes septal growth; that is peptidoglycan insertion, and therefore cell growth only occurs in the septum. Contrarily, during its growth cycle, rod shaped, *B. subtilis* experiences both septal growth and cylinder growth. Therefore, growth differences in the mutant strains may be attributed to teichoic acid polymers regulating or controlling growth along the cell cylinder, but not the septum, thus displaying a more profound defect in *B. subtilis*. If this is true, an appealing group of organisms to study would be those that are rod shaped, but grow that the poles (septal growth mechanism). This includes species such as *Streptomyces* or *Corynebacterium.* It would be exciting to determine the phenotype associated with loss of teichoic acid polymers in these strains. Interestingly, MreB of *Steptomyces coelicolor* is not required for vegetative growth, but is necessary for aerial hyphae formation (Mazza *et al.*, 2006), suggesting that despite not being employed during the vegetative growth phase, cylindrical peptidoglycan insertion plays a role in aerial hyphae formation. Studies of this nature will likely guide our understanding of a potential role of teichoic acid in peptidoglycan insertion or maintenance.

Interpretation of the data generated from the mutants devoid of teichoic acid lead directly to many of the proposed hypotheses. These ideas directly implicate teichoic acid polymers in the most fundamental questions associated to bacterial physiology, bacterial

growth. It would be fascinating if these hypotheses gain merit and despite more than a half a decade of research, the work presented hear initiates further understanding into the core questions of microbial survival.

UNDERSTANDING THE UNUSUAL DISPENSABILITY PARADOX

Chapters two through four describe an unusual paradox surrounding teichoic acid gene dispensability, where early steps, coding for disaccharide synthesis, were found to be dispensable, yet late-acting steps were found to be essential for cell viability. Some rationalization of this observation was uncovered in that deletion of an early step suppresses the lethality associated with deletion of late-steps. Further understanding of this phenomenon was provided in demonstrating that depletion of a late acting enzyme, but not an early acting enzyme, significantly decreases the insertion of peptidoglycan into the cell wall (D'Elia *et al.*, 2009). It has been well established that wall teichoic acid and peptidoglycan synthesis compete for similar components, in particular the limiting supply of undecaprenyl phosphate. Taken together, these data suggest that the underlying mechanism for the lethality associated with the loss of some, but not all teichoic acid biosynthetic genes is an impact on peptidoglycan synthesis. This impact is likely mediated through the sequestration of undecaprenyl phosphate. Similar observations have been seen with a number of exopolysaccharides (Cuthbertson *et al.*, 2005; Reuber and Walker, 1993). Solidifying a mechanistic understanding of these unusual, yet recurrent observations is likely a key aspect in understanding the biological significance of wall teichoic acid polymers and their synthesis.

AN INTERESTING SIDE NOTE: THE LAST UNIDENTIFIED WALL TEICHOIC ACID BIOSYNTHETIC ENZYME

Arguably the 'holy grail' in the teichoic acid field, at least from a drug discovery perspective is the identification and characterization of the last remaining unknown teichoic acid biosynthesis protein: the wall teichoic acid transferase. Given its function is predicted to localize the protein outside the cell, it makes this the most attractive teichoic acid biosynthesis enzyme for a targeted inhibitor study. Despite its importance, no group has been able to identify a protein responsible for this activity. As of yet, bioinformatic approaches have been unsuccessful in this regard. With the explosion of genome sequencing and advancement in genetic tools, there is hope that potential candidates will be identified and tested. Currently one area of active experimentation in our laboratory is in developing tools to identify potential candidate proteins.

FINAL COMMENTS

As described in chapter one, antimicrobial drug discovery throughput has nearly halted in recent decades, resulting in the proliferation of multidrug resistant organism. Highlighted elsewhere (Fischbach and Walsh, 2009; Projan and Shlaes, 2004), a significant cause is the downsizing or withdrawal altogether of most large pharmaceutical companies from the anti-infective discovery area. This departure leaves a sizable niche where academia can make valuable contributions. This idea forms the basis not only for this thesis, but also for the work of the entire laboratory. With this thesis, I hope to have made significant contributions to the understanding of the teichoic acid field particularly in strengthening the notion of wall teichoic acid polymer synthesis as a valid and fruitful therapeutic target. As well as provide insights to further our understanding of Gram-positive cell physiology. Nothing would make me more proud than knowing this work has contributed significantly in these areas.

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APPENDIX – Oligonucleotides used throughout this study.

Table A.1 – Oligonucleotides used in chapter two.

Oligo	Sequence (5'-3')	Site
$spec_{For}$	cacaggaaacagctatgaccatgattaggcgaatggcgattttcgttcg	
$spec_{Rev}$	cattcaaatacagatgcattttatttcccatttttttcaatttttttataatttttttaatc	
erm_{For}	cttagaagcaaacttaagagtg	
erm_{Rev}	gggtctagagtctagggacc	
tarO A	ttttttttt**ctcgag**ttgattcggcacatagtttttaagtaa	XhoI
tarO B	taatcatggtcatagctgtttcctgtgcaactagtaataatgtaaccat	
tarO C	gaaataaaatgcatctgtatttgaatgggattaatagataacaactacc	
tarO D	ttttttttt**ctcgag**gattaaataatcttaagcttatgaattaagg	XhoI
tarB A	ttttttttt**ctcgag**taaaaactaaacttatttcaacttaca	XhoI
tarB B	gatactgcactatcaacacactcttaagtttgcttctaagataaaatttctttattaaaacgttcat	
tarB C	agcttccaagaagctaaagaggtccctagcgcctacggggtatatagataagatggtgacaaaatga	
tarB D	ttttttttt**ctcgag**gtcacatcataggattgcagtttatgg	XhoI
tarD A	ttttttttt**ctcgag**acatgtaataatgaggcaacaattcga	XhoI
tarD B	gatactgcactatcaacacactcttaagtttgcttctaagtgtgccatatgttattacacgtttcat	
tarD C	agcttccaagaagctaaagaggtccctagcgcctacgggggaattatatggtaaagatgctaaataa	
tarD D	ttttttttt**ctcgag**aaagaatatattatgtctacactacct	XhoI
tarF A	ttttttttt**ctcgag**tattccattttatgctgaagcatttgg	XhoI
tarF B	gatactgcactatcaacacactcttaagtttgcttctaagcaatttttaattgtatttttaatcat	
tarF C	agcttccaagaagctaaagaggtccctagcgcctacggggcgaatttgccaaacgatttttaagtga	
tarF D	ttttttttt**ctcgag**gcacaaattgacatatataaaggacgt	XhoI
tarIJ A	ttttttttt**ctgcag**cgttaaaatgctaggaacaaatactca	XhoI
tarIJ B	gatactgcactatcaacacactcttaagtttgcttctaagtccagctagaataccagcgtatttcat	
tarIJ C	agcttccaagaagctaaagaggtccctagcgcctacgggcacgtctagtattggaaaaaggactaa	
tarIJ D	ttttttttt**ctcgag**cggtttcagatgatacataagcttttg	XhoI
tarH A	ttttttttt**ctcgag**agaaaatggcaacaataccgaaattgt	XhoI
tarH B	gatactgcactatcaacacactcttaagtttgcttctaagatttttaatgtttaccgaaacgttcat	
tarH C	agcttccaagaagctaaagaggtccctagcgcctacgggggatgaggcccgcttcgttattaaataa	
tarH D	ttttttttt**ctcgag**gttgaattgtcgtattaaaaaagtttg	XhoI
$tarO_{For}$	cgcc**ggatcc**atggttacattattactagttgc	BamHI
$tarO_{Rev}$	ttttttt**ctcgag**ctattcctctttatgagatgacttacg	XhoI
$tarB_{For}$	caaaaaaa**ggatcc**atgaacgttttaataaagaaattttatcat	BamHI
$tarB_{Rev}$	caaaaaaa**ctcgag**ttttgtcaccatcttatctatatatt	XhoI
$tarD_{For}$	ttttttttt**ggatcc**atgaaacgtgtaataacatatggcaca	BamHI
$tarD_{Rev}$	ttttttttt**ctcgag** tttagcatct ttaccatataattcttg	XhoI
$tarF_{For}$	caaaaaaa**ggatcc**atgattaaaaatacaattaaaaaattgatagaa	BamHI
$tarF_{Rev}$	caaaaaaa**ctcgag**cttaaaaatcgtttggcaaattcg	XhoI
$tarIJ_{For}$	aaaa**ggatcc**atgaaatacgctgg	BamHI

$tarIJ_{Rev}$	aaaa**ctcgag**ttacataatccattt	XhoI
$tarH_{For}$	caaaaaaa**ggatcc**atgaacgtttcggtaaacattaaaaat	BamHI
$tarH_{Rev}$	caaaaaaa**ctcgag**tttaataacgaagcgggcct	XhoI
$confO_{For}$[a]	aactgagaactcttcgccacc	
$confO_{Rev}$[a]	tgtcattttcagtgtataatg	
$confB_{For}$[a]	gattttgttattatcagagtgg	
$confB_{Rev}$[a]	acacgtttcattaagttatcc	
$confD_{For}$[a]	gaggtttacgataatcatacc	
$confD_{Rev}$[a]	gatgacacagtcacaatgacg	
$confF_{For}$[a]	gcatatgtttcatcagaaacc	
$confF_{Rev}$[a]	ccagtataatatctttgatcg	
$confH_{For}$[a]	tatatttcatctgtaagtacg	
$confH_{Rev}$[a]	taacaagctgcaacaataacg	
$SAKO_{For}$[b]	aggttgaggccgttgagc	
$SAKO_{Rev}$[b]	cagcatccttgaacaagg	
$spec_{Up}$[c]	cctgccagtcacgttacg	
$spec_{Down}$[d]	ggagagaatattgaatgg	
erm_{Up}[c]	agatactgcactatcaac	
erm_{Down}[d]	gagtcgcttttgtaaatttgg	

[a]Sequence specific primers designed to anneal to flankings sequences to the $left_{(For)}$ and $right_{(Rev)}$ of each gene studied (eg. $confO_{For}$ – primer designed for the left flank of *tarO*).
[b]Primer designed to anneal $upstream_{(For)}$ and $downstream_{(Rev)}$ of the XhoI site in pSAKO.
[c]Primer designed to the 5' end of the spectinomycin/erythromycin cassette oriented 3'-5'.
[d]Primer designed to the 3' end of the spectinomycin/erythromycin cassette oriented 5'-3'.

Table A.2 – Oligonucleotides used in chapter three.

Oligo	Sequence (5'-3')
erm_{For}	cttagaagcaaacttaagagtg
erm_{Rev}	gggtctagagtctagggacc
tagO A	cccggattcccgctgctgtcttcgc
tagO B	gcactatcaacacactcttaagaagtctcctttggaatt
tagO C	ggaaataattctatgagtcgcccggcttatgtgccggag
tagO D	gaggattacgcgactaaagg
erm_{Up}[a]	agatactgcactatcaac
erm_{Down}[b]	gagtcgcttttgtaaatttgg

[a]Primer designed to the 5' end of the erythromycin cassette oriented 3'-5'.
[b]Primer designed to the 3' end of the erythromycin cassette oriented 5'-3'.

Table A.3 – Oligonucleotides used in chapter four.

Oligo	Sequence (5'-3')	Site
$spec_{For}$	cacaggaaacagctatgaccatgattaggcgaatggcgattttcgttcg	
$spec_{Rev}$	cattcaaatacagatgcattttatttcccatttttttcaatttttttataatttttttaatc	
erm_{For}	cttagaagcaaacttaagagtg	
erm_{Rev}	gggtctagagtctagggacc	
tarA A	ttttttt**ctcgag**aatgataatgtaattattgtc	XhoI
tarA D	ttttttt**ctcgag**tttctctacttaactaaatag	XhoI
tarAe B	gatactgcactatcaacacactcttaagtttgcttctaaggctgtattggatctttcttcaacagtcat	
tarAe C	agcttccaaggagctaaagaggtccctagactctagaccccaaaaataaaaccgaggaaatccttaa	
tarAs B	taatcatggtcatagctgtttcctgtgtattggatctttcttcaacagtcat	
tarAs C	gaaataaaatgcatctgtatttgaatgcaaaaagaaaaataaaaaaggcgaaataa	
tagA A	atgtcaggaatttgttttatattag	
tagA B	taatcatggtcatagctgtttcctgtaggaatattgtgaatagtctctgtttgcat	
tagA C	gaaataaaatgcatctgtatttgaatggaaaaagatcatacaaaacagatttaa	
tagA D	ccaatccccgcttctgttgataaag	
pG $tarA_{For}$	cgcc**ggatcc**atgactgttgaagaaagatcc	BamHI
pLI $tarA_{For}$	aattaaataatcttcacgataagg	
$tarA_{Rev}$	ttttttt**ctcgag**ttatttcgccttttttatttttctttttgc	XhoI
$confA_{For}$ [a]	taagtaattatcattaaacg	
$confA_{Rev}$ [a]	tgtcattttcagtgtataatg	
$SAKO_{For}$ [b]	aggttgaggccgttgagc	
$SAKO_{Rev}$ [b]	cagcatccttgaacaagg	
$spec_{Up}$ [c]	cctgccagtcacgttacg	
$spec_{Down}$ [d]	ggagagaatattgaatgg	
erm_{Up} [c]	agatactgcactatcaac	
erm_{Down} [d]	gagtcgcttttgtaaatttgg	

[a]Primers designed external to the left flank (For) and right flanks (Rev) for *S. aureus tarA*
[b]Primer designed upstream (For) and downstream (Rev) to the XhoI site in pSAKO.
[c]Primer designed to the 5' end of the spectinomycin/erythromycin cassette oriented 3'-5'.
[d]Primer designed to the 3' end of the spectinomycin/erythromycin cassette oriented 5'-3'.