THE STUDY OF A NOVEL (p)ppGpp SYNTHASE (YwaC) FROM *B. subtilis*

BY

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ABSTRACT

Adaptation to any undesirable change in the environment helps to ensure the survival of many microorganisms. During nutrient starvation, bacteria undergo a stringent response characterized by the accumulation of the alarmone (p)ppGpp. This results in the repression of stable RNA species and a change in colony morphology. In Gram-negative bacteria such as *Escherichia coli*, RelA and SpoT synthesize and hydrolyze these nucleotides, respectively, under conditions of nutrient starvation. In Gram-positive bacteria, the bifunctional enzyme Rel is responsible for the accumulation of (p)ppGpp. These enzymes catalyze the transfer of a pyrophosphate moiety from ATP to the 3' end of either GTP or GDP. The overproduction of (p)ppGpp has many diverse consequences on bacterial physiology such as sporulation, virulence, long term persistence of pathogenic bacteria, cell morphology, antibiotic synthesis and fatty acid metabolism. In *Bacillus subtilis* a novel (p)ppGpp synthase, YwaC, is also involved in the accumulation of (p)ppGpp but does not associate with the ribosome. Transcriptional analysis of *ywaC* has implicated it with cell wall stress especially associated with lesions in the teichoic acid biosynthetic pathway. The work described here includes a steady state kinetic analysis of the reaction catalyzed by YwaC. Recombinant YwaC was over-expressed in *E. coli* and purified to homogeneity. Steady-state kinetic experiments were performed utilizing a high-performance liquid chromatography assay. This examination yielded K_m values for GDP and GTP of 5 μM and 6 μM respectively, while the k_{cat} was measured to be 0.13 min^{-1} and 0.11 min^{-1} respectively. As is common with other (p)ppGpp synthases, the low activity of YwaC may be increased in the presence of the appropriate effector molecule. To explore the functional phenotype associated with *ywaC* a deletion strain was made by replacing the gene on the chromosome of *B. subtilis* with a spectinomycin resistance cassette. A variety of antibiotics were used to probe the *ywaC*

deletion strain in an attempt to detect antibiotic sensitivity in comparison to wildtype cells. In addition, the morphology of the *ywaC* deletion strain was investigated using phase contrast confocal microscopy. Length and shape remained the same in a *ywaC* knockout. Growth profiles performed over a 24-hour period showed that the knockout strain grew similarly to wild-type *B. subtilis*. Thus, the phenotype analysis described herein failed to further elucidate the function of YwaC. Nevertheless, rigorous biochemical analysis described here have established the enzymatic role of (p)ppGpp synthesis for YwaC, but there remains much room for further investigation.

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LIST OF ABBREVIATIONS

ACP	acyl carrier protein
AMP	ampicillin
ATP	adenosine triphosphate
CTD	C-terminal domain
DTT	dithiothreitol
ECF	extracytoplasmic function
FPLC	fast protein liquid chromatography
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HEPES	*N*-2-Hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-thiogalactopyranoside
MIC	minimum inhibitory concentration
NTD	N-terminal domain
PIC	paired ion chromatography
(p)ppGpp	5'-triphosphate 3'-diphosphate and guanosine 5'-diphosphate 3'-diphosphate
RAC	ribosomal activating complex
RSH	Rel Spo homolog
SASs	small alarmone synthases
SDS	sodium dodecyl sulfate
SMM	spizizen minimal media
$Spec^r$	spectinomycin resistance cassette
TBAHS	tetrabutyl ammonium sulfate
TCS	two-component system
TGS	ThrRS, GTPase, and SpoT (nucleotide binding domain)

TLC	thin layer chromatography

PREFACE

Kathryn Millar and Amit Bhavsar, former graduate students of the Brown lab, paved the path for discovering that P_{ywaC} is a robust and reliable reporter of lesions in both the peptidoglycan and teichoic acid biosynthetic pathways. Electrospray mass spectrometry was performed and interpreted by Kalinka Koteva in Dr. Gerry Wright's lab, McMaster University. All other research was performed entirely by Heather A. Dalgleish.

CHAPTER 1 – BACKGROUND INFORMATION

1.1 Stress Response and the Alarmone (p)ppGpp

One of the most remarkable attributes of bacteria is their ability to respond to environmental stresses by intricate response mechanisms. Many of these responses require altering transcription by either up regulating genes necessary to fight a particular stress or down regulating genes that are harmful to the cell during that stress. One mechanism that cells utilize to alter transcription is the production of repressor or activator proteins that bind DNA on or near the promoter to prevent binding of RNA polymerase. Another mechanism involves an alternative sigma factor that, in response to a specific stress, replaces the primary sigma unit and redirects RNA polymerase to other locations on the chromosome (Costanzo, Nicoloff et al. 2008). These two mechanisms are not mutually exclusive. During times of starvation, the cell may not have the resources to launch such energy costing responses and it is therefore beneficial to have a more general regulator, which is able to activate individual responses. One such global response is mediated by the alarmone guanosine 5'-triphosphate 3'-diphosphate and guanosine 5'-diphosphate 3'-diphosphate ((p)ppGpp). Extensive research has proven that virtually all bacteria and plants synthesize ppGpp and pppGpp, which are regulatory analogs of GDP and GTP (Potrykus and Cashel 2008). In 1969, Cashel and Gallent first detected the accumulation of (p)ppGpp during amino acid starvation and the subsequent inhibition of stable RNA species such as ribosomal and transfer RNAs in *Escherichia coli* (Cashel 1969). In conditions of stress, accumulation of (p)ppGpp triggers the 'stringent response' that induces a global change in bacterial metabolism. This alarmone has profound effects on many cellular processes such as replication, transcription, translation, and differentiation. Although (p)ppGpp has primarily been

implicated in the stringent response, it is likely involved in general homeostatic growth control as well.

The sequenced genomes of eubacteria and plants contain one or more variants of *rsh* (Rel Spo homolog) genes. These genes encode large RSH proteins (~750 amino acid), which contain a catalytically active N-terminal domain and a regulatory C-terminal domain as depicted in Figure 1.1A (Potrykus and Cashel 2008). The naming of RSH proteins originated from RelA and SpoT from *E. coli*. Evidently, there are two similar RSH proteins in other beta- and gamma-proteobacteria. However, most other bacteria contain only a single RSH protein, which is named Rel with species name, such as Rel*Bsc* (Potrykus and Cashel 2008). Small molecular weight proteins with only synthase activity have also been discovered in *Streptococcus mutans* and *Bacillus subtilis* (Lemos, Nascimento et al. 2007; Nanamiya, Kasai et al. 2008). One such protein discovered in *B. subtilis*, YwaC, will be discussed in depth in this work. Similar sequences coexist typically in genomes of the class *Firmicutes* (e.g., bacilli, staphylococci, *Listeria*, clostridia, streptococci) along with the full-length RSH protein (Potrykus and Cashel 2008). These small synthase fragments do not yet have a function. RSH synthases catalyze the transfer of a pyrophosphate moiety from ATP to the 3' end of either GTP or GDP with dependence of the divalent cation, Mg^{2+}, for catalytic activity. The reaction is carried out by a nucleophilic attack from the 3'-OH of the GDP/GTP ribose moiety on the β-phosphorus atom of ATP. RSH (p)ppGpp hydrolases are Mn^{2+}-dependent pyrophosphohydrolases with a conserved His-Asp (HD) motif (Aravind and Koonin 1998; Hogg, Mechold et al. 2004). YwaC is lacking this (p)ppGpp pyrophosphohydrolase domain completely from the polypeptide sequence, suggesting that the possible function of this protein is limited to (p)ppGpp synthesis (Figure 1.1B).

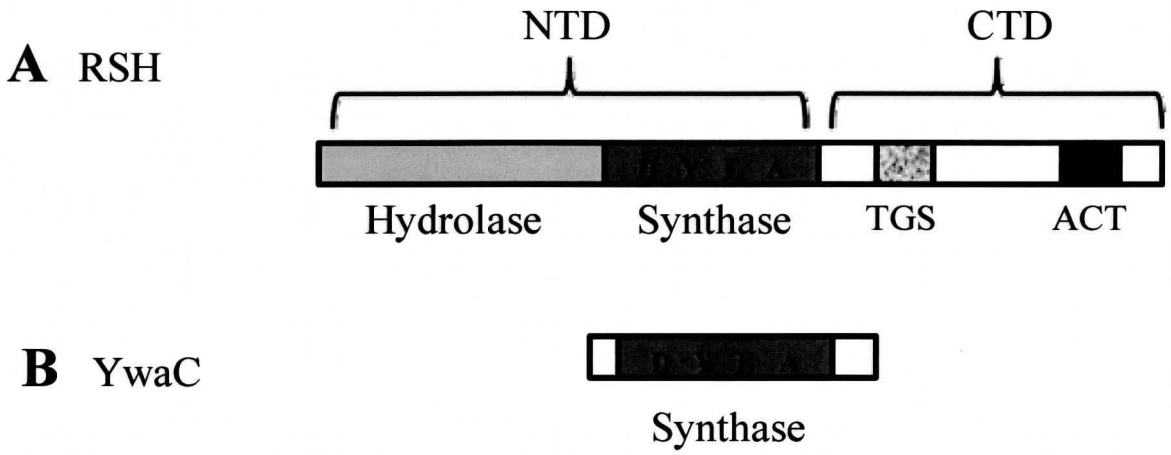


Figure 1.1 Domain structures of a bacterial RSH homologue and YwaC.
(A) Conserved domains of the N-terminal domain (NTD) and C-terminal (CTD) halves of a RSH protein (~750 amino acid residues).
(B) Conserved synthase domain located within the YwaC polypeptide (210 amino acid residues) and the lack of both a regulatory CTD and the conserved hydrolase domain. The letters D, Y, E, and A represent a few of several functionally significant amino acid residues that are conserved in YwaC (Asp^{264}, Tyr^{308}, Glu^{323} and Ala^{335}).

1.2 Activity of RSH Proteins - Gram-negative vs. Gram-positive organisms

The N-terminus of generic RSH proteins contains catalytic activity domains for hydrolase and synthase. In Gram-negative bacteria like *E. coli* and *Pseudomonas aeruginosa*, RelA and SpoT are two distinct genes expressed to maintain an appropriate level of (p)ppGpp in the cell (Chatterji and Ojha 2001). The main synthesis of (p)ppGpp is catalyzed by RelA as it contains an inactive HD domain sequence and therefore does not perform hydrolysis of (p)ppGpp. Whereas SpoT is specialized with pyrophosphohydrolase activity, and also has weak synthesis activity suggesting its role may be largely for replenishing GDP and GTP pools in the cell (Xiao, Kalman et al. 1991). RelA is dependent on intact mature ribosomes and uncharged tRNA for its activity (Haseltine and Block 1973; Martinez-Costa, Fernandez-Moreno et al. 1998). During amino acid starvation, there is a higher ratio of deacylated to acylated tRNA. When the translational machinery binds a deacylated tRNA at its A-site, translation is stalled. RelA is able to recognize and bind such a stalled ribosome resulting in strong stimulation of the synthesis of (p)ppGpp. In the absence of ribosomes, deacylated tRNA, and template mRNA, the activity of RelA in *E. coli* decreases 30-fold (Knutsson Jenvert and Holmberg Schiavone 2005). This suggests that binding to the ribosome creates a conformational change in the protein, allowing it to bind with higher affinity to its substrates and catalyze the production of (p)ppGpp more effectively. RelA activity has been strongly linked to amino acid starvation. However, SpoT, which has both synthase and hydrolase activity in *E. coli*, has been shown to induce (p)ppGpp accumulation during other stress conditions such as fatty acid (Baysse, Cullinane et al. 2005), nitrogen , carbon (Calderon-Flores, Du Pont et al. 2005) and iron starvation (Vinella, Albrecht et al. 2005). In a careful study conducted by Battesti and colleagues, it was confirmed that SpoT is involved in fatty acid starvation through direct interactions between the TGS nucleotide binding domain (Figure 1.1A) and Acyl Carrier Protein (ACP), a key cofactor used in fatty acid

metabolism (Battesti and Bouveret 2006). These results demonstrate the complexity of regulation of RSH proteins and the possibility that they are involved in more than just amino acid starvation and possibly have other roles depending on the species and its niche. In Gram-positive bacteria, the synthesis and subsequent hydrolysis of (p)ppGpp is catalyzed by a bifunctional enzyme, Rel, that has both synthase and hydrolase activity within the N-terminal domain. Bifunctional RSH homologs have been discovered in most Gram-positive organisms studied such as *Streptococcus equisimilis* (Mechold, Murphy et al. 2002), *Bacillus brevis* (Sy and Akers 1976), and *Mycobacterium tuberculosis* (Avarbock, Avarbock et al. 2005).

1.3 Regulation of RSH activity

Regulating the balance between opposing activities of RSH enzymes is crucial. Uncontrollable, fully active synthase and hydrolase activities would catalyze a futile cycle of (p)ppGpp synthesis and hydrolysis. If there was an over production of (p)ppGpp, this would send cells into the stringent response, inhibit growth and, in *E. coli*, change gene expression to curtail unnecessary activities in nongrowing cells. If there is too little (p)ppGpp from excess hydrolase, cells would be less equipped to manage nutritional stress.

Synthase activation (RelA and bifunctional RSH enzymes) seems to occur by a common signal as described previously. This process involves the inability of tRNA aminoacylation to keep up with the demands of protein synthesis and the ribosome stalls. Despite the availability of detailed ribosomal structures, little is known about the interaction between RelA or RSH and ribosomes, except that ribosomal mutants of the L11 protein (termed RelC) abolish activation (Gropp, Strausz et al. 2001). Antibiotics such as tetracycline, which interfere with the A-site,

render RelA inactive due to the lack of interaction with the ribosome (Knutsson Jenvert and Holmberg Schiavone 2005). For RelA and Rel*Mtb* point mutations in the CTD as well as CTD deletions, abolish activation for reactions in the presence of ribosomal activation complex (Avarbock, Avarbock et al. 2005).

For SpoT and RSH bifunctional enzymes, notions of how the CTD alters the balance of hydrolase/synthase activity is speculative due to the lack of structures for full-length RSH enzymes. Hogg and colleagues have recently crystallized the NTD from *Streptococcus dysgalactiae ssp. equisimilis* (Rel*Seq*), which has provided insight into the biochemical regulation of these bifunctional enzymes (Hogg, Mechold et al. 2004). The Rel*Seq* NTD structure displays the head of the hydrolase catalytic domain neighboring the tail of the synthase catalytic portion. This hinge could allow physical contact between the TGS region and hydrolase and/or synthase sequences. This structure suggests that the avoidance of a (p)ppGpp futile cycle may be an intrinsic feature of the catalytic half of the protein. The N-terminal domain contains two active sites located 30 Å apart. The crystals resolve two mutually exclusive active site conformers: monomer 1 and monomer 2 capture the "hydrolase_OFF/synthetase_ON" and "hydrolase_ON/synthetase_OFF" states of the enzyme, respectively. Substrate binding to either site is argued to trigger a signal repression cascade to the adjoining site that is located 30 Å apart (Hogg, Mechold et al. 2004). The CTD domain also plays a regulating role since removal of the C-terminal region leads to a 50-fold decrease in hydrolase activity in tandem with a 25-fold increase in synthase activity (Battesti, A. and E. Bouveret 2006). Possibly altered CTD structure by ACP or RAC effectors may trigger an allosteric switch between the two NTD conformers either by physical contacts or by inducing a

conformational cascade over the full length of the RSH protein. In either case, the net effect is an enzyme active state that favors either hydrolase or synthase, but not both.

1.4 Physiological effects of (p)ppGpp

The production of the alarmone (p)ppGpp has a gamut of effects on the physiology of bacteria. The various effects differ between organisms and environment, but the resounding fact remains that (p)ppGpp plays a significant role to the survival of bacteria during unfavorable conditions. One difficulty has been understanding why (p)ppGpp has such diverse effects on different organisms and whether or not these effects are centrally connected. The novel small (p)ppGpp synthase, YwaC, and those recently identified in other organisms could possibly give new insight into the role this alarmone has in signaling and stress response.

1.4.1 (p)ppGpp and long term persistence of *M. tuberculosis*

M. tuberculosis is able to survive and persist in the human host, creating many difficulties for both vaccine- and drug- based strategies for controlling the spread of tuberculosis. It has been shown that the Rel protein is required for long-term survival of *M. tuberculosis* inside the host (Dahl, Arora et al. 2005). A deletion of Rel in *M. smegmatis*, the non-pathogenic model organism for tuberculosis research, also severely affected long-term persistence in a mouse model and caused drastic alterations to cellular morphology. In the absence of *relA*, *M. smegmantis* exhibits an increased sensitivity to the anionic detergent SDS (Dahl, Arora et al. 2005). This could be due to altered cell wall in the mutant. This morphological defect could contribute to a decrease in long-term persistence in the mouse model because the cells may not be able to combat the host defense systems. In addition, cells lacking *relA* are presumably unable to enter the stringent, and subsequently a dormant, state inside the host.

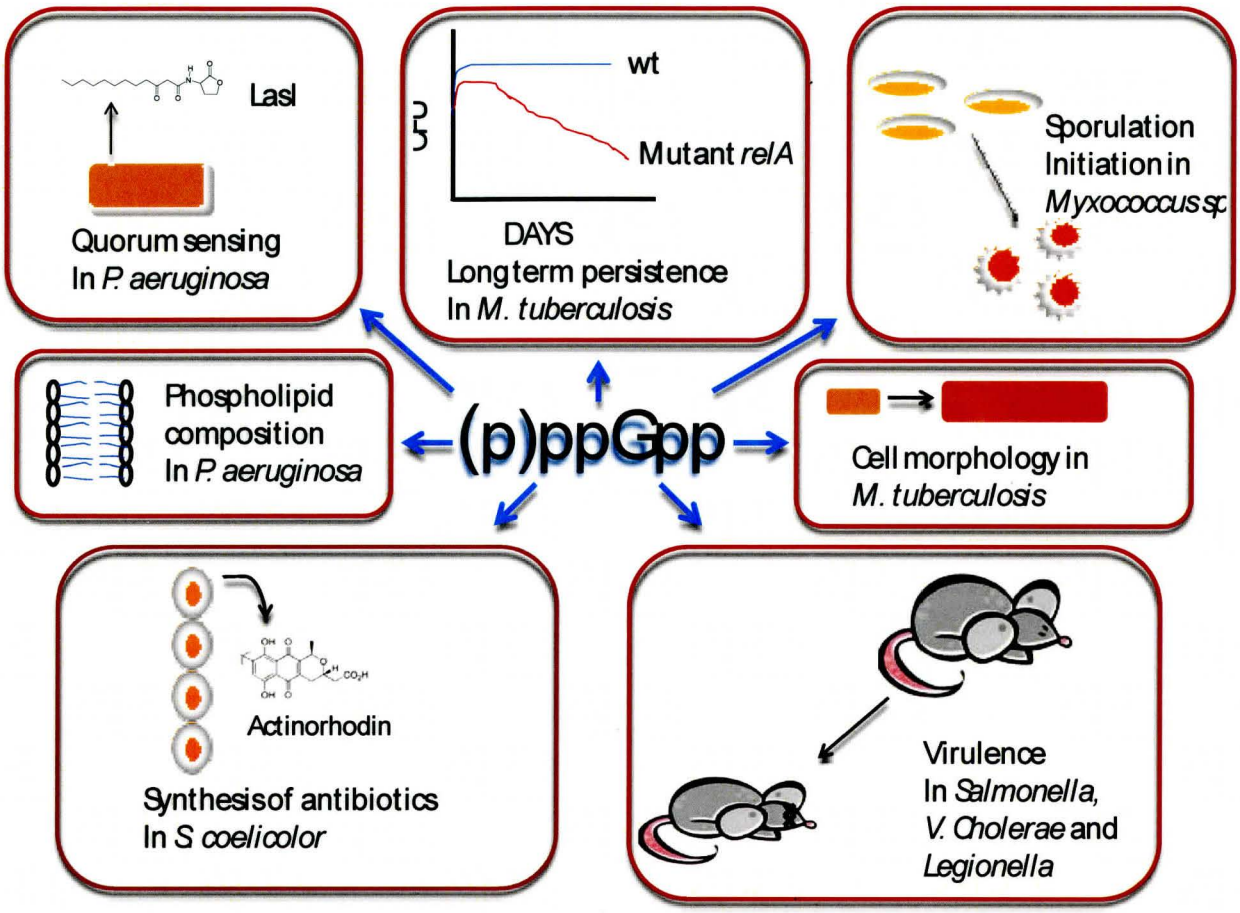


Figure 1.2 Various Diverse Physiological Processes in Bacteria that are Affected by (p)ppGpp Accumulation.

1.4.2 (p)ppGpp and initiation of sporulation in *Myxococcus sp.*

Some bacteria survive during times of extreme nutrient starvation by sporulation, which is the differentiation into a compact and durable entity. When *Myxobacteria* are deprived of essential nutrients, they undergo an energy consuming developmental change in which ~100,000 cells aggregate to form a multicellular fruiting body. The individual cells within the fruiting bodies become environmentally resistant and dormant myxospores (Harris, Kaiser et al. 1998). The species *Myxococccus xanthus* was shown to have elevated amounts of (p)ppGpp during carbon or amino acid starvation. In addition, earlier reports suggested that an increased level of (p)ppGpp is a definite requirement for the initiation of development of fruiting bodies (Harris, Kaiser et al. 1998). Furthermore, when an ectopic copy of RelA from *E. coli* was introduced, the expression of specific genes responsible for fruiting body development was detected. The rise in (p)ppGpp activates the population sensing pathway, the goal of which is to determine whether a sufficient number of starved cells exist to form a fruiting body (Crawford and Shimkets 2000). These results suggest the involvement of (p)ppGpp in development in *M. xanthus* (Harris, Kaiser et al. 1998; Crawford and Shimkets 2000). Also, these results support a model that states, individual cells monitor their nutritional status by means of changes in the intracellular concentration of (p)ppGpp such that accumulation of this nucleotide initiates the developmental process. As the cells become more starved for nutrients, (p)ppGpp also increases, signaling the cell to make fruiting bodies. Again a direct correlation was made between nutrient stress and (p)ppGpp accumulation.

1.4.3 (p)ppGpp effects the pathogenicity of bacteria

Pathogenic bacteria are very responsive to a wide range of environmental conditions including growth phase, temperature, osmolarity and signals within the intestinal or intracellular environments (Pizarro-Cerda and Tedin 2004). Pathogenic bacteria such as *Vibrio cholerae* and *Salmonella* show a RelA-dependent regulation of virulence factors during amino acid starvation (Crawford and Shimkets 2000; Haralalka, Nandi et al. 2003). Opportunistic pathogens such as *Legionella* show a similar adaptive response to amino acid starvation (Hammer and Swanson 1999). Of course, in order for a pathogen to gain entry into a host it is of vital importance for it to adhere to the host surface and colonize. A study done in *Listeria monocytogenes*, a known human pathogen, showed that this bacterium was not able to maintain binding to the host surface with a defective *relA* in murine models (Taylor, Beresford et al. 2002). Transcription of *relA* is induced following initial adhesion to the host. This suggests that the ability to mount a stringent response and undergo physiological adaptation to nutrient deprivation after attachment is essential for the subsequent growth of the adhered bacteria. Possibly during these stages for the adhered bacteria, (p)ppGpp induces expression of genes necessary for biofilm formation (Taylor, Beresford et al. 2002). Other reports of this type of behavior were seen in *S. typhimurium* where a RelA/SpoT knockout has attenuated virulence (Song, Kim et al. 2004). These results clearly implicate (p)ppGpp in virulence of some pathogenic bacteria.

1.4.4 (p)ppGpp is involved in antibiotic production

To survive in extreme conditions, and to obtain an edge in competition over other organisms, some bacteria synthesize antibiotics and secondary metabolites. Bacteria of the genus *Streptomyces* are of particular interest because they synthesize a wide array of industrially

and medically important antibiotics. Strains with a relaxed phenotype (lacking *relA*) show a severe impairment in antibiotic production. In *S. coelicolor*, a *relA* null strain was unable to produce actinorhodin in tandem with a lack of (p)ppGpp (Chakraburtty and Bibb 1997). In addition, the relaxed phenotype involved a marked delay in the onset and extent of morphological differentiation. Production of secondary metabolites such as actinorhodin, are generally confined to stationary phase when nutrients are limited (Chakraburtty and Bibb 1997). Strains lacking *relA* are possibly unable to enter stationary phase and produce the antibiotics necessary to combat other bacteria. This phenotype is also seen in *B. subtilis*, where both (p)ppGpp and GTP regulate the production of bacilysin (Inaoka, Takahashi et al. 2003). Obviously, these organisms require (p)ppGpp for production of these antibiotics during nutrient starvation, however the synthesis pathway of secondary metabolites is extremely complex and further investigation into the exact role (p)ppGpp has in the pathway is necessary.

1.4.5 (p)ppGpp and Quorum sensing in *P. aeruginosa*

P. aeruginosa has the ability to occupy a wide range of environmental niches and can therefore respond to different nutritional conditions. This pathogenic bacterium is able to infect many hosts including humans, nematodes and insects and thus there are many processes related to virulence in *P. aeruginosa* that could be impacted by the stringent response. One of these processes is quorum sensing. Van Delden et al. in 2001 reported a link between quorum sensing and the stringent response by overexpressing an ectopic copy of *relA* from *E. coli* and looking at the activation of quorum sensing (van Delden, Comte et al. 2001). This was further investigated in an infection model that suggested that the stringent response is a mediator of quorum sensing in *P. aeruginosa* (Erickson, Lines et al. 2004). During establishment of host infection, *P. aeruginosa* could be exposed to nutritional stress prior to reaching a critical cell density.

Therefore, the stringent response might prematurely activate the production of quorum-sensing-regulated virulence factors (van Delden, Comte et al. 2001). This pathway may help *P. aeruginosa* to adapt to nutritional deficiencies during infection.

1.5 YwaC and Cell Wall Stress

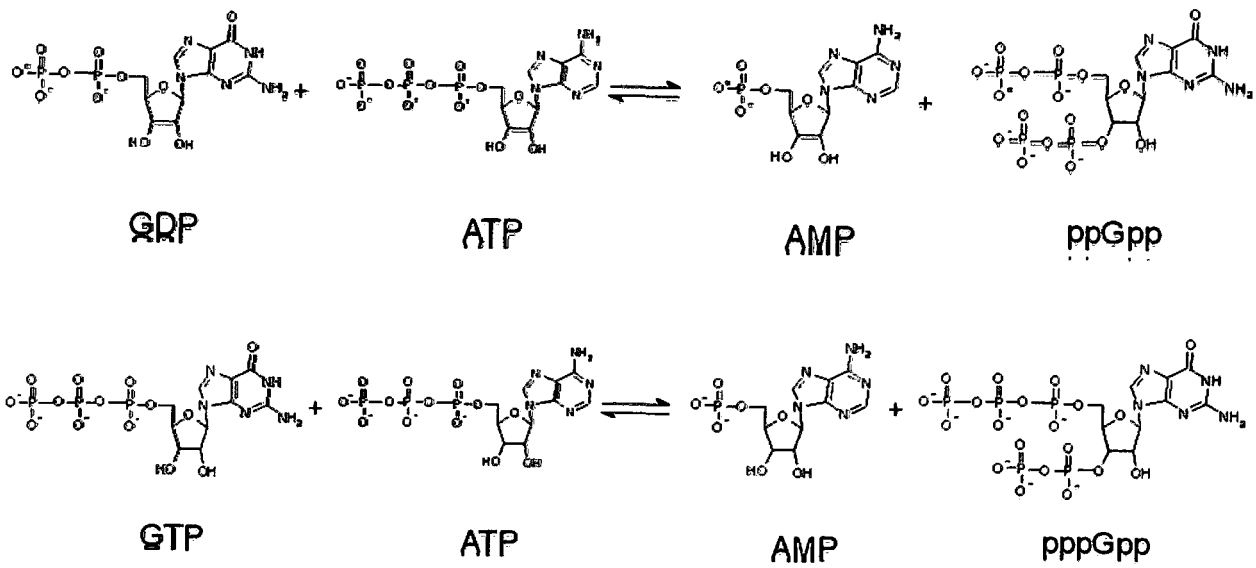
In *B. subtilis* 168, the 24.5 kDa protein, YwaC, was found to be similar through sequence homology to the (p)ppGpp pyrophosphokinase family of enzymes. Several functionally significant amino acid residues such as those in Rel*Seq* (Hogg, Mechold et al. 2004) – namely Asp^{264}, Tyr^{308}, Glu^{323} and Ala^{335} were conserved in YwaC (Figure 1.1B). Work done by Nanamiya, H. et al. in 2008 confirmed YwaC as having (p)ppGpp synthase activity. Nanamiya et al. also identified another putative (p)ppGpp synthase, YjbM, as having similar activity. Before this work it was believed that RelA was the sole enzyme involved in the accumulation of (p)ppGpp in *B. subtilis*. This group of researchers initially did careful functional complementation studies of a RelA mutant in *E. coli* and showed that accumulation of (p)ppGpp could be restored in the cell by overexpression of YwaC (Nanamiya, Kasai et al. 2008). They also performed *in vitro* enzymatic assays using thin layer chromatography (TLC) to detect nucleotide formation and consumption. These assays again show that YwaC is a (p)ppGpp synthase capable of utilizing either GDP or GTP as substrates along with ATP (Scheme 1.1). Sequence investigation, however, showed that a common domain in the RelA-SpoT family, the (p)ppGpp phosphohydrolase domain, is completely absent in the YwaC polypeptide. This suggested that the function of this enzyme might be limited to synthase activity only (Nanamiya, Kasai et al. 2008).

Unlike the cell wall of Gram-negative bacteria, which is comprised mostly of peptidoglycan, Gram-positive bacteria, in addition, contain large amounts of the polymer teichoic acid, covalently attached to peptidoglycan. In *B. subtilis* 168, the polymer consists of poly(glycerol phosphate). The model for teichoic acid suggests that the polymer is synthesized on the cytoplasmic face of the membrane on an undecaprenol-phosphate molecule. This carrier molecule is also utilized for peptidoglycan biosynthesis. It is thought that the polymer is synthesized in a step-wise manner, through addition at the non-reducing end of *N*-acetylglucosamine-1-phosphate (TagO), *N*-acetylmannosamine (TagA) and 20 to 60 residues of glycerol phosphate (TagB and F) to prenol-phosphate substrate. The activated precursor GDP-glycerol is synthesized by glycerol phosphate citidyltransferase, TagD, and is used as the source for glycerol phosphate. In unpublished microarray studies from the Brown lab, *ywaC* was among nine other genes shortlisted that were highly upregulated in response to lesions in the teichoic acid pathway. A cell-based luminescent promoter-reporter was then constructed that contained the *lux* genes from *Photerhabdus luminescens* transcriptionally fused to the *ywaC* promoter (P_{ywaC}). P_{ywaC} had the most robust response to teichoic acid depletion showing 4-, 12-, and 20-fold increases in luminescence on depletion of TagB, D and F, respectively. The P_{ywaC} *lux* reporter construct was then screened against a commercially available library (Prestwick) of 1120 small molecules annotated for biological activity (D'Elia, Millar et al. 2008 unpublished). The P_{ywaC} reporter responded almost exclusively to cell wall active compounds. The antibiotics that elicited the highest response were separated into two groups with mechanism of actions focusing on specific steps in peptidoglycan biosynthesis. The one group of antibiotics is known to be involved in undecaprenol-phosphate-linked steps (bacitracin, ramoplanin, vancomycin, fosfomycin and fosmidomycin). The other group of antibiotics is involved in peptide

crosslinking steps (cycloserine and beta lactams) (D'Elia, Millar et al. 2008 unpublished). These results suggest a genetic interaction between isoprenoid, peptidoglycan and teichoic acid biogenesis evident through the regulation of expression of the gene *ywaC*.

Through different transcriptomic studies, *ywaC* has been implicated in being regulated by both extracytoplasmic function (ECF) sigma factors σ^M and σ^W regulons (Eiamphungporn and Helmann 2008; Helmann 2006). These two sigma factors are among five others in *B. subtilis* that are often involved in regulating genes associated with some aspect of the cell envelope or transport process. Genes implicated in the σ^W regulon are induced by many cell wall antibiotics such as vancomycin, cephalosporin and alkaline shock (Jordan, Hutchings et al. 2008). Various cell wall antibiotics such as bacitracin, fosfomycin and vancomycin induce the σ^M regulon. Acidic pH, heat, ethanol, and superoxide stress can also induce genes involved in the σ^M regulon. Possibly through these response regulons, *ywaC* is upregulated and subsequently induces the production of (p)ppGpp to help evade cell death. In fact, recent experiments conducted by Nanamiya et al. and collegues showed the involvement of YwaC in the accumulation of ppGpp in *B. subtilis* in response to alkaline shock (Nanamiya, Kasai et al. 2008). A much earlier study done by Ishiguro et al. connected penicillin tolerance to the accumulation of (p)ppGpp and the subsequent inhibition of both phospholipid and peptidoglycan synthesis (Rodionov and Ishiguro 1995). These results further validate that accumulation of (p)ppGpp affects aspects of the cell wall in response to environmental stresses. The actual interactions that (p)ppGpp could make in these stress response regulons is open for much investigation. It is possible that (p)ppGpp is synthesized in response to cell wall stress in order to slow cell growth and protect the cell from lysis that would be exacerbated by rapid growth. Alternatively, (p)ppGpp could be involved in antibiotic resistance mechanisms by upregulating the production of ABC transporters specific to

the antibiotic. Clearly, the regulation of envelope stress in both Gram-negative and Gram-positive bacteria is extremely complex. The regulatory network orchestrating the cell envelope stress response in *B. subtilis* is well investigated. It involves at least three sigma factors (σ^X, σ^W, σ^M), which together target ~150 genes and 4 two-component systems (TCS), three of which are linked to ABC transporters. Bacitracin for example, specifically induces four signaling systems, the ECF σ factor σ^M, and the TCS LiaRS, BceRS and YvcPq (Mascher, Margulis et al. 2003). Many of these response regulons are not mutually exclusive, therefore, this leaves open the possibility that YwaC and other small (p)ppGpp synthases yet to be characterized could be involved in responding to cell wall stress or stresses other than nutritional deprivation.



Scheme 1.1 Reactions catalyzed by YwaC

1.6 Thesis Accomplishments

Although there is some work already published on YwaC, no in depth biochemical analysis or functional analysis has been conducted to date. To this end, the following was performed:

1. Gene *ywaC* was cloned into an over-expression vector and YwaC was purified to adequate homogeneity.
2. A robust and reliable *in vitro* biochemical assay was developed in order to determine steady state kinetic constants.
3. A knockout strain was constructed by replacing *ywaC* with a spectinomycin resistance cassette on the chromosome of *B. subtilis* 168.
4. Experiments were conducted that monitored the effect of a *ywaC* knockout in comparison to wildtype in order to investigate the function of YwaC.

CHAPTER 2 – CLONING AND PURIFICATION OF YwaC

2.1 Introduction

In order to investigate the enzymatic properties of YwaC, it was necessary to clone, over-express and purify relatively large amounts of enzyme. This was accomplished using an *E.coli* over-expression strain (EB 1836) along with pDEST17™ serving as the over-expression vector containing an N-terminal His-tag. The method described below was employed for the purification of YwaC.

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), Imidazole (IMD), L-arabinose and ampicillin (AMP) were obtained from BioShop Canada Inc. (Burlington, ON). Glycerol was purchased from Caledon Laboratory Chemicals (Georgetown, ON). All other compounds were purchased from Sigma (Oakville, ON) unless specified.

2.3 Methods

2.3.1 Construction of YwaC Over-expression Plasmid

All plasmids were propagated in *Escherichia coli* Novablue cells (Novagen, Madison, WI) (Table 2.1). The gene *ywaC* was amplified from *B. subtilis* genomic DNA using primers HD01 and HD02 (Table 2.2). These primers were constructed to amplify solely the *ywaC* gene excluding the start ATG codon. The blunt-ended product was inserted at the *Eco*RV restriction

site of pBluescript SK II+ (Stratgene, La Jolla, CA). Subsequently, *ywaC* was amplified off the plasmid using primers HD03 and HD04 (Table 2.2). The gene product was then cloned into pDEST17™ according to manufacturer's instructions. This construct, called pDEST17™_YwaC (Table 2.1), was confirmed by sequencing (MOBIX, McMaster University). Competent *E. coli* BL21-AI cells (Table 2.1) were transformed using established methods (Sambrook, Fritsch *et al.* 1989) and ultimately used for the over-expression and purification of YwaC.

2.3.2 Over-expression and Purification of YwaC

The over-expression strain (EB1836) was grown on Luria-Bertani (LB)-agar supplemented with ampicillin (AMP) (50 μg/mL). Single colonies were used to inoculate 50 mL of LB containing 50 μg/mL AMP. These cultures were grown at 37 °C shaking at 250 revolutions per minute (rpm). For the large-scale purification of YwaC, 10 mL of the overnight cultures were used to inoculate 1 L of LB containing 50 μg/mL AMP. Cells were grown at 37 °C shaking at 250 rpm until OD_{600} (optical density at 600nm) of approximately 0.35 was attained. At this time, cultures were induced with for 3.5 hrs at 37 °C with 0.2 % arabinose. The cells were harvested by centrifugation (5,000 × *g*) for 15 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 and pelleted again at 3,000 × *g* for 20 min. Cell pellet was frozen at -20 °C. Beyond this point, all steps were performed at 4 °C. The cell pellets were re-suspended in lysis buffer (25 mM HEPES (pH 7.5), 500 mM NaCl, 10% glycerol, 20 mM Imidazole, and 1 mM DTT) and lysed by three passes through a French Press (20,000 psi). To remove cell debris, the lysed cells were centrifuged (14, 000 × *g*) for 1 hour at 4 °C.

Table 2.1 List of Strains and Plasmids

Name	Description	Source
Bacillus subtilis		
EB6	*hisA1 argC4 metC3*	Bacillus Genetic Stock Centre (L6602)
EB 1961	*hisA1 argC4 metC3 ywaC::Spec*^{R}	This work
Escherichia coli		
Novablue	*endA1 hsdR17 (rK12*$^{-}$ *mK12*$^{+}$*) supE44 thi-1 gyrA96 relA1 lac[F'proA*$^{+}$*B*$^{+}$ *lacI*^{q}*ZΔM15::Tn10*	Novagene
BL21-AI	F^{-} *ompT hsdSB(rB*$^{-}$ *mB*$^{-}$*) gal dcm araB::*T7RNAP-*tetA*	Invitrogen
EB 1834	Novablue harboring plasmid *ywaC*-pBluescript SK II+	This work
EB 1835	Novablue harboring plasmid *ywaC*-pDONR201	This work
EB 1836	BL21-AI harboring plasmid *ywaC*-pDEST17	This work
Plasmids		
pBluescript SK II+	General cloning vector	Stratagene
pDONR201	Gateway entry vector	Invitrogen
pDEST17	Gateway T7 expression vector	Invitrogen

Table 2.2 List of Oligonucleotides

Oligonucleotide	Sequence
HD01	5'-GATTTATCTGTAACACATATGGACG-3'
HD02	5'-TTAATCCACTTCTTTCTTAATCC-3'
HD03	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGATTTATCTGTAACACATATG-3'
HD04	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAATCCACTTCTTTCTTAATCC-3'
HD05	5'-CGTCCTCATACGTTAACCGC-3'
HD06	5'-TAATCATGGTCATAGCTGTTTCCTGTGTTCGTCATCTCCTTTAACGGAACT-3'
HD07	5'-GAAATAAAATGCATCTGTATTTGAATGAAAAGACGGCACCCAAGTGCCGTC-3'
HD08	5'-CCTGACTTATTACGAGAAA-3'
HD09	5'-ACAGGAAACAGCTATGACCATGATTA-3'
HD10	5'-CATTCAAATACAGATGCATTTTATTTC-3'
HD11	5'-AACCAAACAAATAAGGGTGAG-3'
HD12	5'-AAGAAACCGAAAACCATTAAG-3'

Chromatography was performed on a fast-protein liquid chromatography (FPLC) system (Amersham-Pharmacia) in a cold room at 4 °C. The eluted protein was monitored at 280nm by the absorbance detector. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze fractions at all steps of this procedure. The clarified lysate was loaded onto a 1 mL (0.7 × 2.5 cm) HisTrap™ HP chelating column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) which was equilibrated at 1 mL/min with buffer containing 25 mM HEPES (pH 7.5), 500 mM NaCl, 10 % glycerol, 20 mM IMD, and 1 mM DTT. Initially, unbound protein was washed from the column in 40 column volumes (C.V.). Then a linear IMD gradient, from 20 mM to 1 M IMD over 60 C.V. was employed. The column was then re-equilibrated for 10 C.V.. YwaC eluted from the column at an IMD concentration of approximately 500 mM, which is around 20 C.V. into the gradient. Appropriate fractions were pooled and buffer exchanged using centrifugal concentrators with a molecular weight cut-off of 5-10 kDa (Amicon, Fisher Scientific, Napean, ON). The buffer used to exchange contained 25 mM HEPES (pH 7.5), 150 mM NaCl, 10 % glycerol and 1 mM DTT. Remaining protein was pooled, frozen into small aliquots, and stored at -80 °C.

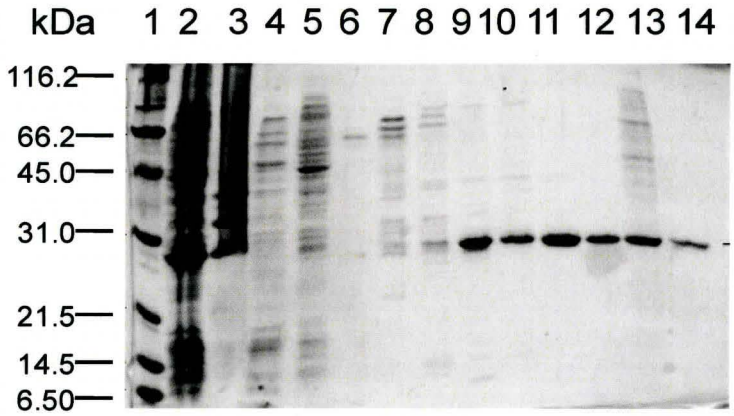


Figure 2.1 Purification of His-tagged YwaC. Protein samples at various steps of the purification of YwaC were analyzed by discontinuous SDS-PAGE using 3 % stacking and 15 % separating polyacrylamide gel visualized with Coomassie Blue stain. Samples were boiled for 5 minutes in 2X loading buffer (62.5 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). Protein was eluted from a 1 mL His-trap affinity column using a linear gradient of imidazole up to 1M. *Lane 1*, Broad band molecular weight ladder; *Lane 2*, clarified lysate; *Lane 3*, insoluble pellet; *Lane 4 – 14*, His-Trap affinity column fractions which eluted around 500 mM imidazole.

2.3.3 Determination of Protein Concentration

Pure protein was quantified using the procedure outlined by Gill and von Hippel (Gill and von Hippel 1989). In this method, protein concentration is determined using the calculated molar absorptivity and measuring the absorbance of the protein denatured with 7 M guanidinium hydrochloride at a wavelength of 280 nm.

2.4 Results and Discussion

A one-column procedure was employed to purify YwaC to appropriate purity for *in vitro* biochemical studies. A distinct band representing pure YwaC at 24.5 kDa is depicted in lanes 9-14 of Figure 2.1. This procedure yielded approximately 5 mg of purified enzyme from each 1 L culture.

CHAPTER 3 – *In vitro* BIOCHEMICAL CHARACTERIZATION OF YwaC

3.1 Introduction

Most steady state kinetic characterization has been conducted on RelA from *E. coli* and the bifunctional enzymes from *M. tuberculosis*, *B. brevis* and *stearothermophilus* (Justesen, Lund *et al.* 1986; Sy and Akers 1976; Fehr and Richter 1981). The RSH proteins from these organisms were reported to possess millimolar K_m values for both GDP and GTP. No steady-state kinetic characterization has been conducted to date on YwaC or other small alarmone synthases (SASs) from organisms in the genomic class *Firmicutes* as discussed in Section 1.1. Recent work done by Nanamiya et al. in 2008 confirms that YwaC does indeed have (p)ppGpp pyrophosphokinase activity.

With pure recombinant YwaC, it was necessary to confirm that our pooled enzyme catalyzed the (p)ppGpp pyrophosphokinase reaction. The product formed was analyzed by mass spectrometry to confirm the presence of (p)ppGpp. In addition, a reliable and robust *in vitro* biochemical assay was constructed to identify the steady state kinetic constants for the forward reaction.

3.2 Materials

N-2-Hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), dithiothreitol (DTT) and urea were obtained from BioShop Canada Inc. (Burlington, ON). Radiolabeled [8-^{3}H] GDP (ammonium salt) (11.9 Ci/mmol) was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Radiolabeled [α-^{32}P] GTP (3000 Ci/mmol) was obtained from Perkin Elmer (Waltham, MA). Glycerol and acetonitrile were bought from Caldon Laboratory Chemicals (Georgetown, ON). Guanosine tetraphosphate (ppGpp) was purchased from TriLink

Biotechnologies (San Diego, CA). Potassium phosphate was obtained from EMD (Darmstadt, Germany). All other compounds were purchased from Sigma (Oakville, ON) unless specified.

3.3 Methods

3.3.1 Development of the YwaC HPLC Enzymatic Assay

A stopped assay was used to monitor the reaction catalyzed by YwaC. A typical reaction contained 25 mM HEPES (pH 7.5), 1 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, 1 mM GDP, 2 μCi [8-^{3}H] GDP 10 μM YwaC in a total volume of 50 μL. After 20 min at room temperature, 150 μL of 8 M urea (6 M final concentration) was added to stop the reaction.

The amount of [8-^{3}H] ppGpp produced during the reactions was monitored by high performance liquid chromatopgraphy (HPLC) (Waters 600 Pump and Controller, Waters 486 Tunable Absorbance Detector, Waters 717 Autosampler, Waters busSAT/IN module, Milford, MA) using a NovaPak® C_{18} 3.9 × 150 mm column (Waters, Milford, MA) set to an analogue full scale of 25, 000. The scintillation cocktail (Ultima-Flo M, Canberra Packard Canada, Mississauga, ON) was pumped at a flow rate of 3 mL/min. After injection of the sample onto the column (50 μL), reaction components were separated using the following eluates: 15 mM potassium phosphate, and 10 mM tetrabutylammonium hydroxide (TBAHS) pH 7.2 (A); and 35 mM potassium phosphate, 10 mM TBAHS, and 30 % Acetonitrile (pH 7.2) (B). The following parameters were used for elution of the material from the PIC column (1 mL/min): 2 min. at 0% B; a linear gradient of 0 to 100% B in 5 min.; 5 min. at 100 % B; 1 min. linear gradient of 100 to 0% B ; and re-equilibrated for 5 min. at 0 % B. The amount of [8-^{3}H] GDP or [α-^{32}P] GTP

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 ppGpp was the product of the enzyme-catalyzed reaction, its identity was confirmed by molecular mass analysis using negative ion electrospray mass spectrometry (Q-trap LC-MS/MS, Applied Biosystems). Approximately 3 mL of separated reaction material was collected from the C_{18} column and then speed vacuumed to a volume of 200 µL. The concentrated ppGpp fraction obtained from the PIC column was loaded onto a 120 Ultrahydrogel column 7.8 × 300 µm (Waters, Milford, MA). Separated reaction material was collected from the size exclusion column by eluting with ddH_20 for 30 minutes with detection set at 250 nm (Waters 600 Pump and Controller, Waters 486 Tunable Absorbance Detector, Milford, MA). The purified sample was prepared for mass spectrometry by removal of TBAHS using a Savant SC210A Speed-Vac Plus (Halbrook, NY).

3.3.3 Steady State Analysis of YwaC

All steady state analysis reactions were performed at room temperature (22 °C). Initially YwaC at a concentration of 0.1 µM was incubated with various GDP concentrations (2.5 to 50 µM) and 2 µCi [8-^{3}H] GDP in assay buffer (25 mM HEPES, 1 mM DTT, 10 mM $MgCl_2$, pH 8.0) for 20 to 120 minutes. Quenched reactions were resolved by paired ion chromatography on a Waters 600 high-performance liquid chromatography system (Milford, MA) as described in

Section 3.3.1. For steady state kinetic analysis with GTP as the substrate the concentration of YwaC was 1 μM. Enzyme was incubated with various GTP concentrations (3 to 100 μM) and 1 μCi [α-^{32}P] GTP in the same assay buffer outlined above for 1 to 30 minutes. Separation of nucleotides was conducted as described in Section 3.3.1. All reactions were performed while keeping product formation below 10 % to allow for proper analysis of steady state kinetic constants.

3.4 Results

3.4.1 Mass spectrometry of ppGpp

When the product of YwaC was purified and subjected to negative ion electrospray mass spectrometry (Figure 3.1), a molecular weight of 599.1 was obtained. This value is near the calculated molecular weight of ppGpp of 599.2 acid free. This confirms that the peak visualized by the HPLC software is indeed ppGpp and that it was appropriate for this peak to be measured in order to monitor the ppGpp pyrophosphokinase activityof YwaC.

3.4.2 Linearity of the HPLC assay

An HPLC assay was used to monitor the (p)ppGpp pyrophosphokinase activity of YwaC due to its high through-put and reproducibility. Because the detection using absorbance at 260 nm was insufficiently sensitive to monitor the production of (p)ppGpp in this assay, an in-line radioactivity detector was used to measure the shift of tritium label from [8-^{3}H] GDP to ppGpp.

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 0.2 min^{-1} was calculated from this experiment, which is comparable to the actual k_{cat} (0.13 ± 0.06, table 3.1). Under these conditions only 1 to 5% of substrate is being converted to product. For the determination of steady state constants, a more appropriate physiological concentration of enzyme was employed.

3.4.3 Steady-state Analysis of YwaC

The kinetic constants k_{cat} and K_m were determined from a plot of initial velocity versus [GDP] by use of Sigma Plot 2000 software to fit the data to a single rectangular two-parameter hyperbolic function. At steady state, a k_{cat} of 0.13 ± 0.06 min^{-1} and a K_m of 5 μM were observed with GDP as the substrate and a k_{cat} of 0.11± 0.03 min^{-1} and K_m of 6 μM were determined with GTP. The K_m values are not comparable to the K_m of 700 μM previously reported for the bifunctional full length Rel from *Mycobacterium tuberculosis* (Avarbock, Avarbock et al. 2005). However, the k_{cat} for Rel*Mtb* with the ribosomal activating complex (RAC) at 8.4 s^{-1} compared to without at 0.4 s^{-1} is quite dramatic (Avarbock, Avarbock et al. 2005). Therefore although the k_{cat} values for YwaC are slow, this leaves open the concept of an effector molecule that could speed the activity of the reaction as is the case for Rel enzymes during the stringent response. In addition, although YwaC has the same activity as the synthase domain of the bifunctional

enzyme Rel*Mtb*, the K_m values could be quite different due to the physiological function of YwaC.

The specificity constant k_{cat}/K_m, which describes the coming together of enzyme and substrate to form a productive complex, is the apparent rate constant at a low substrate concentration (Northrop 1998). The specificity constant for YwaC with GDP is 4.4×10^2 and 3.1×10^2 with GTP as the substrate.

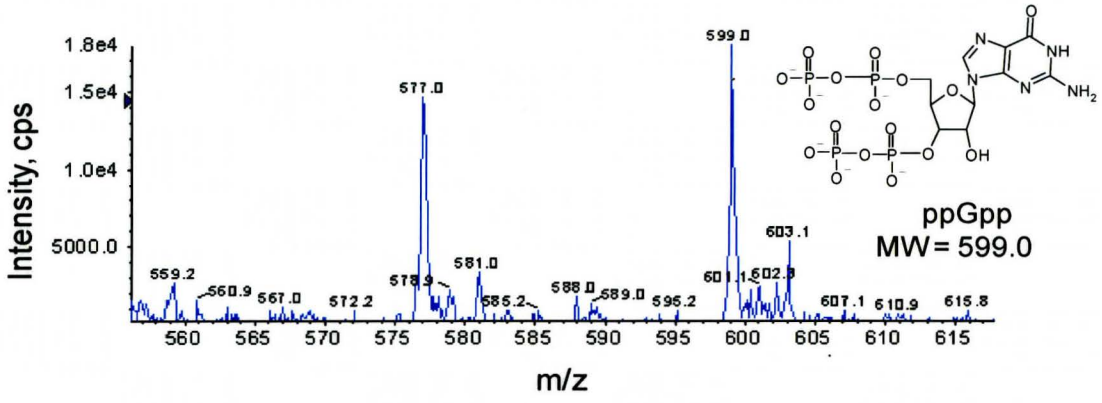


Figure 3.1 Conformation of Enzymatic Production of ppGpp by Electrospray Mass Spectrometry. The nucleotide ppGpp was purified initially from a C_{18} PIC column attached to an HPLC (see Section 3.3.1) and concentrated to approximately 5 μM. This sample was subjected to electrospray mass spectrometry on a LC quadropole mass spectrometer. A molecular weight of 599.0 was observed, which is comparable to the calculated molecular mass of ppGpp (599.2 acid free). The second peak at 577 may represent a fragmentation product. Mass spectrometry was performed and interpreted by Kalinka Koteva, Dr. Gerry Wright's lab, Biochemistry Department, McMaster University.

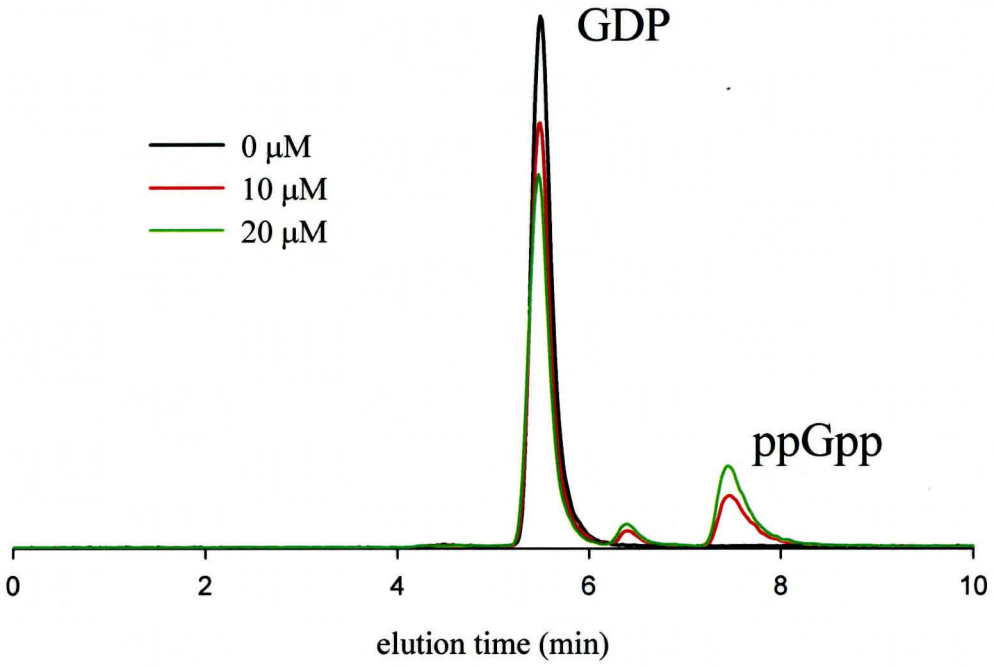


Figure 3.2 HPLC Chromatogram Illustrating Radioactive Detection of ppGpp. The highlighted peaks represent [8-^{3}H] ppGpp produced for 0, 10 and 20 μM of YwaC after 60 minute incubation measured by the Radiomatic Scintillation Detector (Packard). The larger peaks represent [8-^{3}H] GDP. The conditions were as decribed in Section 3.3.1. Briefly, either 0, 10, or 20 μM of YwaC was used with 1 mM ATP and 1mM GDP. For quantification of the ppGpp the integration of those peaks were performed using the Millenium^{32} HPLC software (Waters).

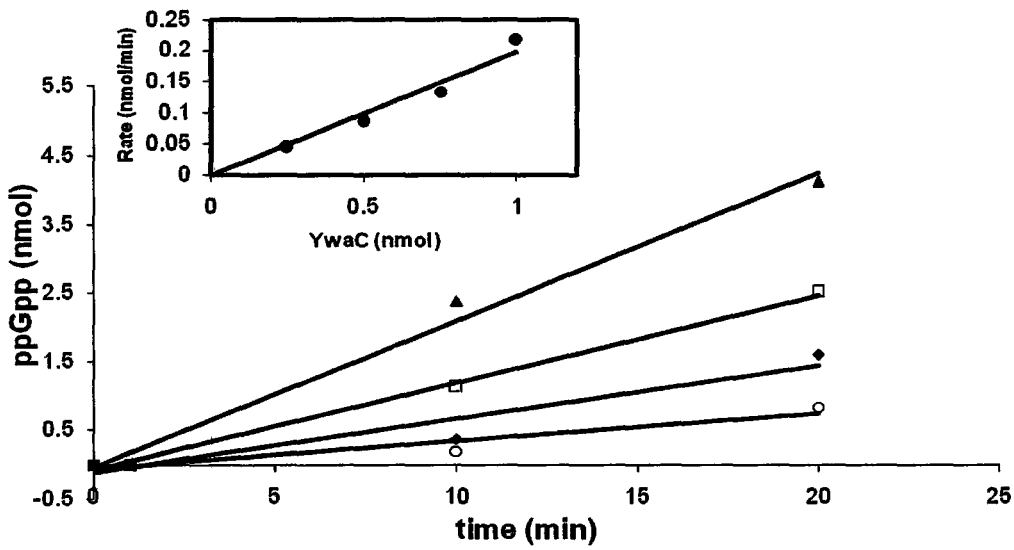


Figure 3.3 Linearity of the YwaC HPLC Assay. Reactions with increasing amounts of enzyme over 20 minutes were monitored by HPLC, equipped with an in-line radioactivity detector set to an analogue full scale of 10,000. The scintillation cocktail (Ultima-Flo M, Canberra Packard Canada, Mississauga, ON) was pumped at a flow rate of 3 mL/min. The volume of reaction injected was 50 µL. The amount of enzyme added to the reaction were 20 µM (▲), 15 µM (□), 10 µM (◆), and 5 µM (○). The concentration of ATP and GDP were both 1 mM. Lines were drawn using linear regression in Microsoft excel. The slope of the line is equal to the turnover number (0.2 min^{-1}).

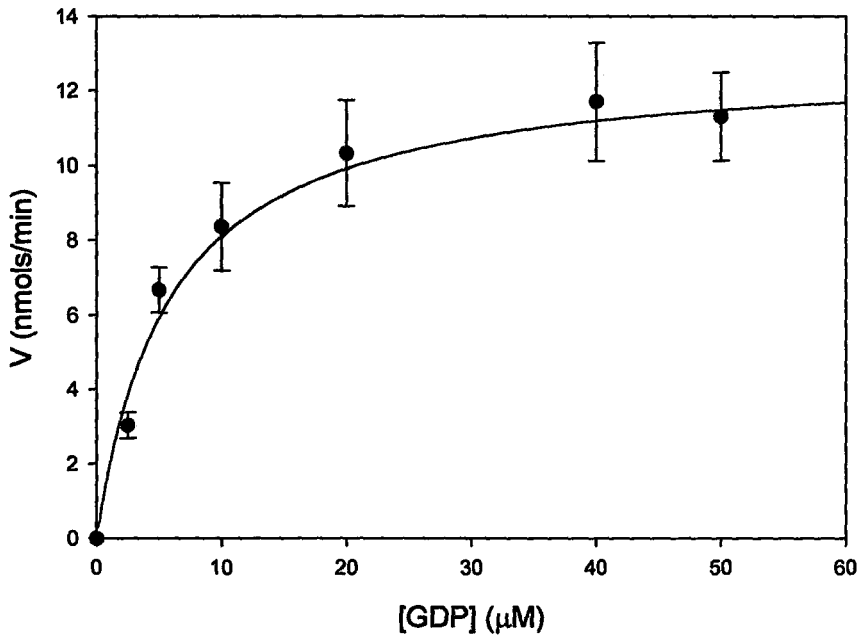


Figure 3.4 Michaelis-Menten plot describing the steady-state relationship between YwaC and GDP as the substrate. Reactions contained 0.1 μM YwaC, 750 μM ATP, 2 μCi [8-^{3}H] GDP, 10 mM $MgCl_2$, 25 mM HEPES (pH 7.5), and 2.5, 5, 10, 20, 40 or 50 μM of GDP. All reactions were performed in a reaction volume of 50 μL and quenched with 6 M urea. The data were fitted to a single rectangular two-parameter hyperbolic function on Sigma Plot 2000.

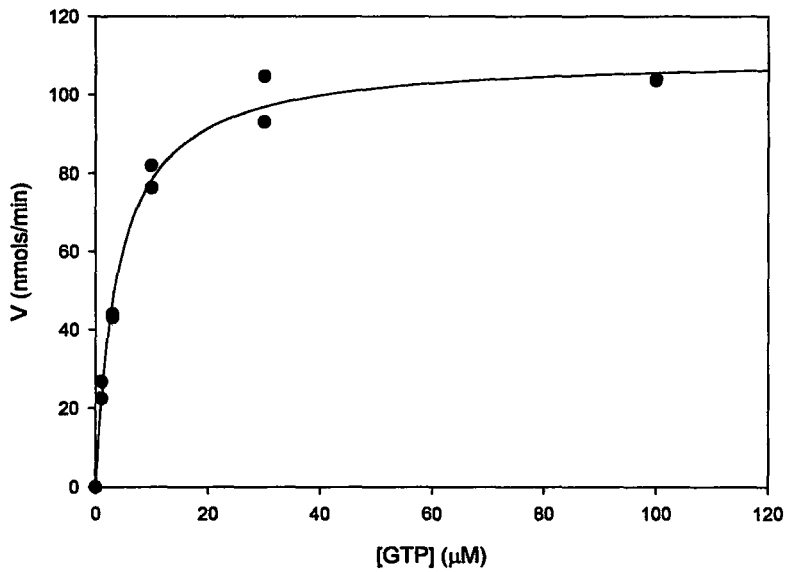


Figure 3.5 Michaelis-Menten plot describing the steady-state relationship between YwaC and GTP as the substrate. Reactions contained 1 μM YwaC, 750 μM ATP, 1 μCi [α-^{32}P] GTP, 10 mM $MgCl_2$, 25 mM HEPES (pH 8.0), and 3, 5, 10, 30, or 100 μM of GTP. All reactions were performed in a reaction volume of 50 μL and quenched with 6 M urea. The data were fitted to a single rectangular two-parameter hyperbolic function on Sigma Plot 2000.

Table 3.1 List of Kinetic Constants for YwaC

Substrate	K_m[a] (μM)	k_{cat}[a] (min^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
GDP	5 ± 0.9	0.13 ± 0.06	4.4×10^2
GTP	6 ± 0.4	0.11 ± 0.03	3.1×10^2

[a] Standard error in the value based on data fitted to rectangular hyperbolic function

3.5 Discussion

Compared to the extensive research that has been reported on (p)ppGpp as a global alarmone especially in relation to the stringent response, very little kinetic knowledge is known about the enzymes synthesizing and metabolizing (p)ppGpp. Steady state analysis has been done in *B. brevis* and *stearothermophilus*, *M. tuberculosis* and *E. coli* (Sy and Akers 1976; Fehr and Richter 1981; Avarbock, Avarbock et al. 2005; Justesen, Lund et al. 1986). In the class *Firmicutes* small alarmone synthases have been discovered in addition to the bifunctional RSH protein. YwaC is one such protein recently identified in *B. subtilis* that contains (p)ppGpp synthase activity. No kinetic data has been reported on this enzyme and therefore, steady state analysis was conducted.

Through the development of an *in vitro* biochemical assay, YwaC was again confirmed to synthesize both ppGpp and pppGpp in only buffer, Mg^{2+}, ATP and either GDP or GTP as had been previously reported (Nanamiya, Kasai et al. 2008). Steady state kinetic parameters were determined by monitoring initial velocity versus substrate concentration. Data were fit using Sigma Plot 2000 to a single rectangular two-parameter hyperbolic function. The affinity of YwaC for both substrates is virtually the same for both substrates with a slight preference for GDP over GTP. In addition, YwaC appears to synthesize product efficient when either GDP or GTP is present as proven by a similar k_{cat} of 0.13 ± 0.06 min^{-1} for GDP compared to 0.1 ± 0.03 min^{-1} for GDP.

As seen for YwaC, there is a higher affinity for GDP over GTP for the (p)ppGpp synthase domain of the RSH bifunctional proteins in *B. brevis*, *M. tuberculosis*, and *E.coli.* Physiologically this makes sense for *E. coli* as this organism was proven to produce more ppGpp than pppGpp. Some preliminary experiments done by Cashel et al. have reported that high

(p)ppGpp induction, in *E. coli*, inhibits growth about eightfold more severely with ppGpp than with pppGpp (Potrykus and Cashel 2008). The opposite is seen in *S. aureus*, as it produces more pppGpp than ppGpp (Cassels, Olivia 1995). The physiological reason for this difference is not known. It is difficult to predict with certainty which substrate YwaC would prefer *in vivo*. Definitely, YwaC utilizes both GDP and GTP as substrates *in vitro*; however, there is a possibility that other nucleotides may be used as the acceptor. For instance, a ribosome-independent purine nucleotide pyrophosphotransferase purified from *Streptomyces morookaensis*, has a broad substrate specificity as it can utilize both guanine and adenine nucleotides as acceptors (Oki, Yoshimoto 1975). Furthermore, the three dimensional conformation of YwaC *in vitro* may not be the preferred state in the cell and thus *in vivo* the substrate specificity may change drastically depending on its function and the demands of the cell.

The turnover constant (k_{cat}) describes the efficiency of the enzyme to capture the substrate and subsequently convert and release the final product. The k_{cat} for YwaC is very slow when either GDP or GTP as the substrate. Typically, for RSH enzymes the k_{cat} is very slow when the effector molecule is not present, which in the case of the stringent response is the stalled ribosome. Wildtype Rel*Mtb* synthesis activity is activated 2-fold from basal levels by uncharged tRNA alone and 80-fold by a complex of uncharged tRNA, ribosomes, and cognate mRNA (RAC) (Avarbock, Avarbock 2005). The C-terminal fragment of Rel*Mtb* is not activated above basal levels by the RAC and this indicated that the 288 C-terminal amino acids contain an area of Rel*Mtb* regulation. This region of the protein is believed to make contacts with the ribosome during the stringent response creating a conformational change favouring (p)ppGpp synthesis. This binding region on Rel*Mtb* corresponds to the C-terminal domain

(residues 455-744) of RelA which is also involved in regulating activity (Yang and Ishiguro 2001). In the case of Rel*Seq* the C-terminus is also able to bind to the ribosome and sense the presence of nonacylated tRNAs. However, the addition of the C-terminal portion of Rel*Seq* to the catalytic fragments leads to a 50-fold activation of the hydrolase and a simultaneous 25-fold reduction of the hydrolase (Mechold, Murphy 2002). These observations suggest that in both Rel*Mtb* and Rel*Seq* systems, the C-terminus is necessary to respond to amino acid deprivation but the two species have evolved different strategies for utilizing the C-terminus based on their environmental niches. YwaC is completely lacking this C-terminal region as was discussed in Section 1.1, therefore the activation and regulation would presumably be different. YwaC is able to complement for a *relA* null in *E. coli* in terms of the synthesis of (p)ppGpp. However, YwaC is not believed to make contacts with the ribosome and so cannot respond to amino acid starvation. The activity of YwaC may be increased though when in the presence of the appropriate effector molecule. The promoter of *ywaC* has been shown to respond to lesions in cell wall biosynthesis and to antibiotics that are known to target the cell wall (D'Elia, Millar 2008 unpublished). It has also been shown to respond to alkaline shock (Nanamiya, Kasai 2008). If YwaC is involved in responding to cell wall stress, a cell wall intermediate may be a possible candidate for binding to YwaC and increasing its activity in the cell.

CHAPTER 4 – CONSTRUCTION OF *ywaC*::Spec^r *B. subtilis* AND PHENOTYPIC ANALYSIS

4.1 Introduction

The function of *ywaC* is to date unknown. Transcriptional analysis has identified *ywaC* as a member of the ECF sigma factors σ^M and σ^W regulons. ECF sigma factors provide a means of regulating gene expression in response to various extracellular changes such as antibiotics, alkaline shock, and cationic peptides to name a few (Helmann 2006). Recent reports suggest that ppGpp synthesis induced by alkaline shock is dependant largely on the function of YwaC (Nanamiya, Kasai 2008). To investigate the possible role YwaC has in cell wall stress, a knockout was made. Sensitivity to cell wall antibiotics cell morphology was investigated using light microscopy.

4.2 Materials

VENT polymerase was purchased from New England BioLabs (Beverly, MA). Expand high fidelity PCR system was obtained from Roche (Basel, Switzerland). Hot-Star Taq polymerase was purchased from Qiagen (Toronto, ON). Yeast extract and trypticase peptone were both purchased from BD Biosciences (Mississauga, ON). Clear 96-well plates were purchased from Corning International (Corning, NY). Potassium phosphate was obtained from EMD (Darmstadt, Germany). Sodium citrate was purchased from Caledon Laboratory Chemicals (Georgetown, ON). All other compounds were purchased from Sigma (Oakville, ON) unless specified.

4.3 Methods

4.3.1 Construction of *ywaC*::Spec^r in *B. subtilis* 168

The flanking regions 1 kb upstream and downstream of ywaC were amplified by VENT poymerase using primers HD05 and HD06 and HD07 and HD08, respectively (Table 2.2). Primers HD06 and HD07 have sequences included that are complimentary to the spectinomycin resistance cassette. The spectinomycin resistance cassette was amplified by VENT polymerase off the plasmid pUS19 using primers HD09 and HD10. Then using primers HD05 and HD08 a 3 kb product was amplified using ROCHE polymerase, creating a linear piece of DNA including the upstream 1 kb flanks of *ywaC* surrounding the spectinomycin resistance cassette. The 3 kb fragment was transformed into wildtype *B. subtilis* 168 strain EB6 (Table 2.1) and selected on LB medium containing spectinomycin (150 μg/mL) using established methods (Harwood and Cutting 1990). The knockout was confirmed using polymerase chain reaction (PCR) with the primers HD11 and HD12, which proved that Spec^r had replaced *ywaC* on the chromosome creating the strain EB 1961 (Table 2.1).

4.3.2. Microscopy analysis of wild type *B. subtilis* and *ywaC*::Spec^r

Microscope slides (76 ×26 mm) along with micro glass covers (22 × 22 mm) were used to analyze *B. subtilis* using a Qcolor3 Olympus America Inc. microscope. To begin, 20 μL of Poly-L-Lysine (0.01 % w/v) was added to the centre of the slide for 20 minutes. The slides were then washed three separate times with 20 μL of PBS. A single colony was selected from LB-agar plates and the cells were resuspended on the slide in 20 μL of PBS. Once the images were taken, cell size was measured using Image-Pro Express (Media Cybernetics Inc.).

4.3.3. Minimum inhibitory concentration (MIC) determination

Triplicate overnight cultures of EB 6 and 1961 transformants were grown in either LB supplemented with no drug or 150 μg/mL of spectinomycin respectively. An initial 10, 000-fold dilution was made into LB supplemented with no drug into clear 96-well plates (Corning, NY). Cells were grown at 30 °C at 250 rpm until they reached an OD_{600} ~0.4 as determined by the Molecular Devices SpectraMax spectrophotometer. Drug concentrations were added as indicated in Table 4.1. The OD_{600} was taken after 16 hours of growth in the presence of drug.

4.3.4 Antibiotic Sensitivity

Triplicate overnight cultures of EB6 and 1961 transformants were grown in either Spizizen Minimal Media (SMM) supplemented with no drug or 150 μg/mL of spectinomycin respectively. SMM contains 5X Spizizen minimal salts (2g Sodium sulfate, 14g potassium phosphate, 6 g potassium phosphate, 1g sodium citrate dihydrate, 0.2g magnesium sulfate heptahydrate), 20 % glucose, 10 % yeast extract and 20 mg/mL of histidine, arginine and methionine (Harwood and Cutting 1990). Overnight cultures were diluted 100-fold and 190 μL of cells were plated in each well. Cells were grown at 30 °C at 250 rpm. The OD_{600} was monitored using SpectraMax spectrophotometer. Once the cells had reached an OD_{600} ~0.2 antibiotics were added as outlined in Table 4.1. Three cell wall and one isoprenoid synthesis inhibitors were chosen bacitracin, fosfomycin, vancomycin and fosmidomycin, respectively. Tetracycline was chosen as a negative control. Drug stock plates were made such that only 10 μL of compound was added to each well to reach the final concentration desired. The drug

concentrations ranged well above the MIC value to well below. The OD_{600} was read every 2 hours for a 24 hour period. This experiment was performed on three separate occasions.

4.4 Results

4.4.1 *ywaC* Knockout Construction

In order to perform functional studies of *ywaC* it was necessary to make a knockout of this non-essential gene. Specifically designed primers outlined in Table 2.2 were used to amplify 1 kb flanking regions up and down stream of the *ywaC* locus. A 3 kb product was constructed with the spectinomycin resistance cassette linked between the 1 kb flanking fragments. This linear piece of DNA was transformed into competent wildtype *B. subtilis* 168 cells and through double homologous recombination, *ywaC* was replaced with the spectinomycin resistant cassette. Colonies that successfully grew on LB agar plates containing 150 μg/mL of spectinomycin were confirmed through PCR. The forward primer HD11 was constructed to sit on the chromosome above the 1 kb flanks while reverse primer HD12 annealed to $Spec^r$. This process yielded EB 1961, which has $Spec^r$ replacing the *ywaC* locus.

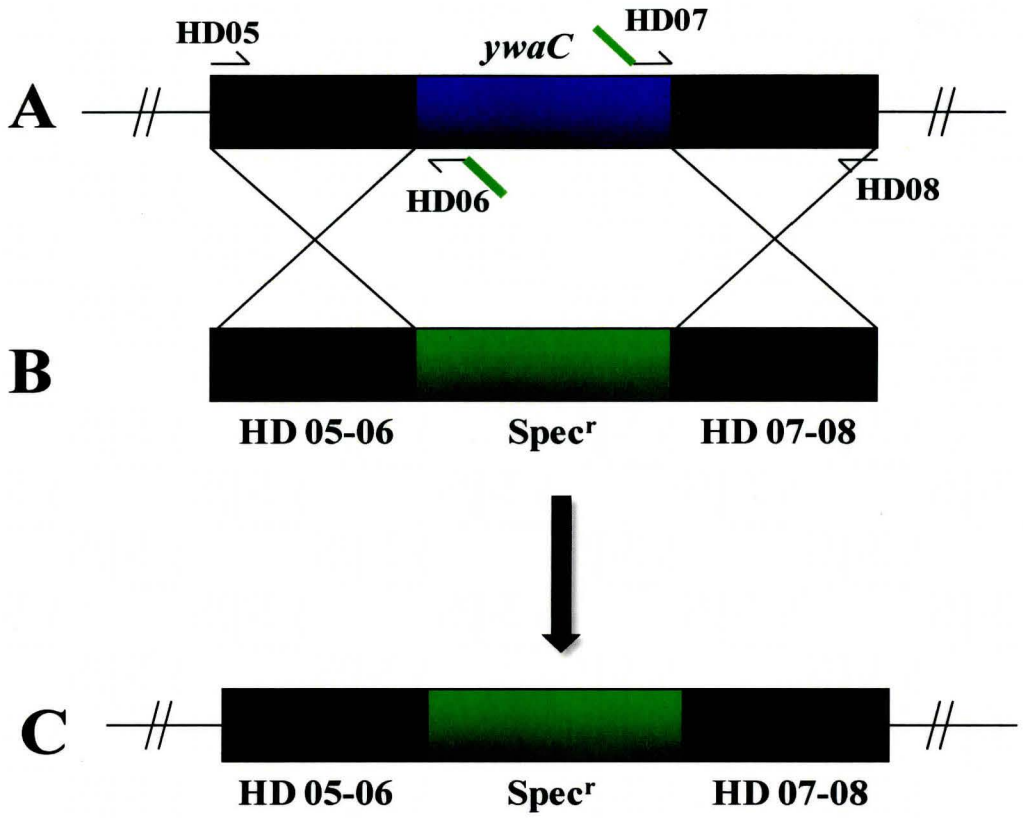


Figure 4.1 Depiction of double homologous recombination allowing for the replacement of endogenous *ywaC* with a spectinomycin resistance cassette.
(A) Illustration of the *B. subtilis* 168 chromosome containing the *ywaC* where HD05/06 and HD07/08 represent the primers used to amplify 1kb regions upstream and downstream of the gene respectively. ▬ Represents the portion of primers HD06 and HD07 that contain the complimentary regions to the spectinomycin resistance cassette.
(B) Character of the 3 kb product obtained through amplification of the 1kb fragments with the spectinomycin resistance cassette shown in green (using primers HD05 and HD08).✕ Represents the double homologous recombination that occurred when the 3 kb product was transformed into wildtype *B. subtilis* competent cells (EB6).
(C) Illustration of the chromosome following the transformation and double homologous recombination with *ywaC* replaced with the spectinomycin resistance cassette on the chromosome (EB 1961).

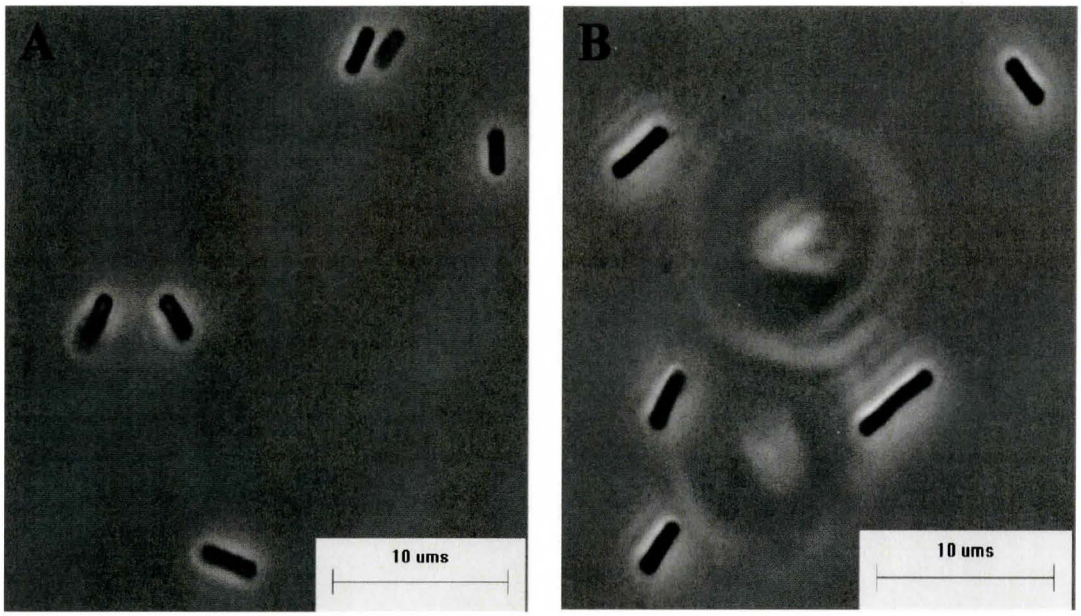


Figure 4.2 Comparison of Colony Morphology Between Wildtype *B. subtilis* and *ywaC* deletion mutant.
Phase contrast microscopy was performed on single colonies obtained from agar plates after 18 hours of growth at 30 °C. (A) wildtype *B. subtilis* cells (EB6). (B) *ywaC*::Spec^r *B. subtilis* (EB1961).

4.4.2 Phase contrast microscopy analysis

The morphology of Wildtype and *ywaC*::Spec^r *B. subtilis* cells was investigated by phase contrast light microscopy. The size and shape of the bacilli in the knockout strain do not appear to be drastically different from wiltype cells (Figure 4.2). Some of the cells in panel B do look slightly longer, but they may just be in very late stages of cell division. The cell wall also does not appear to be altered in the knockout strain.

4.4.3 MIC comparison between wildtype *B. subtilis* and a *ywaC* null

Once a *ywaC* null was successfully constructed, I sought to investigate a phenotypic response to the absence of the gene. One experiment conducted to pursue this was determining the minimum inhibitory concentration (MIC) for both wildtype *B. subtilis* and the *ywaC* knockout strain. If the bacteria were severely impaired by the absence of *ywaC* then this could result in a sensitization to one or more antibiotics. This experiment was conducted on three separate occasions all yielding the same result. Cells were grown at various different concentrations of drug in triplicate. The antibiotics chosen and the corresponding range in concentrations are shown in Table 4.1. Although P_{ywaC} responds selectively to cell wall antibiotics as discussed in Section 1.5, the drugs chosen were from all main classes of antibiotics used clinically. Table 4.2 outlines the MIC values (μg/mL) for each strain against the corresponding antibiotic. The fold enhancement is also shown to compare the MIC values between wildtype and the *ywaC* knockout. The highest fold enhancement is 2-fold for cefotaxime, fosfomycin, cycloserine and fosmidomycin. There is no difference in the MIC value for any antibiotics inhibiting DNA or protein synthesis. Other cell wall inhibitors such as

ampicillin, bacitracin and vancomycin also displayed no difference in MIC value unlike the other cell wall antibiotics outlined previously. Overall, there appeared to be no significant enhancement for the *ywaC* deletion mutant in the presence of the various antibiotics discussed above.

Table 4.1 Antibiotics and the Corresponding range of Concentrations Used.

Drug Class	Antibiotic	Drug (µg/mL)										
Penicillins	Cefotaxime	16	8	4	2	1	0.5	0.25	0.13	0.063	0.031	0.016
	Ampicillin	16	8	4	2	1	0.5	0.25	0.13	0.063	0.031	0.016
Cell Wall	Vancomycin[a]	32	16	8	4	2	1	0.5	0.25	0.125	0.063	0.031
	Bacitracin[a]	1024	512	256	128	64	32	16	8	4	2	1
	Fosfomycin[a]	512	256	128	64	32	16	8	4	2	1	0.5
	Cycloserine	1024	512	256	128	64	32	16	8	4	2	1
Protein Synthesis	Chloramphenicol	16	8	4	2	1	0.5	0.25	0.13	0.063	0.031	0.016
	Tetracycline[a]	16	8	4	2	1	0.5	0.25	0.13	0.063	0.031	0.016
	Erythromycin	32	16	8	4	2	1	0.5	0.25	0.125	0.063	0.031
DNA Synthesis	Norfloxacin	16	8	4	2	1	0.5	0.25	0.13	0.063	0.031	0.016
Miscellaneous	Sulfamethoxazole	1024	512	256	128	64	32	16	8	4	2	1
	Fosmidomycin[a]	8	4	2	1	0.5	0.25	0.125	0.06	0.031	0.016	0.008

a represents the antibiotics used for the experiments discussed in Section 4.3.4

Table 4.2 MIC Values of Various Different Antibiotics for Wildtype and Knockout strains

		MIC (μg/mL)		
Drug Class	**Antibiotic**	**wild type**	***ywaC::spec***	**Fold Enhancement**
Penicillins	Cefotaxime	2	1	2
	Ampicillin	0.063	0.063	1
Cell wall	Vancomycin	0.25	0.25	1
	Bacitracin	256	256	1
	Fosfomycin	256	128	2
	Cycloserine	32	16	2
Protein synthesis	Chloramphenicol	0.5	0.5	1
	Tetracycline	4	2	1
	Erythromycin	0.063	0.063	1
DNA synthesis	Norfloxacin	0.13	0.13	1
Miscellaneous	Sulfamethoxazole	512	512	1
	Fosmidomycin	16	8	2

4.4.4 ywaC Knockout Strain does not Elicit Sensitivity to Cell Wall Antibiotics

The *ywaC* promoter has already proven to respond robustly and selectively to cell wall inhibitors when added around the MIC value at an OD_{600} ~0.2 (D'Elia, Millar et al. 2008 unpublished). Therefore, it became interesting to look at the growth profile of wildtype and *ywaC* knockout cells after the addition of drug at OD_{600} ~0.2. Initially these experiments were conducted in LB media; however, the *ywaC* knockout strain did not display sensitivity to antibiotics when compared to wildtype cells. These experiments were repeated in minimal media to determine if rich media was somehow compensating for the deletion of *ywaC*. SMM was chosen as it has been long reported as appropriate minimal media for *B. subtilis* (Harwood and Cutting 1990). Overnight cultures were diluted 100-fold and plated in triplicate in clear 96-well plates. Once the cells reached an OD_{600} ~0.2, as determined by SpectraMax spectrophotometer, drug was added to each well (Table 4.1). Growth profiles for bacitracin, fosfomycin, vancomycin, fosmidomycin, and tetracycline are shown in Figures 4.3 to 4.7, respectively. Although cells were screened against 11 different concentrations, only three concentrations are shown for each drug. The interest is focused around the MIC value as that is where the largest transcriptional induction of P_{ywaC} was reported (D'Elia, Millar et al. 2008 unpublished).

Bacitracin, which inhibits the dephosphorylation of undecaprenol pyrophosphate, did not affect the growth profile of the *ywaC* knockout even at sub-MIC concentrations such as 256 μg/mL (Figure 4.3). Fosfomycin inhibits MurA, the first enzyme in the peptidoglycan biosynthesis. The addition of fosfomycin at OD_{600} ~0.2 displayed the same growth profile for knockout and wildtype strains (Figure 4.4). Vancomycin inhibits peptidoglycan biosynthesis by binding and sequestering the substrate for transglycosylase and transpeptidase reactions. At

concentrations near MIC value, there was again no effect on the growth profile (Figure 4.5). Fosmidomycin inhibits the methylerythritol phosphate (MEP) pathway by interfering with deoxyxylulose-5-phosphate reductoisomerase (DXR). The product from this pathway can be further metabolized into C_{55} isoprenoid undecaprenyl diphosphate, which is used for both the teichoic acid and peptidoglycan biosynthetic pathways (Walsh 2003). Although this antibiotic induced a large transcriptional response of *ywaC*, the addition of drug at MIC concentrations did not display a large sensitivity response in the knockout (Figure 4.6). There does appear to be a slight decrease in growth for the knockout after addition of drug, however the affect is not significant. Tetracycline binds to the 16S rRNA on the 30S bacterial subunit of the ribosome and blocks rotation of aminoacyl-tRNA into the A-site prematurely stopping translation (Walsh 2003). This antibiotic used as a negative control a P_{ywaC}, did not respond transcriptionally to either DNA or protein synthesis inhibitors. The control proved reliable, as there was no significant change of the growth profile between wildtype and knockout.

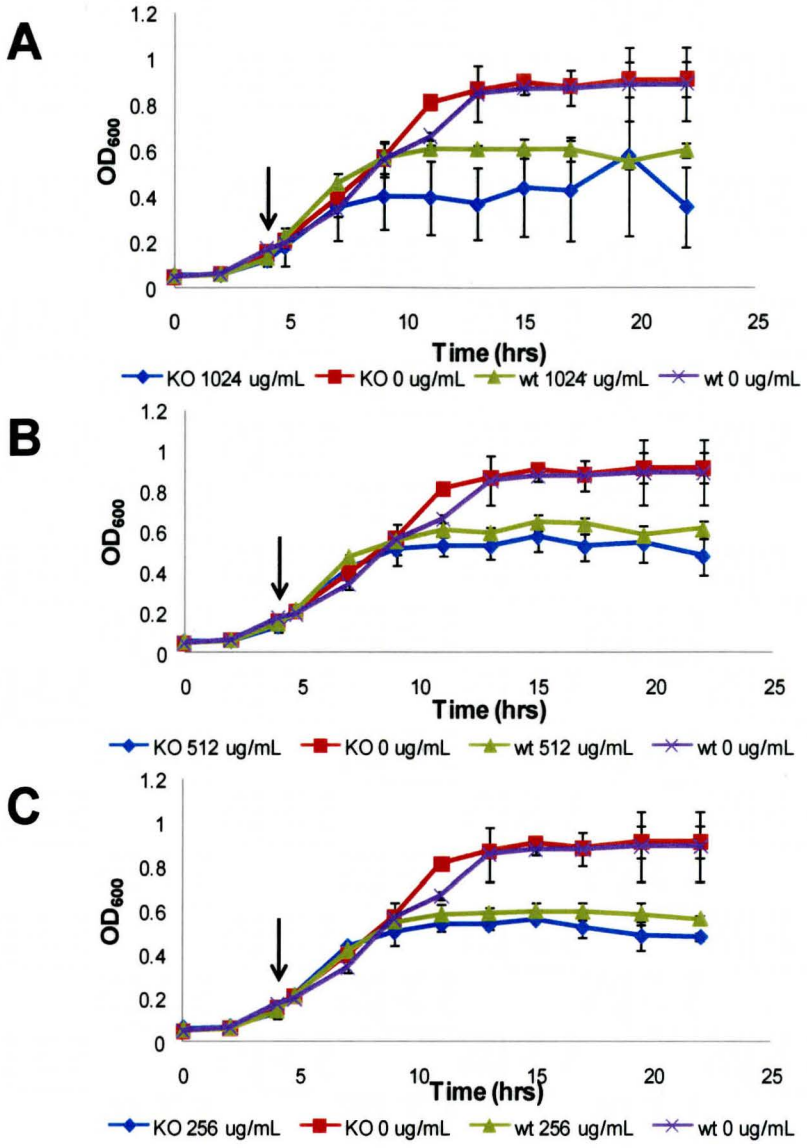


Figure 4.3 Growth Profile of Wildtype *B. subtilis* and *ywaC*::spec^r *B. subtilis* After the Addition of Bacitracin at OD_{600} ~0.2. Colonies grown at 30 °C overnight on LB-agar were used to inoculate 5 mL of SMM. These cultures were grown overnight at 30 °C shaking at 250 rpm. The overnight cultures were diluted 1 in 100 into fresh SMM. These were grown to an OD_{600} of approximately 0.2 at 30 °C. Once cell density was reached, different drug concentrations were added to each well (Table 4.1). The optical density was monitored every two hours by measuring the absorbance at 600 nm in a spectrophotometer (Molecular Devices SpectraMax). Panels A, B, and C represent growth profiles in the presence of 1024, 512, and 256 μg/mL of bacitracin, respectively. Wildtype cultures with and without drug are represented by green and purple traces, respectively. *ywaC* knockout cultures with and without drug are represented by the blue and red traces, respectively. Arrows represent where drug was added along the growth profile.

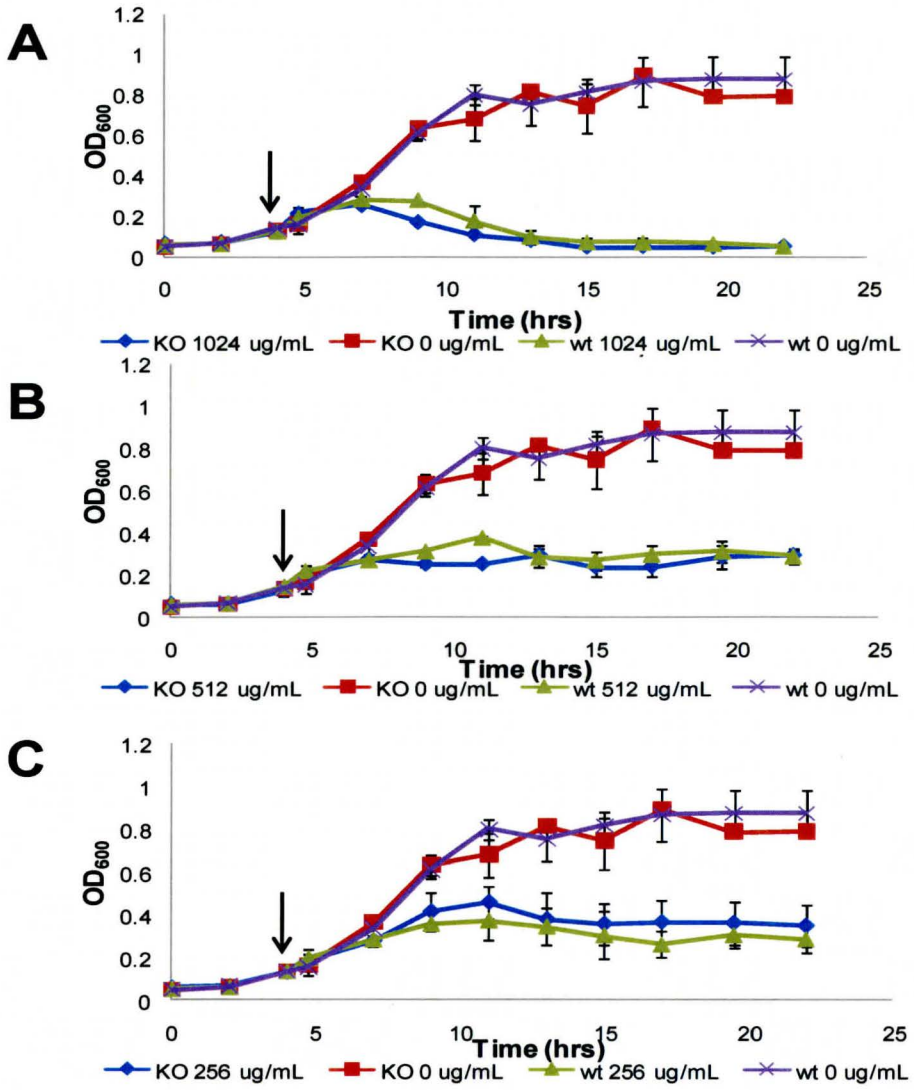


Figure 4.4 Growth Profile of Wildtype *B. subtilis* and *ywaC*::spec^r *B. subtilis* After the Addition of Fosfomycin at OD_{600} ~0.2. Colonies grown at 30 °C overnight on LB-agar were used to inoculate 5 mL of SMM. These cultures were grown overnight at 30 °C shaking at 250 rpm. The overnight cultures were diluted 1 in 100 into fresh SMM. These were grown to an OD_{600} of approximately 0.2 at 30 °C. Once cell density was reached, different drug concentrations were added to each well (Table 4.1). The optical density was monitored every two hours by measuring the absorbance at 600 nm in a spectrophotometer (Molecular Devices SpectraMax). Panels A, B, and C represent growth profiles in the presence of 1024, 512, and 256 µg/mL of fosfomycin, respectively. Wildtype cultures with and without drug are represented by green and purple traces, respectively. *ywaC* knockout cultures with and without drug are represented by the blue and red traces, respectively. Arrows represent where drug was added along the growth profile.

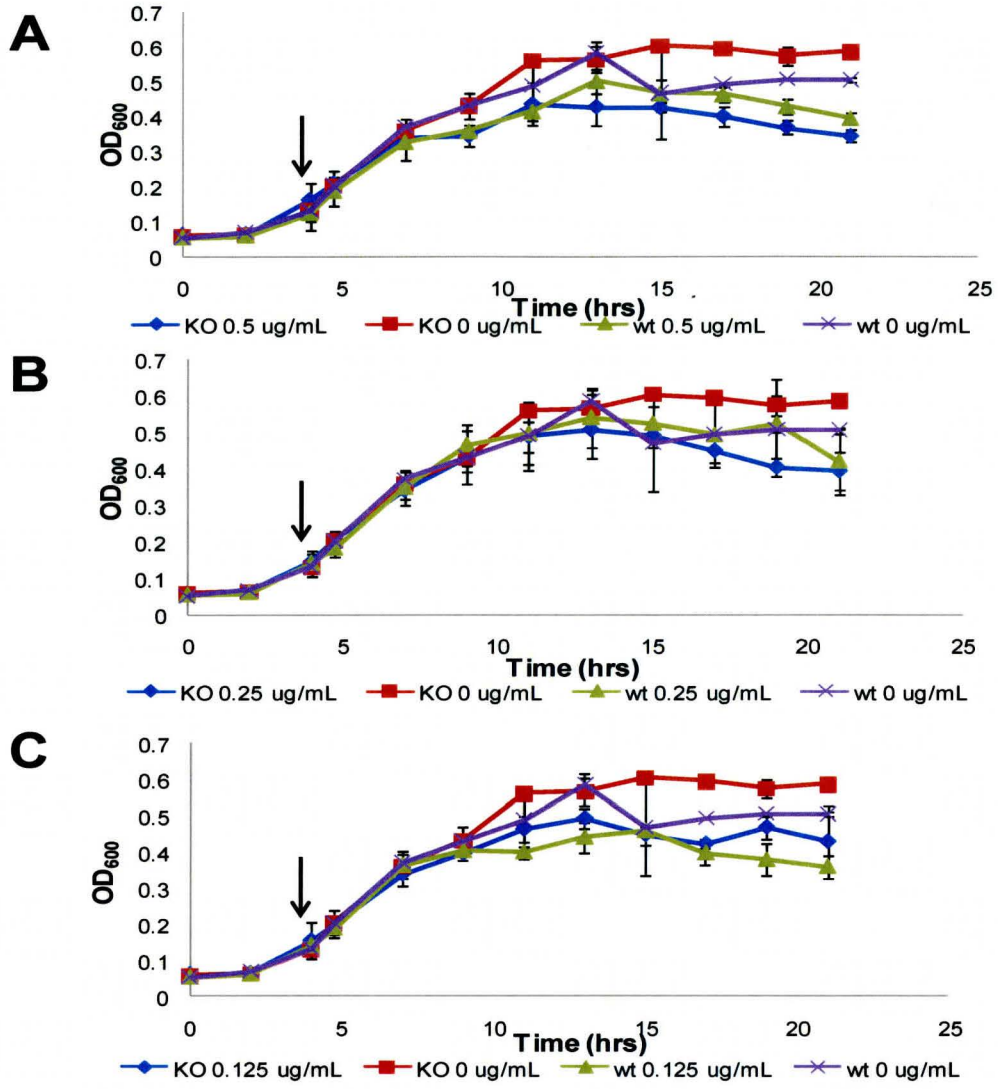


Figure 4.5 Growth Profile of Wildtype *B. subtilis* and *ywaC*::spec^r *B. subtilis* After the Addition of Vancomycin at OD_{600} ~0.2. Colonies grown at 30 °C overnight on LB-agar were used to inoculate 5 mL of SMM. These cultures were grown overnight at 30 °C shaking at 250 rpm. The overnight cultures were diluted 1 in 100 into fresh SMM. These were grown to an OD_{600} of approximately 0.2 at 30 °C. Once cell density was reached, different drug concentrations were added to each well (Table 4.1). The optical density was monitored every two hours by measuring the absorbance at 600 nm in a spectrophotometer (Molecular Devices SpectraMax). Panels A, B, and C represent growth profiles in the presence of 0.5, 0.25, and 0.13 μg/mL of vancomycin, respectively. Wildtype cultures with and without drug are represented by green and purple traces, respectively. *ywaC* knockout cultures with and without drug are represented by the blue and red traces, respectively. Arrows represent where drug was added along the growth profile.

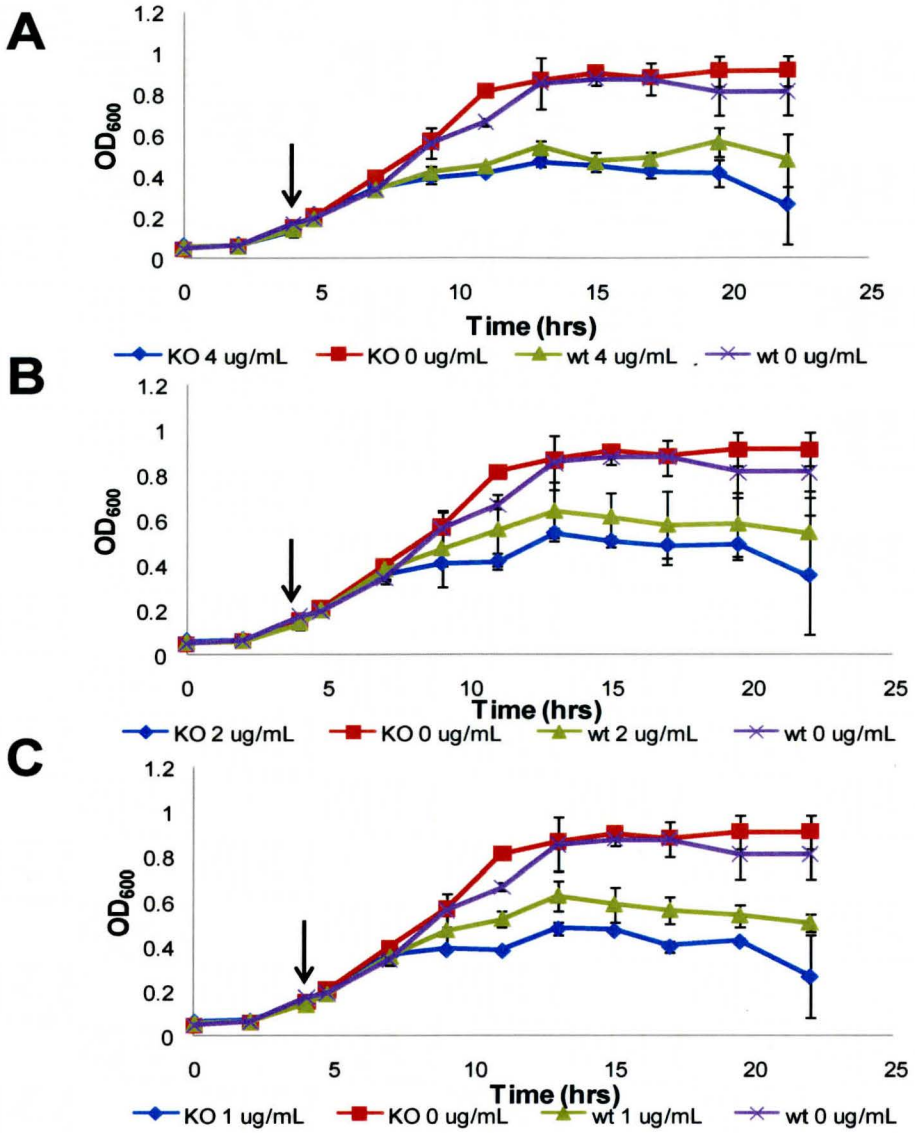


Figure 4.6 Growth Profile of Wildtype *B. subtilis* and *ywaC*::spec^r *B. subtilis* After the Addition of Fosmidomycin at OD_{600} ~0.2. Colonies grown at 30 °C overnight on LB-agar were used to inoculate 5 mL of SMM. These cultures were grown overnight at 30 °C shaking at 250 rpm. The overnight cultures were diluted 1 in 100 into fresh SMM. These were grown to an OD_{600} of approximately 0.2 at 30 °C. Once cell density was reached, different drug concentrations were added to each well (Table 4.1). The optical density was monitored every two hours by measuring the absorbance at 600 nm in a spectrophotometer (Molecular Devices SpectraMax). Panels A, B, and C represent growth profiles in the presence of 4, 2, and 1 μg/mL of fosmidomycin, respectively. Wildtype cultures with and without drug are represented by green and purple traces, respectively. *ywaC* knockout cultures with and without drug are represented by the blue and red traces, respectively. Arrows represent where drug was added along the growth profile.

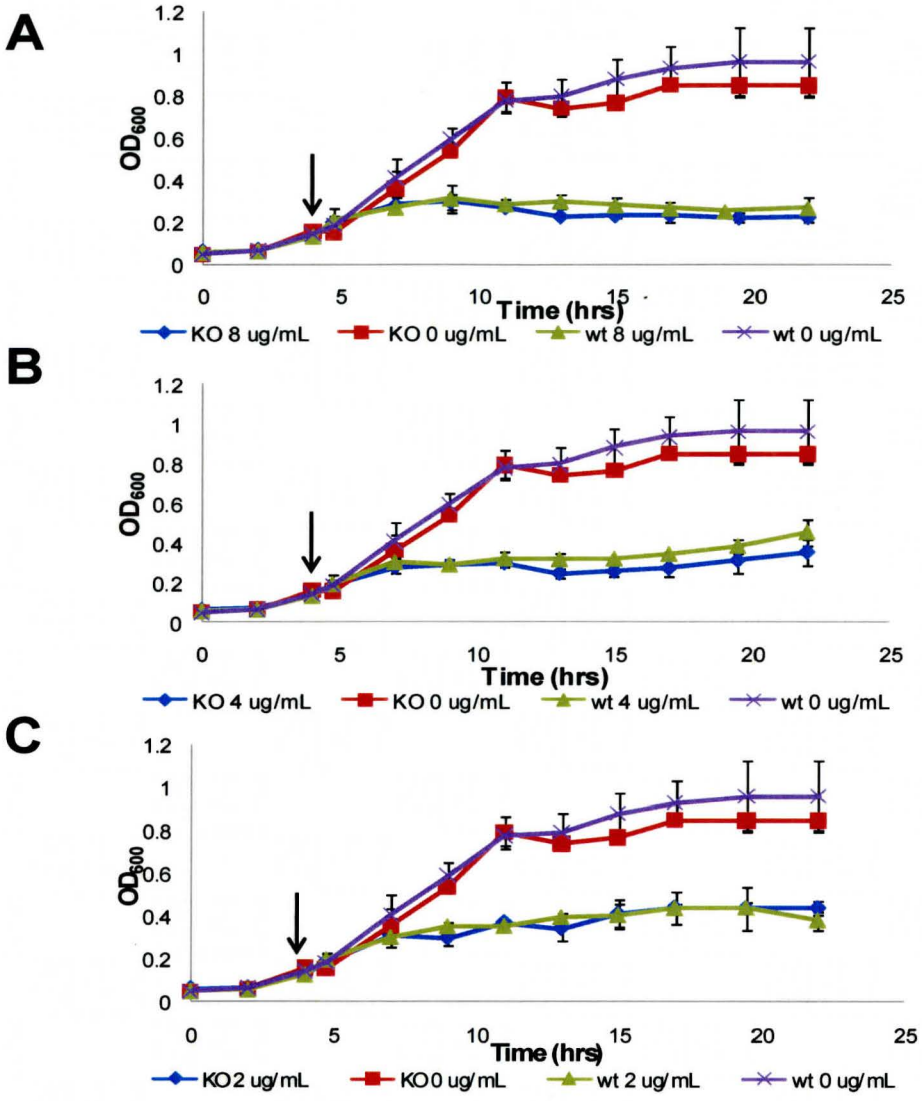


Figure 4.7 Growth Profile of Wildtype *B. subtilis* and *ywaC*::spec^r *B. subtilis* After the Addition of Tetracycline at OD_{600} ~0.2. Colonies grown at 30 °C overnight on LB-agar were used to inoculate 5 mL of SMM. These cultures were grown overnight at 30 °C shaking at 250 rpm. The overnight cultures were diluted 1 in 100 into fresh SMM. These were grown to an OD_{600} of approximately 0.2 at 30 °C. Once cell density was reached, different drug concentrations were added to each well (Table 4.1). The optical density was monitored every two hours by measuring the absorbance at 600 nm in a spectrophotometer (Molecular Devices SpectraMax). Panels A, B, and C represent growth profiles in the presence of 8, 4, and 2 μg/mL of tetracycline, respectively. Wildtype cultures with and without drug are represented by green and purple traces, respectively. *ywaC* knockout cultures with and without drug are represented by the blue and red traces, respectively. Arrows represent where drug was added along the growth profile.

4.5 Discussion

Through this work and that of Nanamiya et al. in 2008, it was shown that YwaC does contain (p)ppGpp pyrophosphokinase activity. Unlike RSH proteins found in sequenced genomes of free-living eubacteria and plants, YwaC does not contain the large C-terminal domain that has been reported to associate with the ribosome during nutrient starvation. Therefore, YwaC is predicted to not bind to the ribosome or respond to amino acid deprivation. This leaves room for much investigation into the activating factors for YwaC and its function. During the stringent response in *E. coli*, cell wall peptidoglycan metabolism is inhibited, specifically during the later steps in polymerization involving the work of the penicillin binding proteins (Ramey and Ishiguro 1978). Inhibition of cell growth has shown to decrease the effect of cell wall antibiotics especially penicillins. To date there has never been a correlation made between the intracellular accumulation of (p)ppGpp and cell wall stress. A luminescent reporter (P_{ywaC}) constructed in *B. subtilis* 168 is robustly upregulated in response to cell wall antibiotics such as bacitracin, vancomycin and fosfomycin among others. It also responds to lesions in the teichoic acid biosynthetic pathway (D'Elia, Millar et al. 2008 unpublished). Since YwaC *in vitro* has proven to be a (p)ppGpp synthase, possibly it functions by responding to cell wall stress, accumulating (p)ppGpp which would slow down cell growth and consequently inhibit the killing effect of cell wall antibiotics. The work herein describes some phenotypic analysis performed to monitor the response of cells when lacking *ywaC*.

The morphological features of wildtype and *ywaC*::Spec^r *B. subtilis* cells were compared using a phase contrast confocal microscope. If *ywaC* has some role in cell wall stress, a knockout may possibly affect the morphology of the cell. However, the knockout strain was of the same shape and length as wildtype cells. The MIC was determined for wildtype versus the

ywaC knockout strain. Bacitracin elicited the best response from the P_{ywaC} reporter system, of the antibiotics tested (D'Elia, Millar et al. 2008 unpublished). When the cells are depleted of *ywaC*, there was no significant change in the presence of bacitracin. For the other cell wall antibiotics, the best response resulted in a 2-fold enhancement, which is not a profound change in sensitivity. Antibiotics targeting protein or DNA synthesis elicited no change in MIC either. The growth profiles of wildtype and knockout cells over a 24-hour period were virtually identical. When the cells were challenged with cell wall antibiotics at an OD_{600} ~0.2, there was still no change in the growth profiles. This OD_{600} was chosen because the P_{ywaC} reporter elicited the highest response to cell wall antibiotics at this growth stage perhaps is due to the increased susceptibility of cells at the onset of log phase. When cells were compromised with cell wall antibiotics in a *ywaC* mutant background, a possible result could have been that cells were unable to respond to cell wall stress as effectively without the production of (p)ppGpp from YwaC and would possibly show more sensitivity to the antibiotic. This phenotype was not seen in the presence of bacitracin, fosfomycin, fosmidomycin or vancomycin at concentrations well above and below the MIC values.

The cell envelope is a crucial cellular structure and its integrity must be ensured at all times and at any costs. Therefore, it is of no surprise that the regulatory network orchestrating cell envelope stress in Gram-positive bacteria is complex (Jordan, Hutchings 2008). Bacitracin, which showed the best response from P_{ywaC}, did not render the knockout cells more sensitive than wildtype. This cyclic dodecylpeptide inhibits the de-phosphorylation of undecaprenol-pyrophosphate to yield undecaprenol-phosphate, the membrane-embedded carrier for teichoic acid and peptidoglycan biosynthesis. This antibiotic specifically induces the ECF σ factor σ^M, and the TCS LiaRS, BceRS and YvcPQ (Mascher, Margulis et al. 2003). The most sensitive

regulatory system in this regulon is the BceRS TCS, while the ECF σ factor σ^M shows the weakest and least sensitive response (Rietkötter, Hoyer et al. 2008). YwaC has been implicated as one of the σ^M-dependant regulator proteins (Eiamphungporn and Helmann 2008). These results suggest a connection between bacitracin and *ywaC*. However, the addition of bacitracin to the cell is obviously recognized by many other factors and the result may be very subtle in a *ywaC* knockout background. The reason σ^M elicits a less sensitive response is possibly because it is a part of a damage-sensing system and is not believed to make direct interactions with bacitracin, rather it responds to the damage caused by addition of the antibiotic (Rietkötter, Hoyer et al. 2008). This makes it very difficult experimentally to decipher the role YwaC may have in the σ^M regulon. In addition, *ywaC* has been implicated as part of the σ^W and σ^X regulons. These two ECF sigma factors along with σ^M have significant regulatory overlap during the cell envelope stress response (Jordan, Hutchings 2008). It is possible that *ywaC* is involved in each one of these regulons in *B. subtilis*. Together the data are consistent with the known complexity of the cell wall stress response, and leave open the notion that the (p)ppGpp synthase, YwaC, could still be an important component.

CHAPTER 5 – SUMMARY AND CONCLUSIONS

Much of the discussion here has been focused on the possible function(s) YwaC has in the cell and its regulation. There is substantial evidence in the literature that suggests YwaC has some role in the cell wall stress response in *B. subtilis*. The work done in this study neither confirms nor denies this to any certainty. Considering the complexity of the cell wall stress response, there is still much investigation needed. The effect of a *ywaC* knockout in *B. subtilis* is subtle in that it does not alter the growth profile of cells even when compromised with cell wall antibiotics.

The ultimate goal of the investigation of this enzyme was to understand the connection between the accumulation of the alarmone (p)ppGpp and the cell wall stress response. Such information will allow for a deeper understanding of the bacterial stress response and may uncover an avenue for an antimicrobial target. One strategy would be to look directly at the levels of (p)ppGpp in the cell after being compromised with various cell wall stressors. This may establish a clearer understanding of what is activating YwaC during cell wall stress. Another possibility is transferring the *ywaC* knockout to more wild soil strains of *B. subtilis*. A wildtype lab strain may behave differently than a strain from the soil. Life for microbes in the soil comes with great adversity due to the number of environmental attacks that occur and the cell wall is the first to defend against them.

In summary, the steady state kinetic constants for the synthesis of (p)ppGpp has been established in this thesis. The K_m values for substrates are in the micromolar range. The k_{cat} values are quite slow, and it is possible that an unknown factor may be required for the efficient (p)ppGpp synthase activity of YwaC *in vivo*. A knockout of *ywaC* was constructed in *B. subtilis*

to investigate the physiological role of YwaC. Morphologically, the cells do not appear altered in the knockout strain compared to wildtype rod shaped cells. The sensitivity of the knockout strain to a wide variety of antibiotics was similar to wildtype *B. subtilis*. Lastly, in the absence of YwaC, the cells do not appear abnormally compromised when faced with cell wall antibiotics during early log phase, a growth condition where maximal production was noted for *ywaC* in response to cell wall-active agents. In aggregate, this work serves as a foundation for future investigations of YwaC, a potential cell wall stress signaling protein.

REFERENCES

Aravind, L. and E. V. Koonin (1998). "The HD domain defines a new superfamily of metal-dependent phosphohydrolases." Trends Biochem. Sci. **23**(12): 469-72.

Avarbock, A., D. Avarbock, et al. (2005). "Functional regulation of the opposing (p)ppGpp synthetase/hydrolase activities of RelMtb from *Mycobacterium tuberculosis*." Biochemistry **44**: 9913-9923.

Battesti, A. and E. Bouveret (2006). "Acyl carrier protein/SpoT interaction, the switch linking SpoT-dependent stress response to fatty acid metabolism." Mol. Microbiol. **62**: 1048-1063.

Baysse, C., M. Cullinane, et al. (2005). "Modulation of quorum sensing in *Pseudomonas aeruginosa* through alteration of membrane properties." Microbiology **151**: 2529-2542.

Calderon-Flores, A., G. Du Pont, et al. (2005). "The stringent response is required for amino acid and nitrate utilization, nod factor regulation, nodulation, and nitrogen fixation in *Rhizobium etli*." J. Bacteriol. **187**: 5075-5083.

Cashel, M. and J. Gallant (1969). "Two compounds implicated in the function of the RC gene of *Escherichia coli*." Nature **221**: 838-841.

Cassels, R., B. Oliva, et al. (1995). "Occurrence of the regulatory nucleotides ppGpp and pppGpp following induction of the stringent response in *staphylococci*." J. Bacteriol. **177**(17): 5161-5.

Chakraburtty, R. and M. Bibb (1997). "The ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2) plays a conditional role in antibiotic production and morphological differentiation." J. Bacteriol. **179**: 5854-5861.

Chatterji, D. and A. K. Ojha (2001). "Revisiting the stringent response, ppGpp and starvation signaling." Curr. Opin. Microbiol. **4**: 160-165.

Costanzo, A., H. Nicoloff et al. (2008). "ppGpp and DksA likely regulate the activity of the extracytoplasmic stress factor sigmaE in *Escherichia coli* by both direct and indirect mechanisms." Mol. Microbiol. **67**: 619-632.

Crawford, E. W., Jr and L. J. Shimkets (2000). "The *Myxococcus xanthus socE* and *csgA* genes are regulated by the stringent response." Mol. Microbiol. **37**: 788-799.

Dahl, J. L., K. Arora, et al. (2005). "The *relA* homolog of *Mycobacterium smegmatis* affects cell appearance, viability, and gene expression." J. Bacteriol. **187**: 2439-2447.

Eiamphungporn, W. and J. D. Helmann (2008). "The *Bacillus subtilis* sigma(M) regulon and its contribution to cell envelope stress responses." Mol. Microbiol. **67**: 830-848.

Erickson, D. L., J. L. Lines, et al. (2004). "*Pseudomonas aeruginosa relA* contributes to virulence in Drosophila melanogaster." Infect. Immun. **72**: 5638-5645.

Fehr, S. and D. Richter (1981). "Stringent response of *Bacillus stearothermophilus*: evidence for the existence of two distinct guanosine 3',5'-polyphosphate synthetases." J. Bacteriol. **145**(1): 68-73.

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.

Gropp, M., Y. Strausz et al. (2001). "Regulation of *Escherichia coli* RelA requires oligomerization of the C-terminal domain." J. Bacteriol. **183**(2): 570-9.

Hammer, B. K. and M. S Swanson (1999). "Co-ordination of *Legionella pneumophila* virulence with entry into stationary phase by ppGpp." Mol. Microbiol. **33**: 721-731.

Haralalka, S., S. Nandi, et al. (2003). "Mutation in the *relA* gene of *Vibrio cholerae* affects *in vitro* and *in vivo* expression of virulence factors." J. Bacteriol. **185**: 4672-4682.

Harris, B. Z., D. Kaiser, et al. (1998). "The guanosine nucleotide (p)ppGpp initiates development and A-factor production in *Myxococcus xanthus*." Genes Dev. **12**: 1022-1035.

Harwood, C. R. and S. M. Cutting. (1990). Molecular biological methods for *Bacillus*. John Wiley and Sons Ltd, West Sussex, England.

Haseltine, W. A. and R. Block (1973). "Synthesis of guanosine tetra- and pentaphosphate requires the presence of a codon-specific, uncharged transfer ribonucleic acid in the acceptor site of ribosomes." Proc. Natl. Acad. Sci. U S A **70**: 1564-1568.

Helmann, J. D. (2006). "Deciphering a complex genetic regulatory network: the *Bacillus subtilis* sigmaW protein and intrinsic resistance to antimicrobial compounds." Sci. Prog. **89**: 243-266.

Hogg, T., U. Mechold, et al. (2004). "Conformational antagonism between opposing active sites in a bifunctional RelA/SpoT homolog modulates (p)ppGpp metabolism during the stringent response [corrected]." Cell **117**: 57-68.

Hutter, B., C. Fischer, et al. (2004). "Panel of *Bacillus subtilis* reporter strains indicative of various modes of action." Antimicrob. Agents Chemother. **48**(7): 2588-94.

Inaoka, T., K. Takahashi, et al. (2003). "Guanine nucleotides guanosine 5'-diphosphate 3'-diphosphate and GTP co-operatively regulate the production of an antibiotic bacilysin in *Bacillus subtilis*." J. Biol. Chem. **278**: 2169-2176.

Jordan, S., M. I. Hutchings, et al. (2008). "Cell envelope stress response in Gram-positive bacteria." FEMS Microbiol. Rev. **32**: 107-146.

Justesen, J., T. Lund, et al. (1986). "The physiology of stringent factor (ATP:GTP 3'-diphosphotransferase) in *Escherichia coli*." Biochimie. **68**(5): 715-22.

Knutsson Jenvert, R. M. and L. Holmberg Schiavone (2005). "Characterization of the tRNA and ribosome-dependent (p)ppGpp-synthesis by recombinant stringent factor from *Escherichia coli*." FEBS J. **272**: 685-695.

Lemos, J. A., V. K. Lin, et al. (2007). "Three gene products govern (p)ppGpp production by *Streptococcus mutans*." Mol. Microbiol. **65**(6): 1568-81.

Martinez-Costa, O. H., M. A. Fernandez-Moreno et al. (1998). "The relA/spoT-homologous gene in *Streptomyces coelicolor* encodes both ribosome-dependent (p)ppGpp-synthesizing and -degrading activities." J. Bacteriol. **180**: 4123-4132.

Mascher, T., N. G. Margulis, et al. (2003). "Cell wall stress responses in *Bacillus subtilis*: the regulatory network of the bacitracin stimulon." Mol. Microbiol. **50**(5): 1591-604.

Mechold, U., H. Murphy, et al. (2002). "Intramolecular regulation of the opposing (p)ppGpp catalytic activities of Rel(*Seq*), the Rel/Spo enzyme from *Streptococcus equisimilis*." J. Bacteriol. **184**(11): 2878-88.

Nanamiya, H., K. Kasai, et al. (2008). "Identification and functional analysis of novel (p)ppGpp synthetase genes in *Bacillus subtilis*." Mol. Microbiol. **67**: 291-304.

Northrop, D. B. (1998). "On the Meaning of Km and V/K in Enzyme Kinetics." J. Chem. Ed. **75**(9): 1153-57.

Oki, T., A. Yoshimoto, et al. (1975). "Purine nucleotide pyrophosphotransferase from *Streptomyces morookaensis*, capable of synthesizing pppApp and pppGpp." Biochim. Biophys. Acta. **410**(2): 262-72.

Pizarro-Cerda, J. and K. Tedin (2004). "The bacterial signal molecule, ppGpp, regulates *Salmonella* virulence gene expression." Mol. Microbiol. **52**: 1827-1844.

Potrykus, K and M. Cashel (2008). "(p)ppGpp: Still Magical?" Annu. Rev. Microbiol. **62**: 35-51.

Ramey, W. D. and E. E. Ishiguro (1978). "Site of inhibition of peptidoglycan biosynthesis during the stringent response in *Escherichia coli*." J. Bacteriol. **135**(1): 71-7.

Rietkötter, E., D. Hoyer, et al. (2008). "Bacitracin sensing in *Bacillus subtilis*." **68**(3): 768-85.

Rodionov, D. G. and E. E. Ishiguro (1995). "Direct correlation between overproduction of guanosine 3',5'-bispyrophosphate (ppGpp) and penicillin tolerance in *Escherichia coli*." J. Bacteriol. **177**: 4224-4229.

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

Song, M., H. J. Kim, (2004). "ppGpp-dependent stationary phase induction of genes on *Salmonella* pathogenicity island 1." J. Biol. Chem. **279**: 34183-34190.

Sy, J. and H. Akers (1976). "Purification and properties of guanosine 5', 3'-polyphosphate synthetase from *Bacillus brevis*." Biochemistry **15**(20): 4399-403.

Taylor, C. M., M. Beresford, (2002). "*Listeria monocytogenes relA* and hpt mutants are impaired in surface-attached growth and virulence." J. Bacteriol. **184**: 621-628.

van Delden, C., R. Comte, et al. (2001). "Stringent response activates quorum sensing and modulates cell density-dependent gene expression in *Pseudomonas aeruginosa*." J. Bacteriol. **183**: 5376-5384.

Vinella, D., C. Albrecht, et al. (2005). "Iron limitation induces SpoT-dependent accumulation of ppGpp in *Escherichia coli*." Mol. Microbiol. **56**: 958-970.

Walsh, C. (2003). Antibiotics – actions, origins, resistance. ASM Press, American Society for Microbiology, Washington, DC.

Xiao, H., M. Kalman, et al. (1991). "Residual guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by spoT null mutations." J. Biol. Chem. **266**: 5980-5990.

Yang, X. and E. E. Ishiguro (2001). "Involvement of the N terminus of ribosomal protein L11 in regulation of the RelA protein of *Escherichia coli*." J. Bacteriol. **183**(22): 6532-7.