KINETIC CHARACTERIZATION OF TARD FROM *S. AUREUS*

THE STUDY OF CTP:GLYCEROL 3-PHOSPHATE CYTIDYLYLTRANSFERASE (TARD) FROM *STAPHYLOCOCCUS AUREUS*

BY

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ABSTRACT

The CTP:glycerol 3-phosphate cytidylyltransferase (TarD) from *Staphylococcus aureus* catalyzes the formation of the nucleotide activated form of glycerol 3-phosphate (CDP-glycerol) used in the construction of teichoic acid, a structure shown to be essential in *Bacillis subtilis* 168. The CTP:glycerol 3-phosphate cytidylyltransferase from *B. subtilis* 168 (TagD) involved in teichioc acid biosynthesis has high sequence identity (69 %) and similarity (86 %) to TarD and its characterization has been well documented. In these studies, TagD was shown to carry out the CTP:glycerol 3-phosphate cytidylyltransferase reaction via a random mechanism where there is negative cooperativity in the binding of substrates but not in catalysis. The work described here illustrates that the kinetic reaction mechanism for TarD is vastly different from TagD in spite of their high sequence similarity. Recombinant TarD was over-expressed in *Escherichia coli* and purified to homogeneity. Steady state bi-substrate experiments were performed utilizing a high-performance liquid chromatography assay in order to deduce the kinetic mechanism for TarD. In this analysis, data were globally best fit to the model that describes the formation of a ternary complex of substrates (CTP and glycerol 3-phosphate) and enzyme before catalysis. This examination yielded K_m values for CTP and glycerol 3-phosphate of 21 ± 4.1 μM and 36 ± 5.8 μM respectively, while the k_{cat} was measured to be 2.6 ± 0.2 s^{-1}. From the pattern observed in product inhibition studies, a classic ordered Bi Bi reaction mechanism was inferred where glycerol 3-phosphate is the initial substrate to bind followed by CTP and the release of CDP-glycerol precedes

the release of pyrophosphate. A K_{eq} of 16 ± 15 was calculated using data obtained from exploring the kinetic parameters of the reverse reaction where data was also fit to the equation that describes the formation of a ternary complex before catalysis. The equilibrium constant was also determined experimentally to be 6. To illustrate the biological role of TarD with respect to TagD, the integration plasmid, pSWEET, was used to introduce a copy of *tarD,* under xylose control, into the chromosome of a strain of *B. subtilis* 168 possessing a temperature sensitive mutation (*tag-12*) mapped to *tagD.* Successful complementation of the temperature sensitive mutant by *tarD* at the restrictive temperature indicated that despite their apparent uniqueness in kinetic mechanism, TarD and TagD have similar roles *in vivo.*

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LIST OF ABBREVIATIONS

AMP	ampicillin
CDP	cytidine diphosphate
CDP-Gro	CDP-glycerol
CDP-Rbo	CDP-ribitol
CMP	cytidine monophosphate
CTP	cytidine triphosphate
DTT	dithiothreitol
EDTA	ethylenedinitrilo tetraacetic acid disodium salt dihydrate
FPLC	fast protein liquid chromatography
GroP	glycerol 3-phophate
HEPES	*N*-2-Hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
HTS	high throughput screening
IPTG	isopropyl-β-thiogalactopyranoside
MRSA	mellicillin resistant *Staphylococcus aureus*
PP_i	inorganic pyrophosphate
RboP	ribitol 5-phosphate
RibP	ribulose 5-phosphate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TRIS	tris (hydroxymethyl) aminomethane
VISA	vancomycin intermediate *Staphylococcus aureus*
VRSA	vancomycin resistant *Staphylococcus aureus*
VRE	vancomycin resistant *Enterococci*

PREFACE

Sections of the work in this thesis have been submitted for publication to under the authorship of David S. Badurina, Michela Zolli-Juran, and Eric D. Brown. The manuscript was written by David S. Badurina and edited by Eric D. Brown. Michela Zolli-Juran, a former graduate student of the Brown lab, constructed the clone used for the over-expression of TarD (pMZ-*tarD*). Mrs. Zolli-Juran also made initial attempts and optimization of the purification of TarD. Electrospray mass spectrometry was performed and interpreted by Kirk Green of the Department of Chemistry, McMaster Univerisity. All other research was performed entirely by David S. Badurina.

CHAPTER 1 – BACKGROUND INFORMATION

1.1 Antimicrobial Resistance

The antibiotic revolution, set off with Fleming's observation of contaminating mold expressing factors that caused the lysis of staphylococcal colonies (see Brock, Madigan et al. 1997), allowed health care professionals to control infections in an efficient manner (Gold and Moellering 1996). With the discovery of penicillin and the windfall of antibiotic discovery in the decades to follow, it was thought that the repertoire of drugs discovered during this time would serve to fight infections indefinitely. Consequently, the research into this field of science abruptly stalled. Antibiotics are introduced into the environment by normal human use, but the major route in which they pollute the ecosystem is through use in agricultural feed (Gold and Moellering 1996). The massive amounts of antibiotics introduced into the ecosystem selects for bacteria that have developed defenses against these drugs. Infected patients must rely on antibiotics that may already be ineffective and are forced to fight up-hill battles in circumventing these infections.

Bacterial pathogens have the innate ability to alter their physiology negating the affects of certain antibiotics. These can include removing the antibiotic out of the cell by efflux pumps, antibiotic alteration so as to render the drug inactive, and modifying antimicrobial targets so that it is undetectable by the drug (Walsh 2000). With the rise of resistance, there have been proposals put forth that address combating this phenomenon.

Compliance in taking antibiotics by patients as well as attempts to circumvent over-prescriptions are bureaucratic solutions used to reduce the prevalence of antibiotic resistance (Hartstein and Mulligan 1999). It has also has been proposed that the widespread use of hygienic practices to reduce the spread of highly resistance bacteria such as the methicillin resistant *Staphylococcus aureus* (MRSA) in the hospital setting could decrease the outbreak of these infections (Hartstein and Mulligan 1999). Other infection control practices for circumventing the problem of resistance pathogens infecting already morbid and seriously ill patients can include laboratory monitoring of the colonization of patients and staff (Hartstein and Mulligan 1999). This preemptive information could prove invaluable for treatment of outbreaks that may occur in the future. Lastly, segregation and decolonization of patients and health care staff are other proposals for the prevention of nosocomial infections (Hartstein and Mulligan 1999).

In the past, the discovery of new classes of drugs such as the aminoglycosides, macrolides, and glycopeptides, as well as the quinolones have helped avert the problem of antimicrobial resistance to some extent (Gold and Moellering 1996). This strategy also includes the research in chemical modifications that will alter older drugs so that they may escape various resistance mechanisms (Gold and Moellering 1996). Given this, however, novel antimicrobial drug discovery has not flourished as it has in the past partly because of the belated research in this field (Gold and Moellering 1996). Coupled to finding new drugs, is research into the discovery of new potential targets for antimicrobials. The use of bacterial genetics and the study of essential pathways and structures in bacterial pathogens are the springs from which these potential targets may

flow (Walsh 2000). This thesis describes the initiation of research into an enzyme that participates in the manufacture of an essential structure in Gram positive bacteria. The direction of this research is dictated by the fact that this enzyme is a potential therapeutic target for antimicrobial drugs.

1.2 Therapeutic relevance of *Staphylococcus aureus*

A considerable proportion (30%) (John and Barg 1999) of the population is colonized with *S. aureus*. This bacterium has continued to be one of the most difficult nosocomial pathogen to treat. These infections have the ability to ultimately cause abscesses, central nervous system infections, endocarditis, osteomyelitis, pneumonia, and urinary tract infections (John and Barg 1999). Furthermore, syndromes caused by endotoxins can include food poisoning, scalded skin syndrome, and toxic shock (John and Barg 1999). *S. aureus* is also considered the most prevalent community or nosocomial acquired pathogen resulting in bacteremia (Diekema 2001). *S. aureus* must also be contended with at points where surgical implements must be inserted and remain in patients for long periods of time (John and Barg 1999). Devices such as dialysis tubing, catheters, and artificial joints pose a particular problem for *Staphylococcal* infection due to the formation of biofilms (Donlan 2000). Biofilms are unique formations of bacteria that have adhered to artificial surfaces present on medical devices. These formations are naturally much more resistant to antibiotics which makes them of great medical concern. For example, it has been shown that biofilms originating from *S.*

aureus are 10 times more resistant to vancomycin to provide a 3-log reduction in growth (Donlan 2000).

Penicillin was the drug of choice to control staphylococcal infection in the early part of the 20th century. In 1945 the first signs of penicillin resistance were reported (Hartstein and Mulligan 1999). Semi-synthetic penicillinase resistant drugs, such as methicillin, started to emerge in order to fight these serious infections. As soon as two years after their introduction, strains were isolated that had natural occurring resistance to methicillin. From 1975 to 1991 the number of *S. aureus* isolates that were methicillin resistant increased from 2.1 % to 35 % (Diekema 2001) finally surpassing 50 % in 1999 (Fridkin 2000). This is of great concern for two reasons. The first is that most MRSA strains are multi-drug resistant; therefore the last resort drug, vancomycin, is the final front on which these infections can be uniformly treated (Smith, Pearson et al. 1999). Secondly, there have been indications that vancomycin intermediate resistant *S. aureus* (VISA) and fully resistant *S. aureus* (VRSA) are on the rise in hospitals around the world. Because it is thought that vancomycin resistance infections result from MRSA infections where large doses of vancomycin have been prescribed (Fridkin 2000), VISA and VRSA could present themselves as particularly deadly pathogens.

Vancomycin resistance in *S. aureus* seems to arise in a manner that is unlike that described for *Enterococci* (Chambers 1997; Fridkin 2000). In vancomycin resistant *Enterococci* (VRE) the *vanHAX* genes encode for products that are responsible for the catalysis of: the reduction of pyruvate to D-lactate (*vanH*); the linkage of D-lactate with D-alanine to produce D-Ala-D-Lac (*vanA*); and the destruction of D-Ala-D-Ala in

peptidoglycan (*vanX*) (Walsh 2000). There are no indications that VISA possesses the *van* genes therefore there must be a novel method of resistance in these organisms (Chambers 1997; Fridkin 2000). The first cases were seen in Japan but have now been encountered in a number of countries including Canada and the United States (Fridkin 2000). With this impeding danger there is no more crucial time to study indispensable biochemical functions in *S. aureus* in order to discover new targets for antimicrobials.

1.3 The Bacterial Cell Wall – Gram positive versus Gram negative

The bacterial cell wall is a dynamic structure protecting the exterior of the cytoplasmic membrane from the surrounding medium. It is intimately involved in cell growth, morphogenesis, division, and making cell-to-cell interactions (Brock, Madigan et al. 1997). It is also responsible for resisting turgor pressure, maintaining cell shape and protecting the underlying protoplast (Brock, Madigan et al. 1997). There are two main classes of bacteria where the most obvious difference is in the structure and organization of the cell wall; these are the Gram negative and the Gram positive bacteria (Figure 1.1). All bacteria possess a layer of peptidoglycan, which makes up part of the bulk of the cell wall. This carbohydrate layer is comprised of *N*-acetylglucosamine-β(1-4)- *N*-acetylmuramic acid subunits crosslinked with pentapeptide chains made from L-alanine, D-alanine, D-glutamic acid and either L-lysine or diaminopimelic acid (Brock, Madigan et al. 1997).

Although both Gram positive and Gram negative bacteria possess a peptidoglycan layer over the cytoplasmic cell membrane, the global organization of the cell wall and the

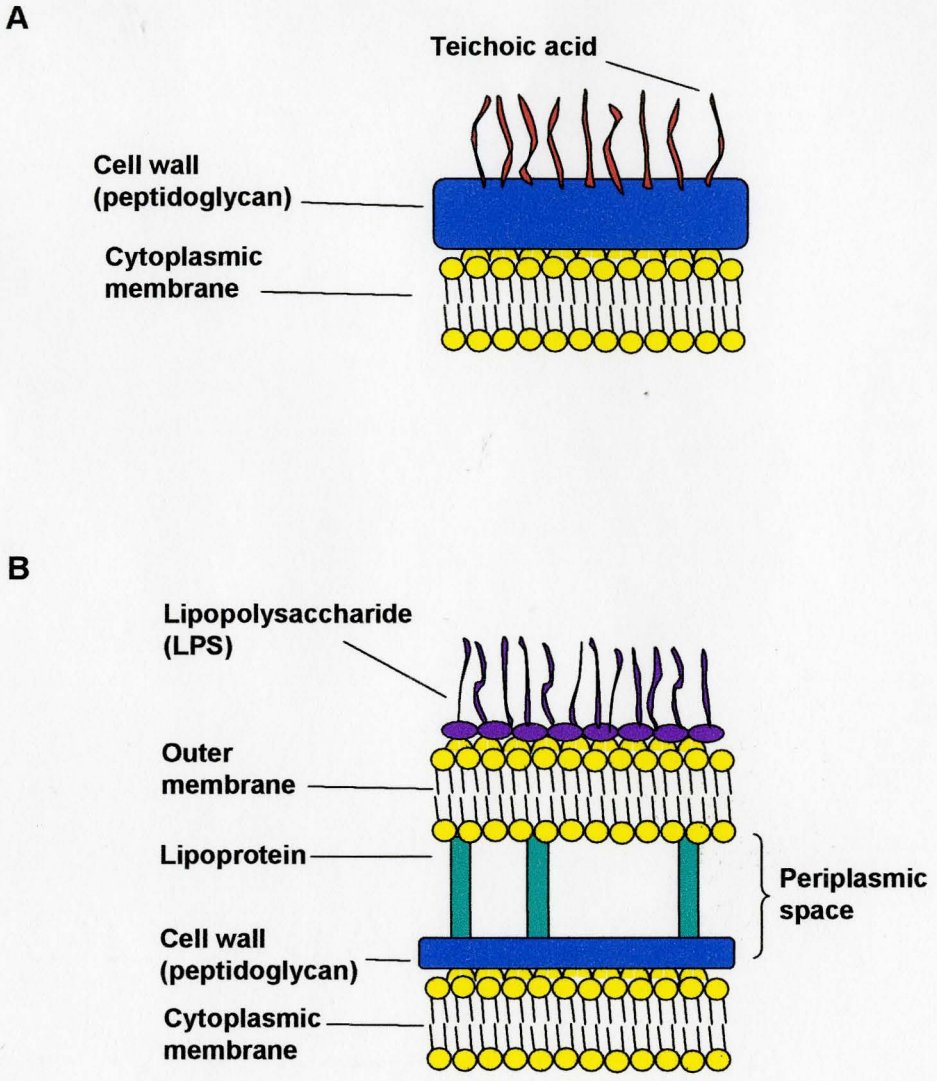


Figure 1.1 Organization of the Cell Wall of Gram positive and Gram negative Bacteria. (A) The schematic diagram of the Gram positive cell wall depicts the thick pepdioglycan layer as well as the teichoic acid polymers. (B) The obvious differences between the two classes of bacteria is the presence of two membranes; LPS on the outer surface; the absence of teichoic acid and the much thinner peptidoglycan layer of Gram negative bacteria.

amounts of each constituent are very distinct in these two classes of microorganisms. Gram negative bacteria possess an outer membrane in addition to their cytoplasmic membrane (Brock, Madigan et al. 1997). These two membranes are separated by the periplasmic space where the peptidoglycan layer is found. Moreover, another sugar polymer, called the lipopolysaccaride (LPS) layer, is attached to the outer membrane by anchorage into the outer membrane via the Lipid A element of LPS (Figure 1.1 B) (Brock, Madigan et al. 1997).

The Gram positive cell wall has a much thicker peptidoglycan layer relative to Gram negative bacteria (Figure 1.1 A). Also, there is no outer membrane nor any LPS in Gram positive bacteria. Lastly, these bacteria, unlike Gram negative bacteria, have major amounts of anionic polymer. Anionic polymers can be either classified as cell wall teichoic acid, lipoteichioc acid or teichuronic acid (Brock, Madigan et al. 1997). Lipoteichoic acid polymer is anchored to the cell envelope of Gram positive bacteria by fatty acids connected to the terminal glycerol phosphate subunit via an ester bond. The biochemical machinery that constructs lipoteichoic acid is distinct from the enzymes that are involved with wall teichoic acid biosyntheis (Perego 1995). The polymer of teichuronic acid is comprised of uronic acid containing molecules and is connected to pepidoglycan via a linkage unit as in teichoic acid. Unlike both wall teichoic acid and lipoteichoic acid, teichuronic acid only appears on the surface of cells in an environment in which there is limited phosphate (Lahooti 1999). This speaks to the fact that the presence of an anionic polymer is indeed important to the survival of the bacteria. This

thesis is concerned with one enzyme that is involved in the production of precursors for the construction of teichoic acid, the major anionic polymer of Gram positive bacteria.

1.4 Teichoic Acid Structure and Biosynthesis

1.4.1 Structure and Function of Teichoic Acid

A substantial amount of the dry weight of the cell wall is comprised of teichoic acid, which is covalently attached to peptidoglycan. There is great diversity in the constituents that make up the teichoic acid polymer among species of Gram positive bacteria. The teichoic acid polymer of *Bacillus subtilus* 168 is composed of 30 to 40 1,3-linked glycerol 3-phosphate (GroP) residues (Figure 1.2 A) while the main anionic polymer in *S. aureus* and *B. subtilus* W23 consists of a chain of repeating, 1,5-linked ribitol 5-phosphate (RboP) moieties (Figure 1.2 B) (Archibald, Hancock et al. 1993). Additionally, the polymer can be derivatized with glucosyl residues at the hydroxyl positions of the polyol. The polymer can alternatively be made up of sugar phosphate residues of different types. Although the diversity of teichoic acid is as broad as their organisms of origin, the linkage unit, which connects the teichoic acid polymer to peptidoglycan at the 6-hydroxyl of *N*-acetylmuramic acid via a phosphodiester bond, is conserved. Despite variations of the disaccharide constituents in the linkage unit, the presence of a short glycerol 3-phosphate chain is ubiquitously conserved between various *Bacillus* strains and *S. aureus* (Kaya, Yokoyama et al. 1983; Kojima, Araki et al. 1983; Kaya, Yokoyama et al. 1984; Archibald, Hancock et al. 1993).

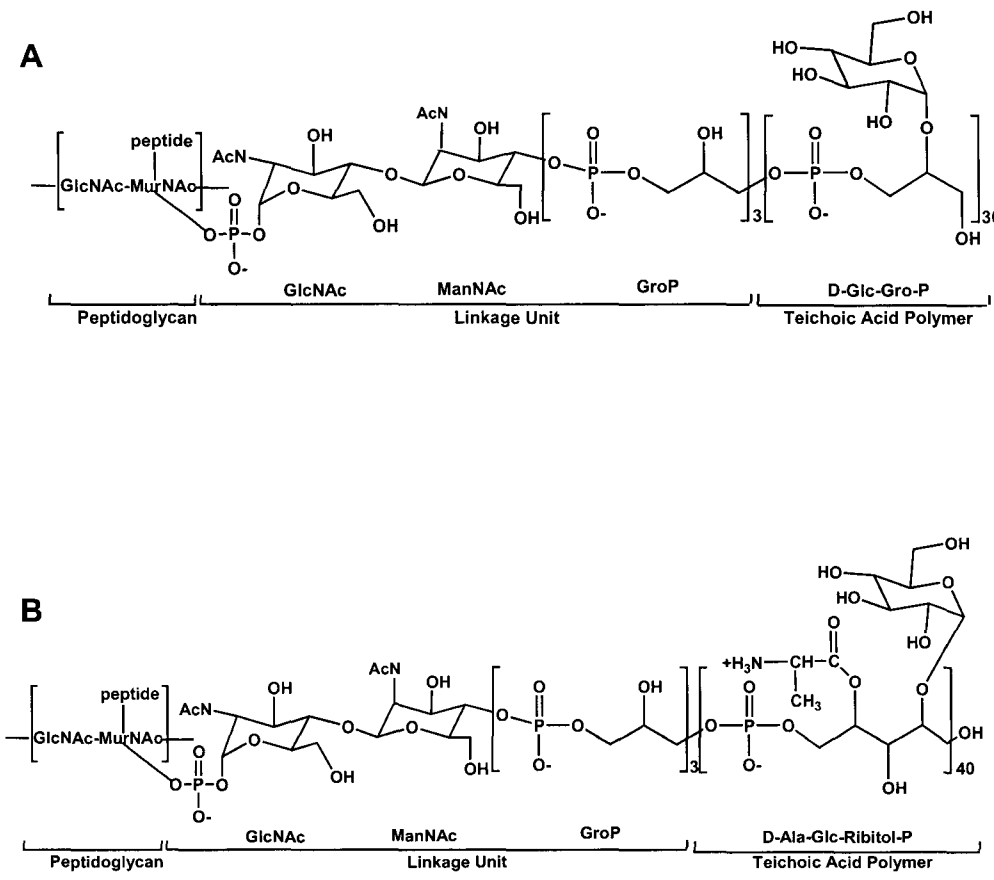


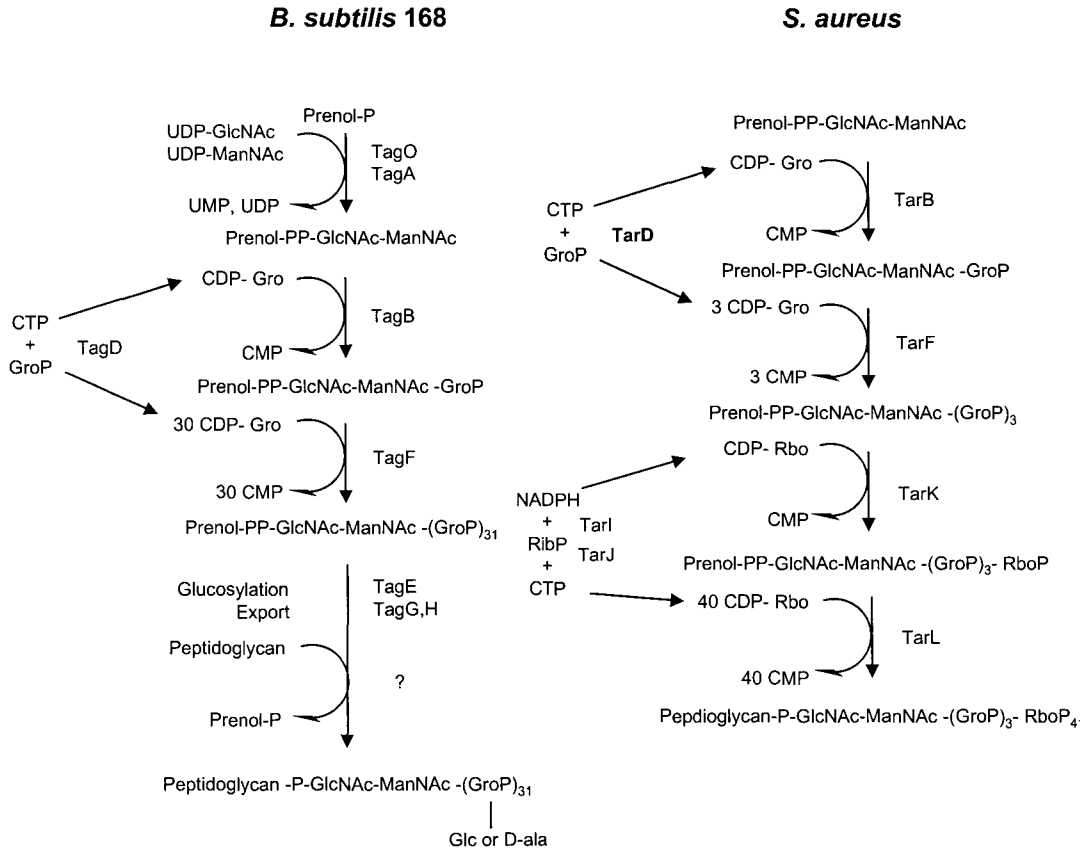
Figure 1.2 Structure of Teichoic Acid from *B. subtilis* and *S. aureus*. Teichoic acid polymer is attached to the 6-hydroxyl of *N*-acetyl muramic acid via a conserved linkage unit. This figure contrasts the glycerol 3-phosphate polymer of *B. subtilis* 168 (A) to the ribitol 5-phosphate polymer of *S. aureus* (B). Although the teichoic acid polymers may differ from organism to organism, the linkage unit always consists of a disaccharide and two to four glycerol 3-phosphate residues.

It had long been thought that teichoic acid was a non-essential structure; however, a number of studies have demonstrated that this chemically diverse anionic polymer is indeed required for cell growth (Briehl, Pooley et al. 1989; Mauel, Young et al. 1989; Mauel, Young et al. 1991; Pooley, Abellan et al. 1991). A number of temperature sensitive mutations were generated by chemical mutagenesis in teichoic acid genes including *tagD*, *tagB* and *tagF* (Briehl, Pooley et al. 1989; Mauel, Young et al. 1991) (biochemical roles discussed in Section 1.4.2). Other studies have been published that have attempted to demonstrate the essentiality of specific enzymes involved in teichoic acid biosynthesis in *B. subtilis* and *S. epidermidis* (Mauel, Young et al. 1989; Lazarevic and Karamata 1995; Fitzgerald and Foster 2000). In these experiments, insertional mutagenesis was attempted to generate teichoic acid mutants but proved impossible. Though its precise function has only been speculated, teichoic acid has been implicated to have a role in cell division in *B. subtilus* 168 (Archibald, Hancock et al. 1993; Bhavsar, Beveridge et al. 2001), helping to maintain the proton gradient across the bacterial membrane, and cell wall turnover by serving as a binding site for autolysins (Pooley and Karamata 1994). Because of its essential nature, this structure and the biosynthetic machinery that constructs it presents itself as a particularly interesting target for antimicrobials.

1.4.2 Genetic Components of Teichoic Acid Biosynthesis

The genetic components involved in the production of poly(glycerol 3-phosphate) teichoic acid in *B. subtilis* 168 are organized in the *tag* (teichoic acid glycerol phosphate)

gene cluster (Mauel, Young et al. 1991). In *B. subtilis* W23 and *S. aureus* these genetic components are responsible for the production of the poly(ribitol 5-phosphate) teichoic acid in these organisms are called *tar* (teichoic acid ribitol phosphate) genes (Scheme 1.1). In strain 168, the genes are organized divergently with *tagDEF, tagAB* and *tagGH* grouped together (Mauel, Young et al. 1991). It is thought that *tagO*, which is located in an area remote from the *tag* cluster, is the *N*-acetyl glucosamine phosphate transferase. Genes *tagA* and *tarA* have homology to the *N*-acetyl mannosamine transferase and their gene products probably catalyze the addition of the final sugar residue of the linkage unit (Abellan 1996). For the completion of the linkage unit, TagB and TarB have been considered (based on sequence homology) candidates for primases that catalyze the addition of one glycerol 3-phosphate residue to the linkage unit (Abellan 1996). In *B. subtilis* 168, TagF has been shown to be the teichoic acid polymerase and is responsible for the addition of the rest of the glycerol 3-phosphates of the polymer (Abellan 1996). In organisms that possess a ribitol 5-phosphate techoic acid polymer, however, TarF is only responsible for addition of enough glycerol 3-phosphate residues as to complete the linkage unit. The addition of the first ribitol 5-phosphate residue is thought to be carried out by TarK (the ribitol 5-phosphate primase) while polymerization of this particular teichoic acid polymer is carried out by TarL (Abellan 1996). TagG and TagH are similar to ATP-binding cassette transport proteins and therefore are thought to transport teichoic acid to the exterior surface of the cell. The two major constituents in these syntheses are glycerol 3-phosphate and ribitol 5-phosphate. These two moieties must be activated through a nucleotidylyltransferase reaction with CTP before being introduced into



Scheme 1.1 Genetic Components Involved in Teichoic Acid Biosynthesis.

teichoic acid. The source of ribitol 5-phosphate for the cytidylyltransferase reaction is from ribulose 5-phosphate (RibP). Ribulose 5-phosphate is first reduced, with NADPH, to ribitol 5-phosphate by TarJ (Abellan 1996). Then, ribitol 5-phosphate is converted to CDP-ribitol (CDP-Rbo) by TarI (Abellan 1996). Genes *tarI* and *tarJ* possess homology to the bifunctional enzyme BscI found in *Haemophilus influenzae* (Zolli 2001). A summary of these pathways is found in Scheme 1.1.

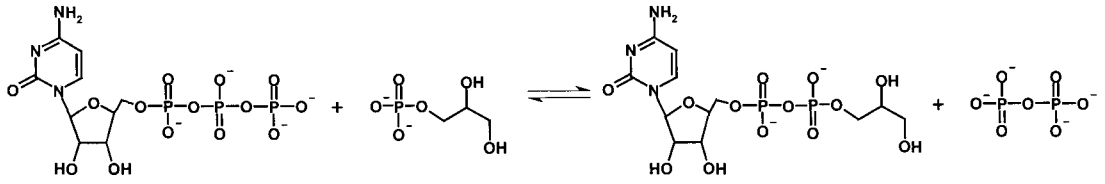
The gene identified as a CTP:glycerol 3-phosphate cytidylyltransferase , called *tagD,* was first classified by analysis of *B. subtilis* cell extracts of a strain possessing the temperature sensitive mutation, *tag-11* (Pooley, Abellan et al. 1991). The temperatrue sensitive mutants were created through chemical mutagenesis and subsequent screening for bacteriophage ϕ29 susceptibility (ϕ29 use teichoic acid for attachement) and the inability to grow at 47 °C (Briehl, Pooley et al. 1989). With a decrease in total cell CDP-glycerol at the non-permissive temperature, it was deduced that the gene in which the mutation was generated, *tagD*, encoded for the CTP:glycerol 3-phosphate cytidylyltransferase. Another temperature sensitive mutant was generated (*tag-12*) which was also mapped to *tagD* (Briehl, Pooley et al. 1989). The sequence of this mutant version of *tagD* indicates that there is an Ile-81 to Asn alteration that is located in a region of the protein that is remote from the active site (data not shown). This temperature sensitive mutant was used in studies where *in trans* complementation of *tag-12* was demonstrated at the non-permissive temperature with *tagD* under the control of xylose at the *amyE* locus (Bhavsar, Zhao et al. 2001). Most recently, a precise deletion of *tagD* in the *tag* locus with a complementing copy of *tagD* under xylose control was

constructed to examine the dispensability of TagD in a defined genetic background and at physiologically relevant temperature (Bhavsar, Beveridge et al. 2001). Controlled deletion of TagD in this strain led to defects in shape, septation and division ultimately followed by cell lysis (Bhavsar, Beveridge et al. 2001). Although these studies have primarily focussed on *B. subtilis* strains, there has been documentation describing the role teichoic acid has in cell wall structure of Gram-positive cocci (Chatterjee, Mirelman et al. 1969; Park, Shaw et al. 1974; Horne and Tomasz 1993). These studies, in addition to the demonstration of the essentiality of *tagF* from *S. epidermidis* (Fitzgerald and Foster 2000), suggest that wall teichoic acid has an essential role in a many Gram-positive pathogens including *S. aureus*. TarD, the CTP:glycerol 3-phosphate cytidylyltransferase of *S. aureus* is the primary focus of this thesis. Of particular relevance to the work here on *S. aureus* TarD, is the presence of glycerol 3-phosphate in the linkage unit despite the existence of ribitol 5-phosphate in the teichoic polymer of *S. aureus*. Hence, TarD has a central role in the activation of glycerol 3-phosphate for incorporation into the linkage unit. Indeed, from genetic data published using *B. subtilis* and a variety of *Staphylococcal* strains and from the role TarD must play in the cell, an essential role for this enzyme seems extremely likely. The preliminary study of this enzyme required for teichoic acid polymer production in the therapeutically relevant *S. aureus* will be the foundation for the design of inhibitors and/or alternate substrates in future endeavors.

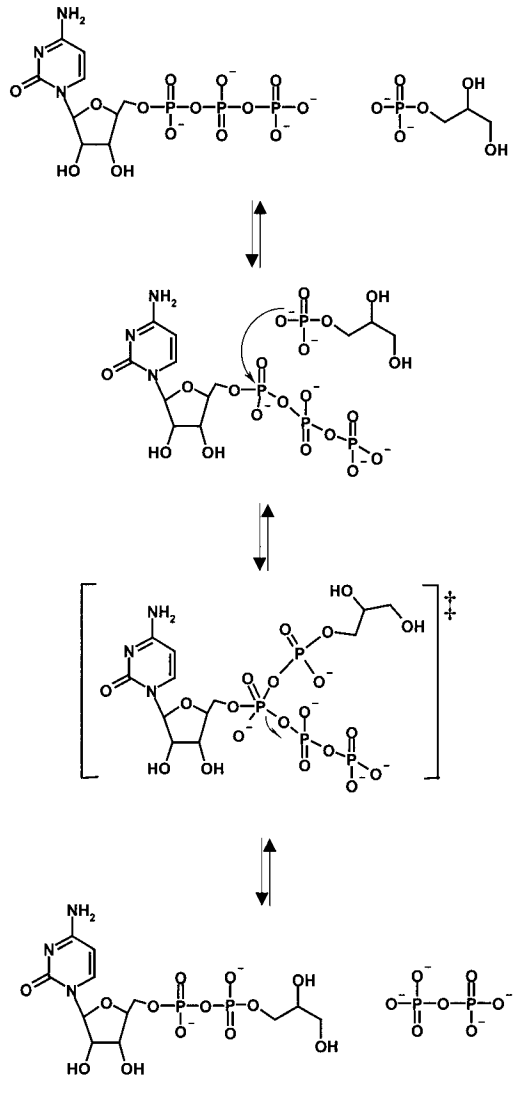
1.5 CTP:glycerol 3-phosphate Cytidylyltransferase – TagD and TarD

TagD is responsible for the production of a nucleotide-activated form of glycerol 3-phosphate, called CDP-glycerol (Scheme 1.2). CDP-glycerol is then used for the production of the linkage unit and, in *B. subtilis* 168, the teichoic acid polymer. The proposed reaction mechanism for this enzyme, where a pentacoordinate phosphate transition state is proposed, is depicted in Scheme 1.3 (Park, Gee et al. 1997).The group led by Claudia Kent has published quite extensively on this enzyme. In addition to important steady state kinetic parameters, this laboratory has established a reaction mechanism for TagD (Park, Sweitzer et al. 1993). The mechanism was inferred from parallel lines obtained in steady state experiments. Though this normally indicates a ping-pong mechanism, with the product inhibition evidence presented by Kent, where each product was a competitive inhibitor with respect to both substrates, a rapid equilibrium random mechanism coupled with negative cooperativity in the binding of substrates was proposed.

To elucidate amino acids important for catalysis, Park *et al* have made a number of mutations in *tagD* (Park, Gee et al. 1997). Based on the results of this mutagenesis study, a HXGH motif was found to be important for activity. This motif is also found in class I aminoacyl-tRNA synthetases and has, in part, been used as a signature for the inclusion of enzymes in the cytidylyltransferase superfamily (or the TagD superfamily) (Bork, Holm et al. 1995). Weber *et al* have solved the structure of TagD with bound CTP to a resolution of 2.0 Å (Weber, Park et al. 1999). This co-crystal structure has served to further illustrate the importance of both the HXGH motif and a RTXGISTT motif in nucleotide binding.



Scheme 1.2 Reaction Catalyzed by TarD and TagD



Scheme 1.3 Reaction Mechanism of TarD and TagD

Most recently, the Kent lab used the intrinsic fluorescence of the three tryptophans of TagD to propose a model where all active sites of the TagD homodimer must be loaded with substrates in a negatively cooperative manner before catalysis can occur (Sanker, Campbell et al. 2001). Furthermore, to illustrate the negative allosteric effects on TagD during CTP binding, NMR spectroscopy was employed (Stevens, Sanker et al. 2001). In these experiments, different structural states of the enzyme were observed as this substrate bound to saturation.

Because of the therapeutic relevance of *S. aureus*, we have focussed our attention to the CTP:glycerol-3-phosphate cytidylyltransferase found in this bacterium. Despite originating from different bacteria, there is great similarity between TarD and TagD. In terms of size, both enzymes are relatively small. TagD is 129 amino acids in length while TarD is slightly larger at 132 amino acids. There is 69 % (89/128) identity and 86 % (112/128) similarity between the two amino acid sequences of these related enzymes. Additionally, by analysis of the structure of TagD, it was observed that 11 of the 12 amino acids in TagD that make interactions with CTP (within 3.5 Å) (Thr-9, His-14, His-17, Lys-46, Gly-92, Arg-113, Thr-114, Ile-117, Ser-118, Thr-119, Thr-120) are identically conserved in TarD. The one amino acid not identical in TarD is a conservative substitution (Phe-10 to Tyr-10). Despite their sequence similarity, the work in this thesis has uncovered some very interesting differences in reaction mechanism and physical properties between these enzymes.

1.6 Thesis Accomplishments

Although there is much work already published on TagD, neither this enzyme nor its homologue, TarD, have been studied as potential targets for therapeutics. To this end, the following was performed:

1. Gene *tarD* was cloned into an over-expression vector and TarD was purified to homogeneity.
2. Steady state and product inhibition experiments were performed in order to discern the reaction mechanism of TarD.
3. The reverse reaction catalyzed by TarD was analyzed in order to calculate the equilibrium constant of the reaction.
4. The integrational plasmid pSWEET was used to determine that *tarD* can complement a temperature sensitive mutation in *tagD* of *B. subtilis* 168 (*tag-12*).

CHAPTER 2 – CLONING AND PURIFICATION OF TARD

2.1 Introduction

In order to study the enzymatic properties of TarD, it was necessary to clone, over-express and purify relatively large amounts of enzyme. This was accomplished using an *E. coli* over-expression strain (W3110) along with a pKK223-3 derivative serving as the over-expression vector. A method similar to the one described below was employed by Kent's laboratory for the purification of TagD (Park, Sweitzer et al. 1993).

2.2 Materials

Restriction enzymes, T4 DNA ligase, and VENT polymerase were purchased from New England BioLabs (Beverly, MA). *N*-2-Hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), dithiothreitol (DTT), ampicillin (AMP), and isopropyl-β-thiogalactopyranoside (IPTG) were obtained from BioShop Canada Inc. (Burlington, ON). Ethylenedinitrilo tetraacetic acid disodium salt dihydrate (EDTA) was purchased from EM Science (Gibbstown, NJ) and ammonium sulfate was obtained from BDH Inc. (Toronto, ON). All other compounds were purchased from Sigma (Oakville, ON) unless specified.

2.3 Methods

2.3.1 Construction of TarD Over-expression Plasmid

All plasmids were propagated in *Escherichia coli* Novablue cells (Novagen, Madison, WI) (Table 2.1). The gene *tarD* was amplified from *S. aureus* genomic DNA using primers: DB01 and DB02 (Table 2.2). The 5' primer (DB01) incorporates silent mutations in *tarD* eliminating two *Nde*I sites found at positions 18 and 27. The blunt-ended product was inserted at the *Eco*RV restriction site of pBluescript SK (-/+) (Stratagene, LaJolla, CA). Subsequently, *tarD* was excised from pBluescript using *Nde*I and *Hin*dIII and inserted into the backbone of pBF-9, a pKK223-3 expression vector derivative described in (Daigle, Hughes et al. 1999), which places the gene under the contol of the *tac* promoter. This construct, called pMZ-*tarD* (Table 2.1), was confirmed by sequencing (MOBIX, McMaster University). Competent *E. coli* W3110 cells (Table 2.1) were transformed using established methods (Sambrook, Fritsch et al. 1989) and ultimately used for the over-expression and purification of TarD.

2.3.2 Over-expression and Purification of TarD

The protein purification method described below is a modification of the procedure found in (Park, Sweitzer et al. 1993). The over-expression strain (EB58) was grown on Luria-Bertani (LB)-agar supplemented with AMP (50 μg/mL). Single colonies were used to inoculate 50 mL of LB containing 50 μg/mL AMP. These cultures were grown overnight at 37 °C shaking at 250 revolutions per minute (rpm). For the large-scale purification of TarD, 10 mL of the overnight cultures were used to inoculate 1 L of LB containing 50 μg/mL ampicillin. Cells were grown at 37 °C shaking at 250 rpm until an optical density (600 nm) of approximately 0.8 was attained. At this time, the cultures

Table 2.1 List of Strains and Plasmids

Name	Description	Source
Bacillus subtilis		
EB4	*hisA1 argC4 metC3 tag-12ϕ29^r*	Bacillus Genetic Stock Centre (L6602)
EB6	*hisA1 argC4 metC3*	Bacillus Genetic Stock Centre (L5087)
EB164	*hisA1 argC4 metC3 amyE::xylR* P_{xylA} *tarD cat86*	This work
EB171	*hisA1 argC4 metC3 tag-12ϕ29^r amyE::xylR* P_{xylA} *tarD cat86*	This work
EB215	*hisA1 argC4 metC3 tag-12ϕ29^r amyE::xylR* P_{xylA} *tarD cat86*	This work
EB249	*hisA1 argC4 metC3 amyE::xylR* P_{xylA} *tarD cat86*	This work
Escherichia coli		
Novablue	*endA1 hsdR17 ($r_{K12}^-m_{K12}^+$) supE44 thi-1 recA1 gyrA96 relA1 lac[F'proA$^+$B$^+$ lacI^{q}ZΔM15::Tn10*	Novagene
W3110	wild type *E. coli* – over-expression strain	Wright Lab (McMaster University)
EB58	W3110 with pMZ - *tarD*	Michela Zolli (Brown Lab, McMaster University)
Plasmids		
pMZ - *tarD*	pKK223-3 derivative[a] over-expression vector for TarD	Michela Zolli (Brown Lab, McMaster University)
pSWEET-*bgaB*	*B. subtilis* vector for integration at *amyE* with *xyl* expression system, *bgaB* from *B. stearothermophilus* and polylinker	Amit Bhavsar (Brown Lab, McMaster Univeristy)
pSWEET-*tarD*	*B. subtilis* vector for integration at *amyE* with *xyl* expression system, *tarD* from *S. aureus* and native *tagD* RBS	This work
pSWEET-*tarD**	*B. subtilis* vector for integration at *amyE* with *xyl* expression, system, *tarD* from *S. aureus* and consensus RBS[b]	This work

[a](Daigle, Hughes et al. 1999)
[b](Vellanoweth and Rabinowitz 1992)

Table 2.2 List of Oligonucleotides

Oligonucleotide	Sequence	Restriction site(s)
DB01[a]	5'-CCGGGCCATATGAAACGTGTAATAACCTATGGCACC TATGACTTACTTC-3'	*Nde*I
DB02[a]	5'-CGCGCGGATCCTTATTTAGCATCTTTACCA-3'	*Bam*HI
DB03[a,b,c]	5'-CACCTTAATTAACATTAG**GAAGGAGCG***TTTCTTTAA* ATGAAACGTGTAA TAACATATGGCACATATGACTTA-3'	*Pac*I
DB04[a,b,c]	5'-CACCTTAATTAACATTAG**TAAGGAGG***TTTCTTTA* ATGAAACGTGTAATAACATATGGCACATATGACTTA-3'	*Pac*I
DB05[a]	5'-GACGGGATCCTTATTTAGCATCTTTACCATATAATTC-3'	*Bam*HI

[a]The underlined sequences indicate the restriction sites
[b]The bold sequences represent the ribosome binding site
[c]The sequence in italics represent the spacer region

were induced for 5 hrs at 37 °C with IPTG (1 mM). The cells were harvested by centrifugation (5,000 × *g*) for 10 min at 4 °C using a Beckman Avanti J-25 centrifuge (Beckman Coulter Inc., Palo Alto, CA). The cell pellet was washed with 0.85 % NaCl (4 °C) and pelleted again at 5,000 × *g* for 10 min. Cell paste was either frozen at –20 °C or lysed immediately. At this point, all steps were performed on ice or at 4 °C. The cell pellets were re-suspended in lysis buffer (25 mM HEPES (pH 8.0), 1 mM DTT and 5 mM EDTA) and lysed by two or more passes through a French Press (20,000 psi). To remove cell debris, the lysed cells were centrifuged (50,000 × *g*) for 1 hour at 4 °C.

Chromatography was performed on an AKTA fast-protein liquid chromatography (FPLC) system (Amersham-Pharmacia) in a cold room at 4°C. The FPLC absorbance detector, set for detection at 280 nm, monitored eluted protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze fractions at all steps of this procedure. The cleared lysate was loaded onto a 50 mL (2.5 x 10 cm) Q Sepharose Fast Flow column (Amersham-Pharmacia) which was equilibrated at 5 mL/min with buffer containing 25 mM HEPES (pH 8.0), 1 mM DTT and 5 mM EDTA. After unbound protein was washed from the column in 5 column volumes (C.V.), a linear salt gradient, from 0 to 0.5 M NaCl over 10 C.V. was used to elute protein in 10 mL fractions. Subsequently the concentration of salt was held at 0.5 M for 4 C.V., raised to 1 M over 2 C.V. and held there for 4 C.V. The column was then re-equilibrated for 4 C.V.. TarD was eluted from this column at a salt concentration of approximately 250 mM, which is approximately 5 C.V. into the gradient. Appropriate fractions were pooled and dialyzed against buffer containing 25 mM HEPES (pH 8.0), 10 mM $MgCl_2$, and 1 mM

DTT for 12 to 24 hours. Alternatively, a buffer exchange was carried out using centrifugal concentrators with a molecular weight cut-off of 5 to 10 kDa (Amicon, Fisher Scientific, Napean, ON).

This protein was loaded onto a 30 mL (2.5 x 6.5 cm) Reactive Blue 4 column (Sigma). The running buffer for this column was identical to the dialysis buffer. After injection, the column was washed for 5 C.V. A step-wise elution with CTP (1 mM) was used to specifically remove TarD from this column in 7 mL fractions at flow rate of 1 mL/min. The CTP was kept at 1 mM for 10 C.V. TarD started to elute from the Reactive Blue column 20 to 30 mL after CTP was introduced. In order to concentrate the fractions collected from the Reactive Blue 4 column, ammonium sulfate was slowly added to the pooled fractions until a concentration of 80 % was obtained. The protein was pelleted by centrifugation (10,000 × *g*) for 1 hour at 4 °C.

The protein pellet was resuspended in 2 to 5 mL of buffer (25 mM HEPES (pH 8.0), 1 mM DTT) then applied to a 130 mL (1.6 x 63.5 cm) Superdex-200 (S200) or S75 gel filtration column (Amersham-Pharmacia) previously equilibrated at 0.5 mL/min with buffer identical to that used for the resuspension of TarD. TarD started to elute from the gel filtration column after approximately 0.5 C.V. Fractions (1.5 mL), collected over 1.2 C.V., containing pure TarD were pooled, frozen in small aliquots, and stored at –80 °C. The gel filtration column step, in addition to eliminating one contaminating protein band, (Figure 2.1) is required to remove CTP and $MgCl_2$ used to elute TarD from the Reactive Blue 4 affinity column.

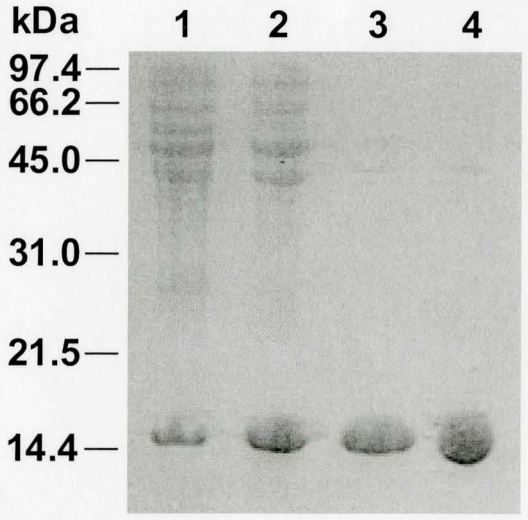


Figure 2.1 Purification of Wild Type TarD. Protein samples at each step of the purification of TarD were analyzed by discontinuous SDS-PAGE using a 3 % stacking and a 15 % separating polyacrylamide gel visualized with Coomassie Blue stain. Approximately 10 μg of protein were loaded in each lane. Samples were boiled for 5 minutes in 2X loading buffer (62.5 mM TRIS-HCl (pH 6.8), 2 % (w/v) SDS, 10 % (v/v) glycerol, 5 % (v/v) 2-mercaptoethanol, 0.05 % (w/v) bromophenol blue). *Lane 1*, cleared lysate; *lane 2*, Q Sepharose ion-exchange column; *lane 3*, Reactive Blue 4 affinity column; *lane 4*, S200 gel filtration column pool.

2.3.3 Determination of Protein Concentration

Protein concentration was determined by methods described by Bradford (Bradford 1976); however, for samples of pure protein the procedure outlined by Gill and von Hippel (Gill and von Hippel 1989) was used. In this method, protein concentration is determined using the calculated molar absorptivity and measuring the absorbance of the protein denatured with 6 M guanidinium hydrochloride at a wavelength of 280 nm.

2.4 Results and Discussion

A three-column procedure was employed to purify TarD to homogeneity from cleared *E. coli* lysate. A clear single band representing pure TarD at 16 kDa is depicted in lane 4 of Figure 2.1. This procedure yielded approximately 15-30 mg of purified enzyme (48 % yield) from each 1 L culture, which ultimately was purified 4.4-fold. The purification of TarD is summarized in Table 2.3

Table 2.3 Purification Table for Wild Type TarD[a]

Step	Volume (mL)	[Protein] (mg/mL)	Total Protein (mg)	Total Activity (μmol/min)	Specific Activity (μmol/min. mg)	Yield (%)	Purification (-fold)
Cleared Lysate	32.0	76	2400	4500	3.2	100	1.0
Q-Sepharose	225.0	1.9	430	3300	9.8	74	3.1
Reactive Blue 4	5.6	27	150	2300	15	52	4.8
S75 Gel Filtration	45.0	3.3	150	2100	14	48	4.4

[a]Describes the purification of TarD from 5L of cell culture.

CHAPTER 3 – REACTION MECHANISM OF TARD

3.1 Introduction

The steady state characterization has been published for the TarD homologue, TagD (Park, Sweitzer et al. 1993), and is described in detail in Sections 1.4 and 1.5. This enzyme has been shown to catalyze the cytidylyltransferase reaction via a random rapid equilibrium mechanism as a homodimer, where there is negative cooperativity in substrate binding but not catalysis (Park, Sweitzer et al. 1993; Sanker, Campbell et al. 2001). Moreover, TagD was reported to possess millimolar K_m values for both substrates (Park, Sweitzer et al. 1993).

With highly pure recombinant TarD, it was necessary to confirm that our pooled enzyme catalyzed the CTP:glycerol 3-phosphate cytidylyltransferase reaction. The product formed was analyzed by mass spectrometry to confirm the presence of CDP-glycerol. Also, to delineate the reaction mechanism for TarD, steady state and product inhibition experiments were performed. In addition to obtaining the kinetic constants for the forward reaction, the reverse reaction was also assayed. With this information, an equilibrium constant was calculated.

3.2 Materials

N-2-Hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), dithiothreitol (DTT), Tris (hydroxymethyl) aminomethane (TRIS), and urea were obtained from

BioShop Canada Inc. (Burlington, ON). Radiolabeled [5-^{3}H] CTP (ammonium salt) (20 Ci/mmol) was purchased from Amersham-Pharmacia (Baie d'Urfé, PQ). Triethylamine (99 %) was purchased from Anachemia Canada Inc. (Montreal, PQ). Pyrophosphate was purchased from Fisher Scientific (Napean, ON). All other compounds were purchased from Sigma (Oakville, ON) unless specified.

3.3 Methods

3.3.1 Development of the TarD HPLC Enzymatic Assay

A stopped assay was used to monitor the reaction catalyzed by TarD. A typical reaction contained 50 mM TRIS-HCl (pH 8.0), 20 mM $MgCl_2$, 1 mM DTT, 125 μM glycerol 3-phosphate, 132 μM CTP, 6.8 μCi [5-^{3}H] CTP (20 Ci/mol) and 1 ng TarD (1.26 nM) in a total volume of 50 μL. After 10 minutes, 150 μL of 8 M urea (6 M final concentration) was added to halt the reaction. The optimal incubation time and enzyme amount was determined from the experiment summarized in Figure 3.3.

The amount of [5-^{3}H] CDP-glycerol produced in the reactions was monitored by high performance liquid chromatography (HPLC) (Waters 600 Pump and Controller, Waters 486 Tunable Absorbance Detector, Waters 717 Autosampler, Waters busSAT/IN module, Milford, MA) using a 4.5 × 50 mm WP QUAT strong ion exchange column (J.T. Baker, Phillipsburg, NJ) and an in-line Radiomatic 150TR flow scintillation analyzer (Canberra Packard Canada, Mississauga, ON) set to an analogue full scale of 100,000 with five point smoothing. The scintillation cocktail (Ultima-Flo M, Canberra Packard Canada, Mississauga, ON) was pumped at a flow rate of 6 mL/min. The HPLC software

was set to a sampling rate of 1 s^{-1} and to a scaling factor of 250 mV. After injection of the sample onto to the column (180 μL), reaction components were separated using the following eluates: water (A); and 0.5 M triethylamine bicarbonate (TEAB) (B). The following parameters were used for elution of material from the ion exchange column (2 mL/min): 2 min. at 4 % B; a linear gradient of 4 to 100 % B in 6 min.; 1 min. linear gradient of 100 to 4 % B; and re-equilibration for 4 min. at 4 % B. The amount of [5- ^{3}H] CDP-glycerol produced was determined by integration of the resolved peaks using the HPLC software (Millenium[32], Waters).

3.3.2 Confirmation of Product Production

To confirm that CDP-glycerol was the product of the enzyme-catalyzed reaction, its identity was confirmed by molecular mass analysis using negative ion electrospray mass spectrometry (Micromass LC quadrupole). Separated reaction material was collected from the ion exchange column described in Section 3.3.1 using HPLC with detecton set at 271 nm (Waters 600 Pump and Controller, Waters 486 Tunable Absorbance Detector, Milford, MA). The program for separation of material used here was identical to that used in Section 3.3.1. The purified sample was prepared for mass spectrometry by removal of TEAB using a Savant SC210A Speed-Vac Plus (Halbrook, NY).

3.3.3 Oligomerization of TarD

A 24 mL analytical S200 gel filtration column (Amersham-Pharmacia) was used to determine the oligomeric state of TarD. The elution volumes of the standards (Sigma) and TarD were determined using the FPLC software (UNICORN, Amersham Biosciences Inc., Piscataway, NJ). The running buffer contained 50 mM HEPES (pH 7.5) and 1 mM DTT. In experiments where substrates were included (1 mM CTP and 1 mM glycerol 3-phosphate), the buffer also contained 6 mM $MgCl_2$. To better describe the hydrodynamic properties of TarD under the conditions of gel filtration described in this section, the Stokes radius was determined from comparison to the elution of standards for which Stokes radii are known (carbonic anhydrase, 2.0 nm; β-amylase, 5.4 nm; alcohol dehydrogenase, 4.6 nm; bovine serum albumin, 3.5 nm (Siegel and Monty 1966)). To calculate the frictional ratio (f/f_o) equation 1 (Brown 1992) was used.

$$f/f_0 = r_s / (3\nu m / 4\pi N_A)^{1/3} \qquad \text{(Eq. 1)}$$

r_s is Stokes radius of the protein, ν is the partial specific volume of the protein (a value of 0.725 ml/g was used which is an average value for soluble proteins), m is the calculated molecular mass, and N_A is Avogadro's number. The value f is defined as the translational frictional coefficient and can be used to provide information on conformation. More convenient is the use of the dimensionless f/f_o value where f_o is the frictional coeifficient of the biomolecule with an *anhydrous* mass and density (Harding and Jumel 1998). The Perrin factor describes the shape and flexibilty of a hydrated biomolecule. To determine the Perrin factor (P) equation 2 was used (Harding and Jumel 1998).

$$P = (f/f_o)\,(1+ \delta /vp_0)^{-1/3} \qquad \text{(Eq. 2)}$$

The degree of hydration is described by δ (average value of 0.35 ml/g for a soluble protein used (Brown 1992)) and p_0 is the density of the solvent (value of 1 used).

3.3.4 Bi-substrate Initial Velocity Experiment

Bi-substrate experiments were all performed at room temperature (22 °C) as described above. The concentrations of glycerol 3-phosphate ranged from 10 to 125 μM while the CTP concentration ranged from 17 to 132 μM. Data were fitted to equations that described two different enzyme reaction mechanisms: the sequential (ternary complex) mechanism equation (equation 3); and the ping-pong (covalent intermediate) mechanism equation (equation 4) using the GraFit 4 software package (Erithacus Software Ltd., UK). This was accomplished using non-linear regression methods.

$$v = V_{max}[A][B] / (K_{ia}K_{mb} + K_{mb}[A] + K_{ma}[B] + [A][B]) \qquad \text{(Eq. 3)}$$

$$v = V_{max}[A][B] / (K_{mb}[A] + K_{ma}[B] + [A][B]) \qquad \text{(Eq. 4)}$$

K_{ma} and K_{mb} are the Michaelis constants for A and B respectively. V_{max} is the maximum velocity and K_{ia} is defined as the dissociation constant of A from the binary complex, EA (Cleland 1970).

3.3.5 Product Inhibition Studies

CDP-glycerol, required for product inhibition studies, was produced enzymatically. The following conditions were used to produce CDP-glycerol in large scale: 50 mM TRIS (pH 8.0), 20 mM $MgCl_2$, 1 mM DTT, 30 mM glycerol 3-phosphate, 30 mM CTP, 0.02 units/μL inorganic pyrophosphatase and 5.3 μM TarD. The progress of the reaction was monitored by detection at 271 nm using HPLC. After the completion of the reaction, the synthesized CDP-glycerol was filtered through a 5 kDa MWCO Amicon Ultrafree-MC filter (Millipore, Bedford, MA) to remove protein. From this synthesis, 98% of the CTP in the assay had been converted to CDP-glycerol. Subsequently, sample purity was measured to be 93 % CDP-glycerol as determined by HPLC (absorbance at 271 nm). While only 2 % of the absorbance at 271 nm in the preparation was attributed to CTP, 5 % was CDP which is a contaminant in CTP purchased from Sigma. The concentration of the CDP-glycerol was calculated by measurement of the absorbance of the sample at 271 nm using a Molecular Devices SpectraMax spectrophotometer (Sunnyvale, CA) (ε for cytidine-phosphate containing molecules is 9,100 $M^{-1}cm^{-1}$ at 271 nm), taking the proportion of CDP-glycerol in the preparation into account.

The concentration of CDP-glycerol, used as an inhibitor, ranged from 0 to 1.2 μM while the concentration of pyrophosphate (PP_i) ranged from 0 to 5 mM. The reaction conditions for these experiments were as follows: 45 mM TRIS-HCl (pH 8.0), 18 mM $MgCl_2$, 0.9 mM DTT and 6.8 μCi [5-^{3}H] CTP. When glycerol 3-phosphate was the varied substrate, CTP was kept at approximately K_m concentration (57 μM). When CTP was the

varied substrate, glycerol 3-phosphate was held at 24 μM, which is also near its K_m. Data were fitted to all inhibition equations in the Enzymes Module of Sigma Plot 2000 (SPSS Inc., Chicago, IL). Ultimately, only the equations for partial competitive inhibition (equation 5) and mixed (non-competitive) inhibition (equation 6) were used for non-linear regression.

$$v = V_{max} / (1 + K_m[S])(1 + [I] / K_{is}) / (1 + [I] / \alpha K_{is}) \quad \text{(Eq. 5)}$$

$$v = V_{max} / (K_m / [S])(1 + [I] / K_{is}) + (1 + [I]/ K_{ii}) \quad \text{(Eq. 6)}$$

K_m is the Michaelis constant for the varied substrate, while K_{is} and K_{ii} are the inhibition constants for the affect the inhibitor has on the slope and intercept (respectively) of double reciprocal plots. The constant α is the factor by which the inhibition constant (K_{is}) changes when the inhibitor binds to the enzyme substrate complex (Segal 1975). To confirm the proposed reaction mechanism, an experiment with varying CTP (17 to 132 μM), pyrophosphate as the inhibitor (0 to 3 mM) and glycerol 3-phosphate at approximately 100 times the K_m concentration (2.4 mM) was performed.

3.3.6 Analysis of the Reverse Reaction of TarD

[5-^{3}H]CDP-glycerol, required as substrate for the reverse reaction, was prepared enzymatically utilizing [5-^{3}H]CTP and an equimolar amount of glycerol 3-phosphate. The reaction was carried out in 50 mM TRIS-HCl (pH 8.0), 20 mM $MgCl_2$, 1 mM DTT, 0.13 units/μL inorganic pyrophosphatase and 62.3 μM TarD. The concentration of

glycerol 3-phosphate and [5-^{3}H] CTP (20 Ci/mmol) was 26 μM (200 μCi of labeled CTP). Because the reaction was carried out to near completion (greater than 97 %), the specific activity of the radiolabeled CDP-glycerol was approximately equal to that of the [5-^{3}H] CTP (20 Ci/mmol). [CDP-glycerol] ranged from 0.25 to 5 μM and [pyrophosphate] was varied from 1 to 10 mM. The amount of [5-^{3}H] CDP-glycerol added into the reaction ranged from 0.25 to 0.68 μCi. The production of CTP was monitored by HPLC as in the analysis of the forward reaction. The data were also fitted to equation 3. With the appropriate kinetic constants established, equation 7 was used to calculate the equilibrium constant (K_{eq}) of this reaction (Segal 1975).

$$K_{eq} = (V_{maxf} K_{iq} K_{mp}) / (V_{maxr} K_{ia} K_{mb}) \quad \text{(Eq. 7)}$$

V_{maxf} and V_{maxr} are the maximum velocities of the forward and reverse reactions. K_{mb} and K_{mp} are Michaelis-Menton constants and K_{ia} and K_{iq} are dissociation constants, all calculated from equation 3 (using the nomenclature that defines substrates as A and B and products as P and Q). The equilibrium constant was also determined experimentally under to following conditions: 50 mM TRIS-HCl pH 8.0, 20 mM $MgCl_2$, 1 mM DTT 125 μM glycerol 3-phosphate, 132 μM CTP, 6.8 μCi [5-^{3}H]CTP and 1 μM TarD. The progress of the reaction was monitored by anion exchange HPLC as described above.

3.4 Results

3.4.1 Mass spectrometry of CDP-glycerol

When the product of TarD was purified and subjected to negative ion electrospray mass spectrometry (Figure 3.1), a molecular weight of 475.7 was obtained. This value is near the calculated molecular weight of CDP-glycerol of 475.3. This confirms that the peak visualized by the HPLC software is indeed CDP-glycerol and that it was appropriate for this peak to be measured in order to monitor the cytidylyltransferase.

3.4.2 Oligomerization of TarD

The oligomeric state of TarD was determined using an analytical S200 gel filtration column. By measuring the elution volume of TarD, the soluble molecular weight of this protein was calculated to be 64-68 kDa (Table 3.1). This implies that TarD is a tetramer. The molecular weight as determined by gel filtration was not altered a great deal by the addition of substrates but when substrates are present there is a slight decrease in calculated molecular weight (63 kDa with 1 mM CTP and 57 kDa with 1mM glycerol-3-phosphate) (Table 3.1). To further describe the oligomeric state of TarD a more detailed analysis of the hydrodynamic properties of TarD was performed. Table 3.2 outlines frictional ratios (f/f_0) for TarD, which depend on protein conformation, flexibility, and degree of solvation. For the assumptions that are inherit in the calculation of this dimensionless ratio refer to Section 3.3.3. From frictional ratios, Perrin factors were calculated to confirm the conformation of TarD and to give an indication that the oligomeric state from gel filtration analysis has meaning and are consistent with the hydrodynamic properties of TarD. The Perrin factor approaches 1.0 as the shape of the protein mimics a sphere in solution. Perrin factors for native TarD, and TarD in the

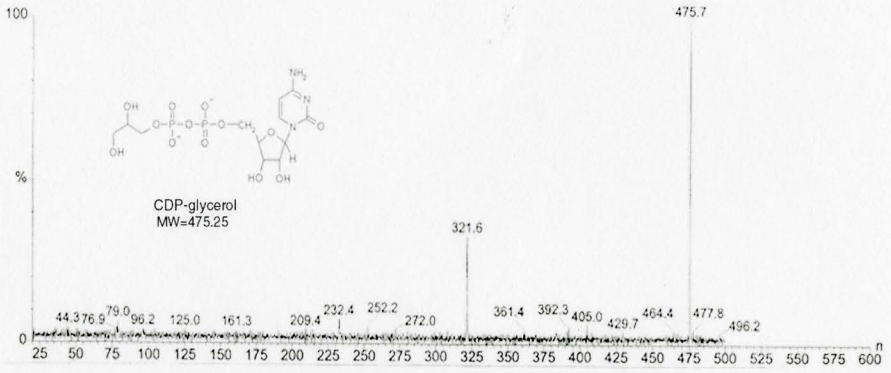


Figure 3.1 Confirmation of Enzymatic Production of CDP-glycerol by Electrospray Mass Spectrometry. CDP-glycerol was purified from a Mono Q column attached to an HPLC (see Section 3.3.1) and concentrated to 10 μM. This sample was diluted 10X and subjected to negative ion electrospray mass spectrometry on a Micromass LC quadropole mass spectrometer. A molecular weight of 475.7 was observed, which is comparable to the calculated molecular mass of CDP-glycerol (475.25). The second peak at 321.6 may represent a fragmentation product. Most likely is that this species is CMP which has a molecular mass of 323.7. Mass spectrometry was performed and interpreted by Dr. Kirk Green, Department of Chemistry, McMaster University.

Table 3.1 Oligomerization of TarD

Conditions	Elution Volumes[a] (mL)	Molecular Weight[b] (kDa)	Stokes Radius[b] (nm)
TarD[c]	13.4	68	3.6
	13.5	64	3.5
TarD – 1 mM CTP[d]	13.5	63	3.5
	13.5	63	3.5
TarD – 1 mM glycerol-3-phosphate[d]	13.7	57	3.3
	13.7	57	3.3

[a]Volumes determined from FPLC software
[b]Determined using standard curve
[c]50 mM HEPES, pH 7.5
[d]50 mM HEPES, pH 7.5, 6 mM $MgCl_2$

Table 3.2 Hydrodynamic Properties of TarD

Conditions	Possible Oligermeric State[a]	Frictional Ratio[b]	Perrin Factor[c]
TarD[d]	Monomer	2.17	1.90
	Dimer	1.72	1.51
	Trimer	1.51	1.32
	Tetramer	1.37	1.20
	Pentamer	1.27	1.11
TarD – 1 mM CTP[e]	Monomer	2.11	1.85
	Dimer	1.68	1.47
	Trimer	1.46	1.28
	Tetramer	1.33	1.17
	Pentamer	1.23	1.08
TarD – 1 mM glycerol-3-phosphate[e]	Monomer	1.99	1.75
	Dimer	1.58	1.39
	Trimer	1.38	1.21
	Tetramer	1.25	1.10
	Pentamer	1.16	1.02

[a]Molecular weight calculated from 1^0 sequence is 15817 g/mol
[b]Calculated from equation 1 using an average Stokes radius for each condition
[c]Calculated from equation 2 using an average Stokes radius for each condition
[d]50 mM HEPES, pH 7.5
[e]50 mM HEPES, pH 7.5, 6 mM $MgCl_2$

presence of CTP or glycerol 3-phosphate were determined in which the oligomeric state was presumed to be from a monomer to a pentamer (Table 3.2). The data displayed in Table 3.2 shows that Perrin factors approach 1.0, indicating a near sphere-like shape, when TarD is presumed to be a tetramer or pentamer (1.20, 1.17 and 1.10 for native TarD, TarD with CTP and TarD with glycerol 3-phosphate, respectively, in a tetrameric state - 1.11, 1.08, and 1.02, for native TarD, TarD with CTP and TarD with glycerol 3-phosphate, respectively, in a pentameric state). The calculation of Perrin factors indicates that TarD is globular (near sphere shaped) as is multimerizes to a tetramer or pentamer. These calculations complement and support the gel filtration results, which indicated that TarD exists as a tetramer in solution. The recently partially solved structure of TarD has four subunits in each asymmetric unit thus providing further confirmation of the oligomeric state of TarD (Yim, Zolli et al. 2001).

3.4.3 Linearity of the HPLC assay

A number of assays were screened in order to assay TarD including the charcoal binding procedure used for TagD (Park, Sweitzer et al. 1993). Many problems were encountered with this system, including low adsorption of nucleotide containing molecules to activated charcoal and non-uniform quenching of scintillation counting, which ultimately resulted in irreproducible data. For this reason, an HPLC assay was used to monitor the cytidylyltransferase reaction. Because the detection using absorbance at 271 nm was insufficiently sensitive to monitor the production of CDP-glycerol in this assay, an in-line radioactivity detector was implemented to measure the shift of tritium

label from [5-^{3}H] CTP to CDP-glycerol. A representative HPLC chromatogram depicting the detection of tritium labeled substrates and products can be found in Figure 3.2.

The linearity of the HPLC assay used in this work was confirmed over a number of enzyme concentrations (Figure 3.3). The velocities at each enzyme concentration were plotted in the inset of Figure 3.3. The slope of this line is equal to the turnover number of the enzyme under these conditions. A turnover of 1.7 s^{-1} was calculated from this experiment, which is comparable to the actual k_{cat} (2.6 ± 0.2, Table 3.3). Under these conditions only 1 to 2% of substrate is being converted to product. From these data, it was decided that utilization of 1 ng of enzyme was most appropriate for all assays.

3.4.4 Bi-substrate Initial Velocity Experiments

Initial velocity experiments were conducted to determine the kinetic mechanism and important kinetic constants for TarD. It is clear from Figure 3.4 that the data generated intersect at a point left of the y-axis. This indicates that TarD catalyzes the formation of CDP-glycerol and pyrophosphate by a sequential (ternary) mechanism as described by Cleland (1970). The reduced χ^2 for the fit of the data to this model was 1.7 × 10^{-5}. When data was fit to the ping-pong mechanism equation, this value almost doubled (3.1 ×10^{-5}). This difference indicates that the data is best fit to the model that describes the formation of a ternary complex. The kinetic constants determined from this experiment are found in Table 3.3. The K_m's for the substrates of TarD were 21 ± 4.1 μM for glycerol 3-phosphate and 36 ± 5.8 μM for CTP, which differs greatly from TagD.

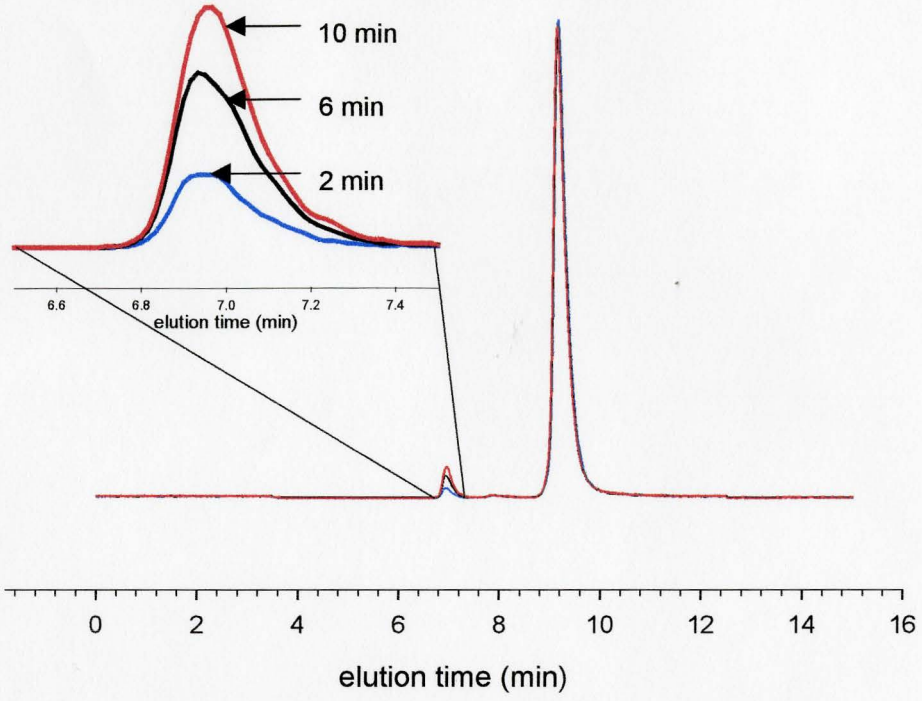


Figure 3.2 HPLC Chromatogram Illustrating Radioactive Detection of CDP-glycerol. A TarD time course is displayed. The highlighted peaks represent [5-^{3}H] CDP-glycerol produced in 2, 6 and 10 minutes measured by the Radiomatic Scintillation Detector (Packard). The larger peaks represent [5-^{3}H] CTP. The conditions were as described in Section 3.3.1. Briefly, 1.26 nM of TarD was used with 125 μM glycerol 3-phosphate and 132 μM CTP. For quantification of CDP-glycerol the integration of these peaks were performed using the Millenium^{32} HPLC software (Waters).

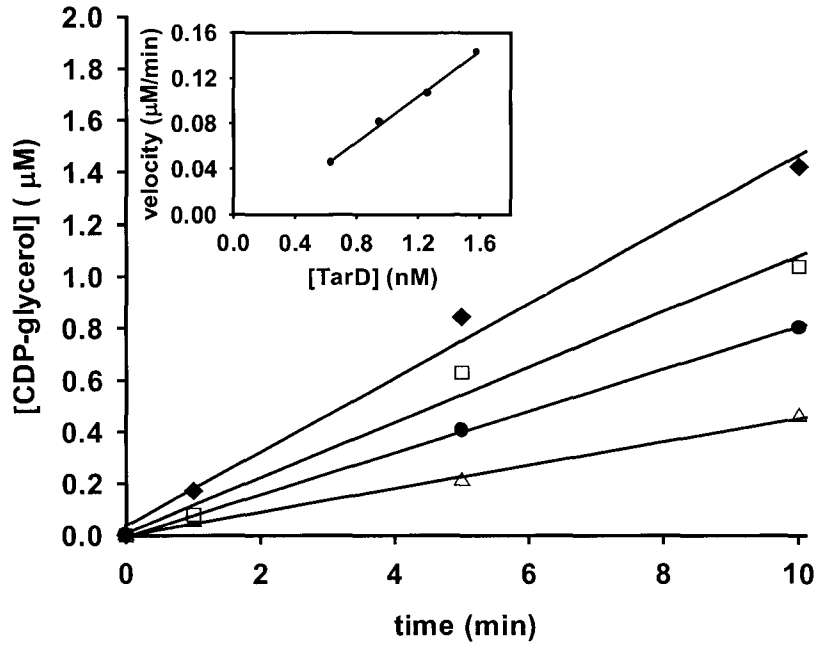


Figure 3.3 Linearity of the TarD HPLC Assay. Reactions with increasing amounts of enzyme over 10 minutes were monitored by HPLC, equipped with an in-line radioactivity detector set to an analogue full scale of 100,000 with five point smoothing. The scintillation cocktail (Ultima-Flo M, Canberra Packard Canada, Mississauga, ON) was pumped at a flow rate of 6 mL/min. The HPLC software was set to a sampling rate of 1 s^{-1} and to a scaling factor of 250 mV. The volume of reaction injected was 180 μL. The amount of enzyme added to the reaction were 1.25 ng (◆), 1.0 ng (□), 0.75 ng (●), and 0.5 ng (△). The concentration of glycerol 3-phosphate and CTP were 125 μM and 132 μM respectively. Lines were drawn using linear regression in Sigma Plot 2000. *Inset*, velocities were plotted *versus* the corresponding enzyme concentration using linear regression. The slope of the line is equal to the turnover number (1.7 s^{-1}).

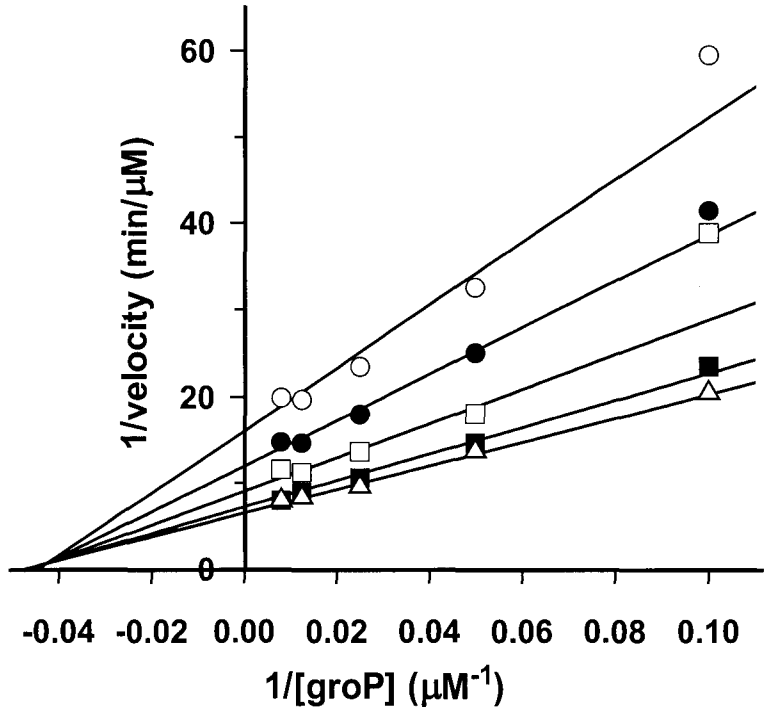


Figure 3.4 Steady State Kinetic Assay of the Forward Reaction. Double reciprocal plots of 1/velocity versus 1/[groP] are shown. The concentration of CTP was fixed at 17 μM (O), 27 μM (●), 47 μM (□), 87 μM (■), and 132 μM (△). The data were fitted by non-linear least squares method to a sequential kinetic model where a ternary complex forms as described by equation 3. The steady state parameters obtained from this bi-substrate analysis are in Table 3.3.

Table 3.3 Summary of Kinetic Constants for the Forward and Reverse Reactions of TarD

	Substrate	K_m (μM)	Dissociation Constant[b] μM	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Forward reaction					
	groP	21 ± 4.1[a]	24 ± 7.8	2.6 ± 0.2	1.3×10^5
	CTP	36 ± 5.8	41 ± 15		7.2×10^4
Reverse reaction					
	CDP-gro	1.4 ± 0.62	0.54 ± 0.38	1.0 ± 0.2	7.1×10^5
	PP_i	9,700 ± 3,800	3,800 ± 3,100		1.1×10^2

[a]Standard error in the value based on data fitted to ternary complex model.
[b]Obtained from equation 3 (K_{ia}).

The *Bacillus* homolgue possesses K_m's in the millimolar range for both substrates (3.9 mM and 3.2 mM for CTP and glycerol 3-phosphate respectively) (Park, Sweitzer et al. 1993). A k_{cat} of 2.6 ± 0.2 s^{-1} was also calculated for TarD. This value is 19-fold lower than the k_{cat} determined for TagD (50 s^{-1}) (Park, Sweitzer et al. 1993).

3.4.5 Product Inhibition Studies

Although initial velocity experiments can discriminate between ping-pong and sequential mechanisms, product inhibition experiments must be performed to precisely ascertain whether the sequential mechanism is random or ordered. Moreover, if the mechanism is ordered, the sequence of binding of substrates and release of products can be discerned. Lineweaver-Burk plots for the product inhibition studies conducted on TarD are depicted in Figures 3.5 and 3.6 where CDP-glycerol and pyrophosphate are utilized as inhibitors respectively. In each of these experiments, the co-substrate was kept at a concentration near its K_m. The data from these experiments were fitted to all inhibition models found in the Sigma Plot 2000 software by non-linear regression (least-squares method). Best-fit analysis determined that CDP-glycerol was a mixed (non-competitive) inhibitor with respect to both glycerol 3-phosphate (Figure 3.5 A) and CTP (Figure 3.5 B) as defined by Segal (Segal 1975) signified by the intersecting of the fitted series of lines to the left of the y-axis. The R^2 and standard deviation of the residuals were 0.928 and 5.1×10^{-3} (velocities were between 5.4×10^{-3} and 6.7×10^{-2} μM/min) respectively when CTP was varied. For data fitted to the non-competitive (full and partial), competitive competitive (full and partial) or uncompetitive competitive (full

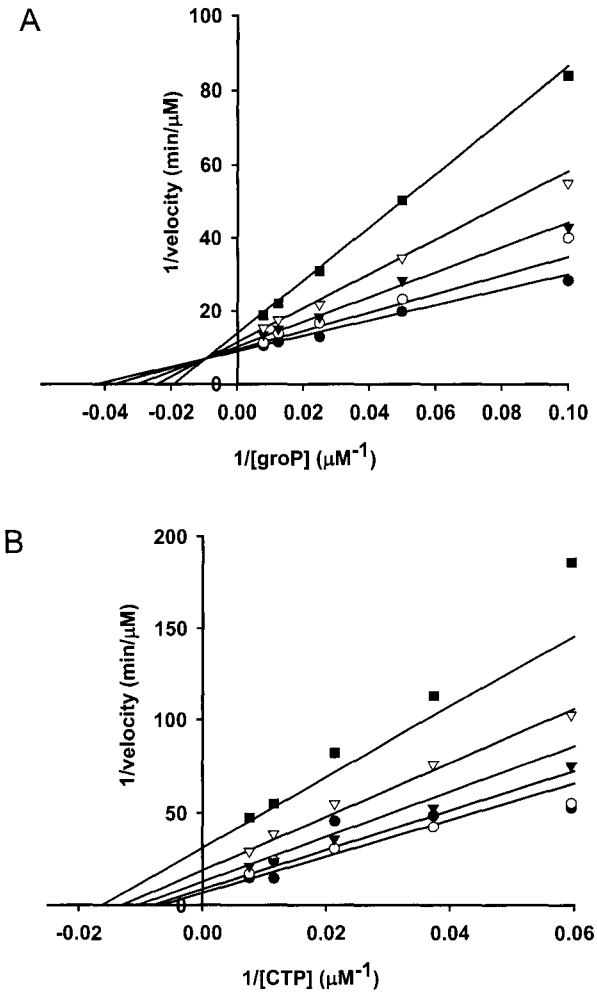


Figure 3.5 Analysis of Product Inhibition of TarD by CDP-glycerol. (A) Initial velocity plots were generated with varying glycerol 3-phosphate and CTP at a fixed concentration, which was approximately at K_m (57 μM). The inhibition data were screened against all the inhibition models in the Sigma Plot 2000 software. From all these possible models, a mixed (non-competitive) inhibition model (equation 6) was determined to be the model that best characterizes these data using non-linear regression. (B) CTP was varied while glycerol 3-phosphate was kept at K_m concentration (24 μM). These data also were best fit to the equation that specifies a mixed inhibition model. CDP-glycerol concentrations used for (A) and (B) were 0 μM (●), 0.1 μM (O), 0.3 μM (▼), 0.6 μM (▽), and 1.2 μM (■). Table 3.4 summarizes the inhibitor constants generated from these plots.

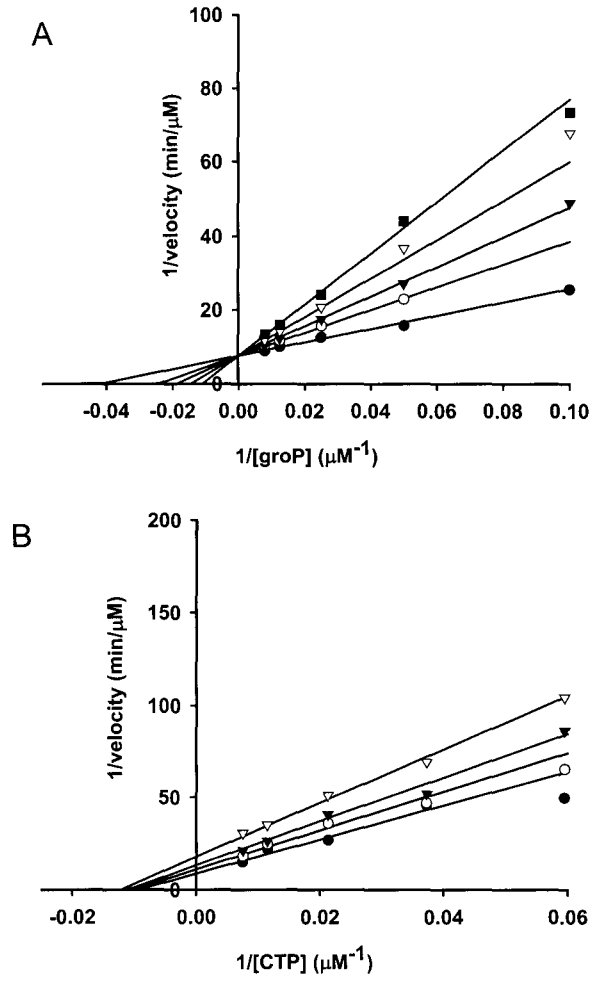
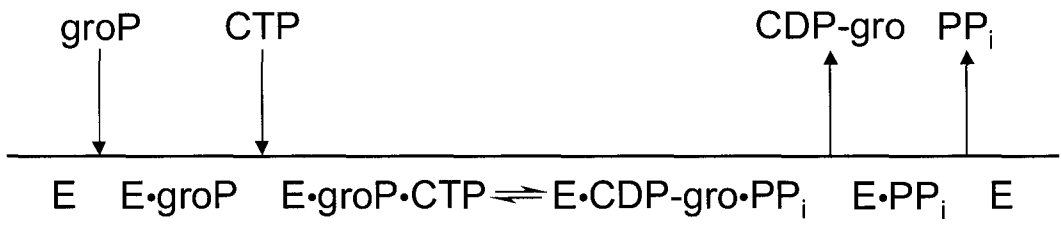


Figure 3.6 Analysis of Product Inhibition of TarD by Pyrophosphate. Lineweaver-Burk plots were used to determine the mode of inhibition of pyrophosphate with respect to the substrates of TarD. (A) Glycerol 3-phosphate was varied while CTP was kept at K_m concentration (57 μM). The model that best describes these data is one where pyrophosphate is a competitive inhibitor with respect to glycerol 3-phosphate (equation 5). (B) When CTP is varied, and glycerol 3-phosphate is kept at K_m level (24 μM), a pattern of double reciprocal plots indicating mixed inhibition is obtained (equation 6). Pyrophosphate concentrations for both plots were 0 mM (●), 0.5 mM (O), 1 mM (▼), and 2 mM (▽). In (A) an additional plot using 5 mM (■) pyrophosphate exists. Table 3.4 summarizes the inhibitor constants generated from these plots.

and partial) models, the standard deviation of the residuals were between 5.2×10^{-3} and 5.8×10^{-3}. The R^2 and standard deviation of the residuals were 0.999 and 2.7×10^{-3} (velocities were between 1.2×10^{-2} and 9.6×10^{-2} μM/min) respectively when glycerol 3-phosphate was varied. For data fitted to the non-competitive (full and partial), competitive competitive (full and partial) or uncompetitive competitive (full and partial) models, the standard deviation of the residuals were between 3.4×10^{-3} and 5.6×10^{-3}. Pyrophosphate was shown to be a mixed inhibitor with respect to CTP (Figure 3.6 B) but competitively (partially) inhibited TarD when glycerol 3-phosphate was the varied substrate (Figure 3.6 A). This is easily recognized by intersection of the lines directly on the vertical axis. The R^2 and standard deviation of the residuals were 0.976 and 2.6×10^{-3} (velocities were between 9.6×10^{-3} and 6.7×10^{-2} μM/min) respectively when CTP was varied. For data fitted to the non-competitive (full and partial), competitive competitive (full and partial) or uncompetitive competitive (full and partial) models, the standard deviation of the residuals were between 2.6×10^{-3} and 3.2×10^{-3}. The R^2 and standard deviation of the residuals were 0.996 and 2.0×10^{-3} (velocities were between 2.3×10^{-2} and 11.3×10^{-2} μM/min) respectively when glycerol 3-phosphate was varied. For data fitted to the non-competitive (full and partial), mixed (full) or uncompetitive competitive (full and partial) models, the standard deviation of the residuals were between 4.9×10^{-3} and 8.7×10^{-3}. Because pyrophosphate and glycerol 3-phosphate directly compete for binding, it can be inferred that both these compounds bind similar (apo) forms of TarD. This product inhibition pattern is a classic example of an ordered Bi Bi reaction

mechanism (Cooper and Rudolph 1996). In this type of mechanism both substrates bind (before any product is released) in a specific order. After catalysis, products are released in a specific order as well. In TarD's case, glycerol 3-phosphate is the first substrate to bind to the enzyme and pyrophosphate is the last product to be released. Scheme 3.1 outlines the proposed mechanism of TarD as established from product inhibition analysis.

The constants of inhibition are summarized in Table 3.4. There were striking differences between inhibition constants for pyrophosphate and CDP-glycerol. With respect to glycerol 3-phosphate, a K_{is} of 0.5 μM and a K_{ii} of 2.3 μM was observed for CDP-glycerol. Similar values were obtained when CDP-glycerol acted as an inhibitor and CTP was the varied substrate. Under these circumstances a K_{is} of 1.3 μM and a K_{ii} of 0.39 μM were observed. The relatively small inhibition constants for CDP-glycerol explains why TarD must be studied at enzyme levels where only 1 to 2% of substrate may be converted into product. On the other hand, the inhibition constants for pyrophosphate were very different. When pyrophosphate was a competitive inhibitor with respect to glycerol 3-phosphate a K_{is} of 470 μM was observed. When CTP was the varied substrate, TarD had a K_{is} and a K_{ii} of 3,400 μM and 2,000 μM (respectively) for pyrophosphate. It is obvious from these values that CDP-glycerol is a much more potent inhibitor than pyrophosphate. Toward confirming the mechanism proposed in Scheme 3.1, the inhibition experiment using pyrophosphate as an inhibitor with varying CTP was repeated with saturating amounts of glycerol 3-phosphate (2.4 mM, about 100-fold K_m). If pyrophosphate and glycerol 3-phosphate both bound to apo-TarD then a large increase in glycerol 3-phosphate would compete out the effect of pyrophosphate as an inhibitor



Scheme 3.1 Ordered Bi Bi Reaction Mechanism of TarD

Table 3.4 Pattern of Product Inhibition for TarD.

Inhibitor	Varied substrate	Co-substrate Concentration (μM)	Type of Inhibition[a]	K_{is} (μM)	K_{ii} (μM)	α^b
CDP-gro	groP	57	mixed	0.5	2.3	
CDP-gro	CTP	24	mixed	1.3	0.39	
PP_i	groP	57	competitive-partial	470		5.2
PP_i	CTP	24	mixed	3,400	2,000	
PPi	CTP	2400	mixed	28,000	1,900	

[a]Determined by non-linear regression methods.
[b]Factor by which the inhibition constant changes when inhibitor binds to enzyme substrate complex.

when CTP is varied (Cooper and Rudolph 1996). This effect was evident in the approximately 8-fold increase in the K_{is} (3.4 mM and 28 mM, respectively) for pyrophosphate with respect to CTP (Table 3.4).

3.4.6 Analysis of the Reverse Reaction

In order to study the reaction catalyzed in the reverse direction, the formation of [5-^{3}H] CTP was measured. Initial velocity experiments were conducted to determine the kinetic constants of this reaction. As expected, the data were best fit to the equation describing a sequential mechanism (equation 3) (Figure 3.7). The K_m's for CDP-glycerol and pyrophosphate, which can be found in Table 3.3, were 1.4 ± 0.62 μM and 9,700 ± 3,800 μM respectively. Moreover, the k_{cat} was calculated to be 1.0 ± 0.2 s^{-1}. Dissociation constants along with K_m's for the substrates of the forward and reverse reaction, calculated from equation 3, are listed in Table 3.3. With these, the equilibrium constant (K_{eq}) of this reaction was calculated using equation 7. In this case, an equilibrium constant of 16 ± 15 was obtained. The experimentally derived K_{eq} was determined to be 6.

3.5 Discussion

The diminishing ability for conventional antibiotics to fight infections arising from pathogens has sparked our interest in the investigation of the CTP:glycerol 3-phosphate cytidylyltransferase from *S. aureus* as a potential therapeutic target. This enzyme is proposed to be involved in the construction of a major anionic polymer of the

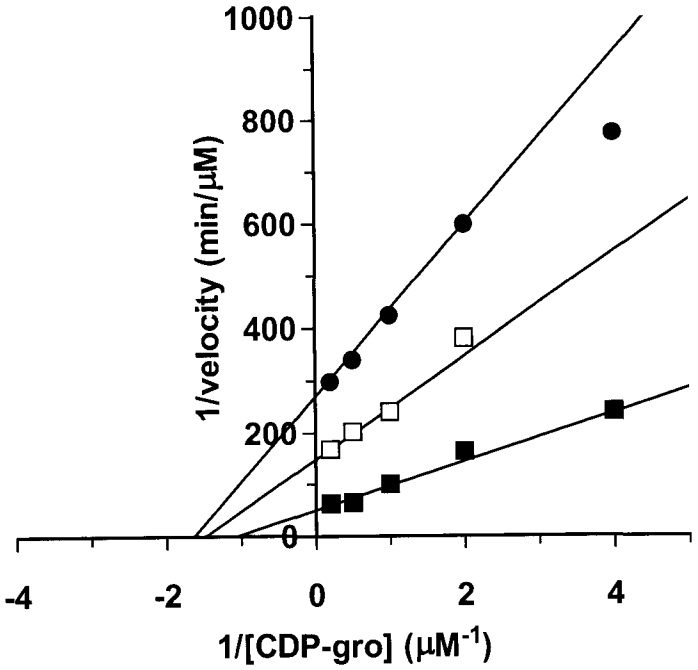


Figure 3.7 Initial Velocity Study of the Reverse Reaction. Double reciprocal plots of initial velocity experiments with varying CDP-glycerol concentration (0.25 to 5 μM) at fixed pyrophosphate concentrations (1 mM (●), 2 mM (□), and 10 mM (■)) are shown. The production of [5-^{3}H] CTP was monitored by HPLC. Data were fitted to a ternary sequential mechanism as described by equation 3.

cell wall, which has been shown to be essential in *B. subtilis* (Briehl, Pooley et al. 1989; Mauel, Young et al. 1989; Mauel, Young et al. 1991; Pooley, Abellan et al. 1991). Therefore, understanding the details of its kinetic mechanism is an essential step required to build therapeutics for this target.

An ordered Bi Bi reaction mechanism has been proposed in this thesis for TarD, where glycerol 3-phosphate is the first to bind in the forward reaction followed by CTP. After the formation of the ternary complex and catalysis, CDP-glycerol is released first followed by pyrophosphate.

In bi-substrate kinetic analysis of a 5 x 5 matirix of substrate concentrations, the data were fit to the equation that describes the formation of a ternary complex between enzyme and substrates (Cleland 1970). To discern the order of binding, steady state experiments were coupled with product inhibition studies. In this analysis, all experiments yielded mixed (non-competitive) inhibition except where pyrophosphate was a partial competitive inhibitor with respect to glycerol 3-phosphate. Further mechanistic evidence of the competitive inhibition pattern observed with pyrophosphate was provided by analysis of the inhibition by this compound using saturating amounts of glycerol 3-phosphate as a co-substrate. Here, the impressive increase in K_{is} of pyrophosphate when CTP is the varied substrate authenticates pyrophosphate as a true competitive inhibitor with respect to glycerol 3-phosphate (Cooper and Rudolph 1996). It follows that glycerol 3-phosphate is the leading substrate and pyrophosphate is the final product to be released.

TarD and TagD are members of the cytidylyltransferase superfamily (also called the TagD superfamily) along with other nucleotidylyltransferases such as rat choline-

phosphate cytidylyltransferase, human ethanolamine-phosphate cytidylyltransferase (Weber, Park et al. 1999) and ATP-sulfurylase (Venkatachalam, Fuda et al. 1999; Ullrich, Blaesse et al. 2001). Also included into this family are the class I aminoacyl-tRNA sythetases (Bork, Holm et al. 1995), and pantothenate synthetase (PanC) (von Delft, Lewendon et al. 2001). This superfamily has been established around a shared and unique nucleotide-binding fold characterized by an HXGH motif. The histidine residues are clear participants in critical interactions with the α and β phosphates of the nucleotide (Weber, Park et al. 1999). These structurally related proteins are thought to optimize interactions to analogous transition states but it has yet to be shown whether all these examples are actually mechanistically related. For example, the differences in steady state reaction mechanism deduced for TagD, PanC and ATP-sulfurylase are considerable. While the Bi Uni Uni Bi ping-pong reaction mechanism of PanC more closely resembles the mechanisms of some amino-acyl tRNA synthetases (Zheng and Blanchard 2001), ATP-sulfurylase has an ordered Bi Bi mechanism with ATP binding first followed by sulfate with the release of pyrophosphate preceding the release of adenosine 5'-phosphosulfate (Lyle, Geller et al. 1994). Interestingly, neither of these mechanisms resembles the unique situation described for this family's namesake TagD, discussed in detail below.

While there is great variability in mechanisms of amino-acyl tRNA synthetases and the members of the cytidylyltransferase superfamily, there are examples of ordered Bi Bi mechanisms for specific nucleotidylyltransferases in the literature that resemble the situation described here for TarD. CTP:phosphocholine cytidylyltransferase from

Streptococcus pneumoniae (LicC) is a member of the GlmU/RmlA nucleotidytransferase family (Brown, Pompeo et al. 1999; Kwak, Zhang et al. 2001) and catalyzes a sequential ordered Bi Bi reaction where CTP is the leading substrate and CDP-choline is the last product to be released. A sequential ordered Bi Bi mechanism has also been suggested for a glucose-1-phosphate thymidylyltransferase from *E. coli* (Zuccotti, Zanardi et al. 2001) as well as an ADP-glucose pyrophosphorylase (named for the reverse reaction) (Kleczkowski, Villand et al. 1993). Both these enzymes also bind the nucleotide first with the release of the nucleotide-containing product last. TarD differs from these mechanisms in that the nucleotide is the second substrate to bind with CDP-glycerol the initial product to be released. Interestingly, the bifunctional uridylyltransferase/uridylyl-removing enzyme from *E.coli* catalyzes a uridylyl transferase to the PII signal transduction protein via an ordered Bi Bi mechanism that has its non-nulceotide-containing substrate (PII) bind first followed by UTP (Jiang, Peliska et al. 1998). Although the binding of substrates is similar to TarD, the release of products in the uridylyltransferase resembles other nucleotidylyltransferases discussed here, with the nucleotide-containing product released last.

Despite remarkable sequence identity (69 %) and similarity (86 %) between TarD and TagD, they have very different kinetic properties. These properties are summarized in Table 3.5. While TarD exists as a tetramer and catalyzes the cytidylyltransferase reaction via an order Bi Bi mechanism, TagD, exists as a dimer, and was shown to carry out the CTP:glycerol 3-phosphate cytidylyltransferase reaction via a random mechanism where there is negative cooperativity in the binding of substrates but not catalysis (Park,

Table 3.5 Comparison of the Kinetic Constants of TarD and TagD.

	Identity and Similarity	Soluble Molecular Weight	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$) ($x10^{-4}$)	Kinetic Mechanism
TagD	68% (89/128) 86% (112/128)	30,000	3200 (groP) 3900 (CTP)	50	1.6 (groP) 1.3 (CTP)	Rapid Eq. Random neg. cooperativity
TarD		64,000	21 (groP) 36 (CTP)	2.6	13.0 (groP) 7.2 (CTP)	Ordered Bi Bi

Sweitzer et al. 1993; Sanker, Campbell et al. 2001). This mechanism is unparalleled in the literature for other TagD family members or other nucleotidylyltransferases to date. A model was proposed where substrates randomly bind to a TagD homodimer with decreasing affinity from submicrolar K_d values for the first substrate molecule to millimolar affinity for the final substrate molecule. That the dimer must be loaded with substrates before catalysis can occur was also proposed and consistent with millimolar K_m values for both CTP (3.9 mM) and glycerol 3-phosphate (3.2 mM)(Park, Sweitzer et al. 1993). In contrast, the K_m values determined in this work for TarD (36 μM and 21 μM for CTP and glycerol 3-phosphate, respectively) are more than 100-fold lower than those described for TagD. Indeed, the TarD and TagD proteins appear to differ both in substrate binding affinity and steady state kinetic mechanism. The next chapter describes the investigation into the question of whether, in spite of the differences in steady state reaction mechanism the function of TagD can be replaced by TarD *in vivo*.

CHAPTER 4 – COMPLEMENTATION OF A TEMPERATURE SENSITIVE MUTANT IN *TAGD* (*TAG*-12) BY *TARD*

4.1 Introduction

Previously published work and the experiments outlined above suggest that TarD and TagD have very distinct reaction mechanisms. Based on their genetic and structural similarity this was very surprising. In order to confirm the role TarD may play in the cell, complementation of a temperature sensitive mutation in *tagD* of *B. subtilis* 168 was attempted. This work was carried out using an integration plasmid, pSWEET, designed and tested by (Bhavsar, Zhao et al. 2001). These investigators have used this vector to conditionally complement a temperature sensitive strain with wild type *tagD*. The vector exploits the xylose promoter for the controlled transcription of the gene in question. In addition to controlling the presence or absence of gene products, this vector can be conveniently integrated into the *B. subtilis* 168 chromosome. Flanking the *xyl* promoter regulatory sequences and the cloned gene are regions homologous to *amyE* at which double recombination can occur. In this work, a heterologous complementation experiment was performed to confirm that TarD can replace the activity of TagD and that their physiological roles in the living cell are similar.

4.2 Materials

Restriction enzymes, T4 DNA ligase, and VENT polymerase were purchased from New England BioLabs (Beverly, MA). Ampicillin (AMP) was obtained from

BioShop Canada Inc. (Burlington, ON). All other compounds were purchased from Sigma (Oakville, ON) unless specified.

4.3 Methods

4.3.1 Construction of pSWEET – *tarD*

pSWEET-*bgaB* (Bhavsar, Zhao et al. 2001) was digested with *Pac*I and *Bam*HI to remove the *bgaB* gene. The plasmid backbone was then gel purified. Two pSWEET-*tarD* constructs were prepared, which differed in their ribosome binding site (RBS). The primers DB03 and DB05 (Table 2.2) were used to amplify *tarD* from *S. aureus* genomic DNA so that the native *tagD* RBS was upstream of the gene. So as to place a consensus RBS (Vellanoweth and Rabinowitz 1992) upstream of the *tarD* gene, the primers DB04 and DB05 were used (Table 2.2). The amplified products were cloned into the *Eco*RV restriction site of pBluescript SK (+/-) (Stratagene). This plasmid was digested with *Pac*I and *Bam*HI and the band corresponding to *tarD* was gel purified. Gene *tarD* was ligated into digested pSWEET and the product was used to transform Novablue *E. coli* cells. Wild-type *B. subtilus* (EB6) and a temperature sensitive (*ts*) strain (EB4) were transformed with pSWEET-*tarD* with the native *tagD* or consensus RBS by the method described in (Cutting and Youngman 1994). EB164 and EB249 designate wild type strains with integrated pSWEET plasmids with native *tagD* and *B. subtilis* consensus RBS respectively. EB171 and EB215 represent the temperature sensitive strains with linearized pSWEET integrated at *amyE* with the native *tagD* and consensus RBS respectively. Strain genotypes are listed in Table 2.1. Integration of the plasmid at *amyE*

was verified by growth on LB-agar supplemented with 10 μg/mL chloramphenicol and using a starch plate assay (Cutting and Horn 1990).

4.4 Results

4.4.1 Complementation of *tag*-12 by *tarD* – plate assay

Although TagD and TarD catalyze identical reactions they apparently perform this reaction by different mechanisms. Therefore, the ability of *tarD* to complement a temperature sensitive mutation in *tagD* (*tag*-12 (Briehl, Pooley et al. 1989)) was tested. The integration plasmid, pSWEET, was used to introduce a copy of *tarD* into wild type *B. subtilus* 168 and the *ts* strain at the *amyE* locus. Furthermore, two different ribosome binding sites (RBS) were used in this system: the RBS upstream of *tagD* in *B. subtilis*; and a consensus RBS (Vellanoweth and Rabinowitz 1992). Figure 4.1 displays the growth of different strains at the permissive temperature (30 °C) or non-permissive temperature (47 °C) with or without 0.2 % xylose. All strains were able to grow at 30 °C despite the presence or absence of xylose. However, when strains are grown at 47 °C without xylose all those that were temperature sensitive were unable to grow. When the inducer was present, only the temperature sensitive strains possessing a complementing chromosomal copy of *tarD* grew (EB171 and EB215). Strain EB171, which has the native *tagD* RBS, only grew where there were cells plated in high density, but strain EB215, which has the consensus RBS, is able to form single colonies. Although there are slight differences in the growth of the two complemented strains, the reasons for this

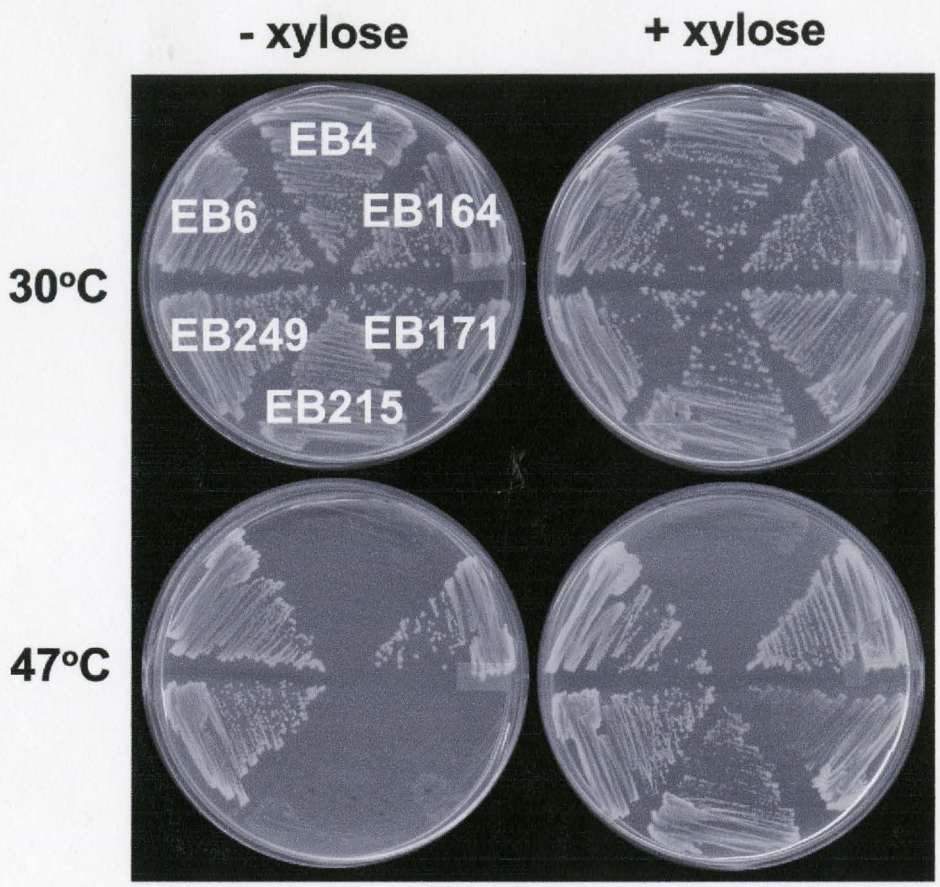


Figure 4.1 Complementation of the Temperature Sensitive Mutation in *tagD* of B. subtilis (*tag-12*) with a Chromosomal Copy of *tarD* Using pSWEET - Plate Assay. *B. subtilis* 168 strains were plated on LB-agar in the presence or absence of xylose (0.2 %) and either incubated at 30 °C (permissive temperature) or 47 °C (non-permissive temperature) overnight. In brief, strain designations are: EB6, wild type, *tag*$^+$; EB4, temperature sensitive, *tag*-12; EB171 *tag-12* P_{xyl} *tarD* native RBS; EB164, *tag*$^+$ P_{xyl} *tarD native RBS;* EB215, *tag*-12 P_{xyl} *tarD* consensus RBS; EB 249, *tag*$^+$ P_{xyl} *tarD* consensus RBS. Refer to Table 2.1 for strain genotypes.

difference would be only speculative. More investigation of this system must be done in order to uncover the source of the difference.

4.4.2 Complementation of *tag*-12 by *tarD* – liquid culture assay

An experiment similar to the plate assay was performed in liquid culture and is displayed in Figure 4.2. Again, in this study, all temperature sensitive strains were unable to grow unless the production of TarD was induced with xylose. Little difference could be seen between the strains differing in ribosome binding sites. Not only do these two experiments give strong genetic evidence that both *tarD* and *tagD* have similar roles in the cell; namely the production of CDP-glycerol for use in the biosynthesis of teichoic acid, but they also illustrate the fact that this enzyme is a particularly potent target. The absence of a glycerol 3-phosphate cytidylyltransferase not only halts the growth of the cells but also causes a lytic phenotype characterized by the decrease in cell density after the temperature shift in Figure 4.2.

4.5 Discussion

The contrasts between the related enzymes TarD and TagD illustrated throughout this thesis are very interesting. It is extraordinary that two enzymes, so similar in primary structure, would exist in different multimeric states and catalyze identical reactions in such vastly different manners. However, the ability of *tarD* to complement a temperature sensitive mutation in *tagD* confirms that in the living cell, these two enzymes have similar roles. TarD involved in teichoic acid synthesis in *S. aureus* has to create enough

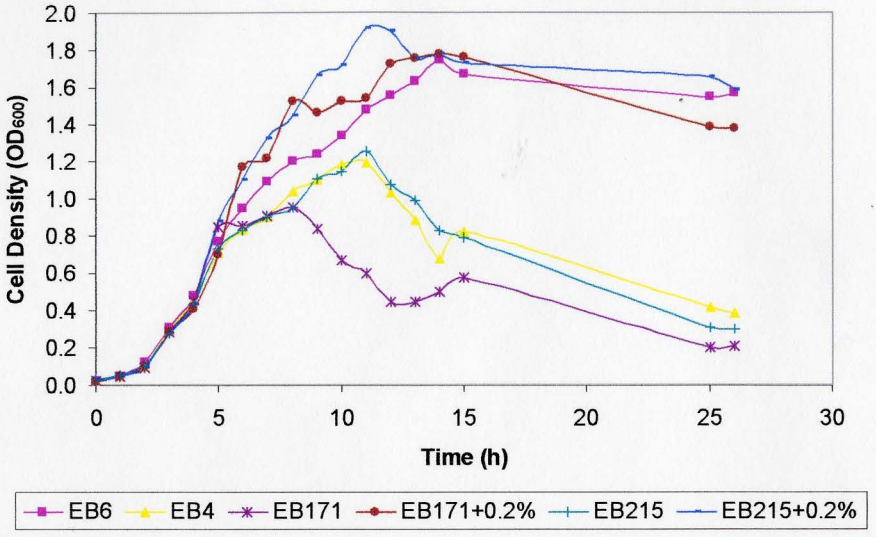


Figure 4.2 Complementation of the Temperature Sensitive Mutation in *tagD* of *B. subtilis* (*tag-12*) with a Chromosomal Copy of *tarD* Using pSWEET - Liquid Culture Assay. Colonies grown at 30 °C overnight on LB-agar were used to inoculate 2 to 3 mL of LB. These cultures were grown overnight at 30 °C shaking at 250 rpm. The overnight cultures were used to inoculate 100 mL of LB (1 in 100). These were grown to an OD_{600} of approximately 0.5 at 30 °C. Once this cell density was reached, the temperature was raised to 47 °C. Aliquots (0.5 mL) were removed every hour and diluted with an equal amount of water before measuring the absorbance at 600 nm in a spectrophotometer (Molecular Devices SpectraMax).

CDP-glycerol to complete the linkage unit. In *B. subtilis* 168 enough CDP-glycerol must be present to supply the construction of the entire teichoic acid polymer. There seems to be no indication (other than the formation of single colonies with the presence of a consensus RBS upstream of *tarD*) that this small difference in physiological role is affecting the ability of *tarD* to complement a deficiency in *tagD*. Given this, a possible inherent species specificity of the cytidylyltransferase reaction may not be apparent in this complementation experiment.

CHAPTER 5 – SUMMARY AND CONCLUSIONS

Much of the discussion here has been focussed on making a comparison between TarD and TagD. It has been demonstrated that on every front that has been investigated in this thesis and the evidence presented by Kent in the literature that these highly related enzymes perform their function differently. Without attempting to put the *B. subtilis* enzyme through experiments identical to the ones described in this thesis, it is impossible to discern with certainty whether the differences in assays used for the study of these enzyme is the cause of the different pictures portrayed. Alternatively, these two enzymes may indeed behave differently and we have uncovered a case of species specificity in the catalysis performed by two related cytidylyltransferases.

The ultimate goal of the investigation of this enzyme as a target for antimicrobial small molecules is to identify lead inhibitors. This may be accomplished on two fronts. The first is to use glycerol 3-phosphate as a scaffold to design alternate forms of this substrate. These compounds may prove to be inhibitors or interesting alternate substrates. Either way, much information of binding of the actual substrates can be attained in these experiments. The second manner in which possible inhibitors may be identified is through high throughput screening (HTS). With the newly formed HTS lab at McMaster University the HPLC assay for TarD may be adapted into a colourimetirc assay such as the malachite green assay, where inorganic phosphate may be monitored (from hydrolysis of pyrophosphate with inorganic pyrophosphatase). Therefore, all the tools for this type of screen are available.

In summary, the reaction mechanism for TarD has been established in this thesis. An ordered Bi Bi mechanism for TarD has been illustrated by initial velocity and product inhibition studies. The K_m values for substrates in the forward direction are in the micromolar range. Moreover, the reverse reaction catalyzed by TarD was also analyzed. From these experiments Michaelis-Menten constants were calculated for substrates in the reverse reaction as well as a K_{eq} of 16 while the experimental value of K_{eq} was calculated to be 6. In order to illustrate the physiological role of TarD a temperature sensitive mutation in *tagD* was successfully complemented by *tarD*. This experiment also illustrates that *tarD* can substitute for *tagD*. This work in total has served as a foundation for the rigorous search for potent inhibitors of this potential therapeutic target.

REFERENCES

Abellan, F.-X. (1996). Determinants Genetiques du Poly(Ribitol-phosphate), le Polymere Anionique Majeur de la ouche *Bacillus subtilis* W23: Seuencage des Genes *tar* et Analyse Comparative des Souches W23 et 168. Faculte des Sciences. Lausanne, L'Universite de Lausanne: 127.

Archibald, A. R., I. C. Hancock, et al. (1993). Cell wall structure, synthesis and turnover. in *Bacillus subtilis* and other gram-postive bacteria. A. L. Sonenshein, J. A. Hoch and R. Losick. Washington, DC, American Society for Microbiology: 381-410.

Bhavsar, A. P., T. J. Beveridge, et al. (2001). "Precise deletion of *tagD* and controlled depletion of its product, glycerol 3-phosphate cytidylyltransferase, leads to irregular morphology and lysis of *Bacillus subtilis* grown at physiological temperature." J. Bacteriol. **183**(22): 6688-93.

Bhavsar, A. P., X. Zhao, et al. (2001). "Development and characterization of a xylose-dependent system for expression of cloned genes in *Bacillus subtilis*: conditional complementation of a teichoic acid mutant." Appl. Environ. Microbiol. **67**(1): 403-10.

Bork, P., L. Holm, et al. (1995). "The cytidylyltransferase superfamily: identification of the nucleotide- binding site and fold prediction." Proteins **22**(3): 259-66.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal. Biochem. **72**: 248-54.

Briehl, M., H. M. Pooley, et al. (1989). "Mutants of *Bacillus subtilis* 168 thermosensitive for growth and wall teichoic acid biosynthesis." J. Gen. Microbiol. **135**: 1325-1334.

Brock, T. D., M. T. Madigan, et al. (1997). Biology of Microorgansims. Upper Saddle River, NJ, Predice-Hall Inc.

Brown, E. D. and J. Wood (1992). "Redesigned purification yields a fully functional PutA protein dimer from *Escherichia coli*." J. Biol. Chem. **267**(18): 13086-92.

Brown, K., F. Pompeo, et al. (1999). "Crystal structure of the bifunctional N-

acetylglucosamine 1-phosphate uridyltransferase from *Escherichia coli*: a paradigm for the related pyrophosphorylase superfamily." EMBO. J. **18**(15): 4096-107.

Chambers, H. F. (1997). "Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications." Clin. Microbiol. Rev. **10**(4): 781-791.

Chatterjee, A. N., D. Mirelman, et al. (1969). "Properties of a novel pleiotropic bacteriophage-resistant mutant of *Staphylococcus* aureus H." J. Bacteriol. **100**(2): 846-53.

Cleland, W. W. (1970). Steady State Kinetics. in The Enzymes. P. D. Boyer. New York, Acedemic Press Inc. **2:** 1-65.

Cooper, B. F. and F. B. Rudolph (1996). Product Inhibition Applications. Contemporary Enzyme Kinetics and Mechanism. D. L. Purich. San Diego, CA, Academic Press Inc.: 183-205.

Cutting, S. M. and P. B. V. Horn (1990). Genetic Analysis. Molecular Biological Methods for *Bacillus*. C. R. Harwood and S. M. Cutting. Toronto, John Wiley and Sons: 27-60.

Cutting, S. M. and P. Youngman (1994). Gene transfer in Gram-positive bacteria. in Methods for General and Molecular Bacteriology. P. Gerhardt, R. G. E. Murray, W. A. Wood and N. R. Krieg. Washington DC, American Society for Microbiology: 348-364.

Daigle, D. M., D. W. Hughes, et al. (1999). "Prodigious substrate specificity of AAC(6')-APH(2"), an aminoglycoside antibiotic resistance determinant in *enterococci* and *staphylococci*." Chem. Biol. **6**(2): 99-110.

Diekema, D. J. (2001). "Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999." Clin. Microbiol. & Infect. **7**(3): 152-7.

Donlan, R. M. (2000). "Biofilm formation: a clinically relevant microbiological process." ASAIO Journal **46**(6): S47-52.

Fitzgerald, S. N. and T. J. Foster (2000). "Molecular analysis of the *tagF* gene, encoding

CDP- Glycerol:Poly(glycerophosphate) glycerophosphotransferase of *Staphylococcus epidermidis* ATCC 14990." J. Bacteriol. **182**(4): 1046-52.

Fridkin, S. K. (2000). "Vancomycin-intermediate and -resistant *Staphylococcus aureus*: what the infectious disease specialist needs to know." Diag. Microbiol. Infect. Dis. **38**(1): 59-67.

Gill, S. C. and P. H. von Hippel (1989). "Calculation of protein extinction coefficients from amino acid sequence data." Anal. Biochem. **182**(2): 319-26.

Gold, H. S. and R. C. Moellering (1996). "Drug Therapy: Antimicrobial-Drug Resistance." N. Eng. J. of Med. **335**(19): 1445-1453.

Harding, S. E. and K. Jumel (1998). Light Scattering. in Current Protocols in Protein Science. J. E. Coligan, B. M. Dunn, D. W. Speicher and P. T. Wingfield. New York, NY, John Wiley & Sons, Inc. **1:** 7.8.1-7.10.15.

Hartstein, A. I. and M. E. Mulligan (1999). Methicillin-Resistant *Staphylococcus aureus*. in Hospital Epidemiology and Infection Control. C. G. Mayhall. Philadelphia, PA, Lippincott Williams and Wilkins**:** 347-364.

Horne, D. S. and A. Tomasz (1993). "Possible role of a choline-containing teichoic acid in the maintenance of normal cell shape and physiology in *Streptococcus oralis*." J. Bacteriol. **175**(6): 1717-22.

Jiang, P., J. A. Peliska, et al. (1998). "Enzymological characterization of the signal-transducing uridylyltransferase/uridylyl-removing enzyme (EC 2.7.7.59) of *Escherichia coli* and its interaction with the PII protein." Biochemistry **37**(37): 12782-94.

John, J. F. and N. L. Barg (1999). *Staphylococcus aureus*. in Hospital Epidemiology and Infection Control. C. G. Mayhall. Philadelphia, PA, Lippincott Williams and Wilkins**:** 325-345.

Kaya, S., K. Yokoyama, et al. (1983). "Structural and biosynthetic studies on linkage region between poly(galactosylglycerol phosphate) and peptidoglycan in *Bacillus coagulans*." Biochem. Biophys. Res. Commun. **111**(1): 312-8.

Kaya, S., K. Yokoyama, et al. (1984). "N-acetylmannosaminyl(1-4)N-acetylglucosamine, a linkage unit between glycerol teichoic acid and peptidoglycan in cell walls of several *Bacillus* strains." J. Bacteriol. **158**(3): 990-6.

Kleczkowski, L. A., P. Villand, et al. (1993). "Kinetic mechanism and regulation of

ADP-glucose pyrophosphorylase from barley (*Hordeum vulgare*) leaves." J. Biol. Chem. **268**(9): 6228-33.

Kojima, N., Y. Araki, et al. (1983). "Structure of linkage region between ribitol teichoic acid and peptidoglycan in cell walls of *Staphylococcus aureus* H." J. Biol. Chem. **258**(15): 9043-5.

Kwak, B. Y., Y. M. Zhang, et al. (2001). "Structure and mechanism of CTP:phosphocholine Cytidylyltransferase (LicC) from *Streptococcus pneumoniae*." J. Biol. Chem. **12**: 12.

Lahooti, M. (1999). "Transcriptional analysis of the *Bacillus subtilis* teichuronic acid operon." Microbiol. **145**(Pt 12): 3409-17.

Lazarevic, V. and D. Karamata (1995). "The *tagGH* operon of *Bacillus subtilis* 168 encodes a two-component ABC transporter involved in the metabolism of two wall teichoic acids." Mol. Microbiol. **16**(2): 345-55.

Lyle, S., D. H. Geller, et al. (1994). "Kinetic mechanism of ATP-sulphurylase from rat chondrosarcoma." Biochem. J. **301**(Pt 2): 349-54.

Mauel, C., M. Young, et al. (1991). "Genes concerned with synthesis of poly(glycerol phosphate), the essential teichoic acid in *Bacillus subtilis* strain 168, are organized in two divergent transcription units." J. Gen. Microbiol. **137**(Pt 4): 929-41.

Mauel, C., M. Young, et al. (1989). "The essential nature of teichoic acids *in Bacillus subtilis* as revealed by insertional mutagenesis." Mol. Gen. Genet. **215**(3): 388-94.

Park, J. T., D. R. Shaw, et al. (1974). "Mutants of *staphylococci* with altered cell walls." Ann. N Y Acad. Sci. **236**(0): 54-62.

Park, Y. S., P. Gee, et al. (1997). "Identification of functional conserved residues of CTP:glycerol-3- phosphate cytidylyltransferase. Role of histidines in the conserved HXGH in catalysis." J. Biol. Chem. **272**(24): 15161-6.

Park, Y. S., T. D. Sweitzer, et al. (1993). "Expression, purification, and characterization of CTP:glycerol-3- phosphate cytidylyltransferase from *Bacillus subtilis*." J. Biol. Chem. **268**(22): 16648-54.

Perego, M. (1995). "Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis*. Identification of genes and regulation." J. Biol. Chem. **270**(26): 15598-606.

Pooley, H. M., F. X. Abellan, et al. (1991). "A conditional-lethal mutant of *Bacillus*

subtilis 168 with a thermosensitive glycerol-3-phosphate cytidylyltransferase, an enzyme specific for the synthesis of the major wall teichoic acid." J. Gen. Microbiol. **137**(Pt 4): 921-8.

Pooley, H. M. and D. Karamata (1994). Teichoic acid synthesis in *Bacillus subtilis*: genetic organization and biological roles. Bacterial Cell Wall. J.-M. Ghuysen and R. Hakenbeck. New York, Elsevier Science: 187-198.

Sambrook, J., E. F. Fritsch, et al. (1989). Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.".

Sanker, S., H. A. Campbell, et al. (2001). "Negative cooperativity of substrate binding but not enzyme activity in wild-type and mutant forms of CTP:glycerol-3-phosphate cytidylyltransferase." J. Biol. Chem. **276**(41): 37922-8.

Segal, I. H. (1975). Rapid Equilibrium Partial and Mixed-Type Inhibition. Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems. New York, NY, John Wiley and Sons,Inc.: 161-226.

Segal, I. H. (1975). Steady State Kinetics of Multireactant Enzymes. Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems. New York, NY, John Wiley and Sons,Inc.: 505-626.

Siegel, L. M. and K. J. Monty (1966). "Determination of molecular weights and frictional ratios of proteins in impure systems by use of gel filtration and density gradient centrifugation. Application to crude preparations of sulfite and hydroxylamine reductases" Biochim. Biophys. Acta. **112**(2): 346-62.

Smith, T. L., M. L. Pearson, et al. (1999). "Emergence of Vancomycin Resistance in *Staphylococcus aureus*." N. Eng. J. Med. **340**(7): 493-501.

Stevens, S. Y., S. Sanker, et al. (2001). "Delineation of the allosteric mechanism of a cytidylyltransferase exhibiting negative cooperativity." Nat. Struct. Biol. **8**(11): 947-52.

Ullrich, T. C., M. Blaesse, et al. (2001). "Crystal structure of ATP sulfurylase from *Saccharomyces cerevisiae*, a key enzyme in sulfate activation." EMBO J. **20**(3): 316-29.

Vellanoweth, R. L. and J. C. Rabinowitz (1992). "The influence of ribosome-binding-site elements on translational efficiency in *Bacillus subtilis* and *Escherichia coli* in vivo." Mol. Microbiol. **6**(9): 1105-14.

Venkatachalam, K. V., H. Fuda, et al. (1999). "Site-selected mutagenesis of a conserved

nucleotide binding HXGH motif located in the ATP sulfurylase domain of human bifunctional 3'- phosphoadenosine 5'-phosphosulfate synthase." J. Biol.Chem. **274**(5): 2601-4.

von Delft, F., A. Lewendon, et al. (2001). "The crystal structure of *E. coli* pantothenate synthetase confirms it as a member of the cytidylyltransferase superfamily." Structure (Camb.) **9**(5): 439-50.

Walsh, C. (2000). "Molecular mechanisms that confer antibacterial drug resistance." Nature **406**(6797): 775-81.

Weber, C. H., Y. S. Park, et al. (1999). "A prototypical cytidylyltransferase: CTP:glycerol-3-phosphate cytidylyltransferase from *Bacillus subtilis*." Structure Fold. Des. **7**(9): 1113-24.

Yim, V. C., M. Zolli, et al. (2001). "Crystallization and preliminary X-ray diffraction studies of glycerol 3- phosphate cytidylyltransferase from *Staphylococcus aureus*." Acta Crystallogr. D Biol. Crystallogr. **57**(Pt 6): 918-20.

Zheng, R. and J. S. Blanchard (2001). "Steady-state and pre-steady-state kinetic analysis of *Mycobacterium tuberculosis* pantothenate synthetase." Biochemistry **40**(43): 12904-12.

Zolli, M., D. Kobric, et al. (2001). "Reduction precedes cytidylyl transfer without substrate channeling indistinct active sites of the bifunctional CDP-ribitol synthase from *Haemophilus influenzae*." Biochemistry **40**(16): 5041-8.

Zuccotti, S., D. Zanardi, et al. (2001). "Kinetic and crystallographic analyses support a sequential-ordered Bi Bi catalytic mechanism for *Escherichia coli* glucose-1-phosphate thymidylyltransferase." J. Mol. Biol. **313**(4): 831-43.