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The Role Of Cyclic Di Amp In Speb Virulence And Pathogenesis In *Streptococcus Pyogenes*

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THE ROLE OF CYCLIC DI AMP IN SPEB VIRULENCE AND PATHOGENESIS IN *STREPTOCOCCUS PYOGENES*

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ABSTRACT

The second messenger molecule, c-di-AMP, plays a critical role in pathogenesis and virulence in group A Streptococcus or *S. pyogenes.* However, relatively little is known about its underlying molecular mechanisms in the c-di-AMP signal transduction pathways. This study aims to understand the role of c-di-AMP in SpeB virulence regulation and pathogenesis to decipher the c-di-AMP signaling mechanism in *S. pyogenes*. SpeB is one of the major virulence factors crucial for the pathogenesis of severe invasive GAS diseases. It is a cysteine protease that can cleave host proteins and own surface proteins. In *S. pyogenes*, c-di-AMP is synthesized by a diadenylate cyclase DacA and degraded by phosphodiesterases (PDEs) GdpP and Pde2. Mutations in these two enzyme-encoding genes dysregulate the c-di-AMP level and alter gene expressions in the downstream processes. Previous studies showed that the c-di-AMP degradation encoding gene *gdpP* influences SpeB processing and virulence in GAS (Cho & Kang, 2013). In this study, I found that the deletion of the c-di-AMP synthase gene, *dacA*, and degrading gene *pde2* abolish the ability of *S. pyogenes* to express SpeB at the transcriptional level, and both Δ*dacA* and Δ*pde2* mutants are severely attenuated by losing their virulence to cause lesions in a mouse subcutaneous infection model. Further, I demonstrated that c-di-AMP regulates SpeB at the transcriptional level via the KtrAB potassium transporter.

I found that the deletion of *ktrB* restores SpeB expression in the Δ*dacA* mutant. KtrB is a subunit of the K^+ transport system KtrAB that forms a putative high-affinity K^+ importer. KtrB forms a membrane K^+ channel, and KtrA acts as a cytosolic gating protein that controls the

transport capacity of the system by binding ligands, including c-di-AMP. However, the null pathogenicity of the Δ*dacA* mutant in a murine subcutaneous infection model is not restored by *ktrB* deletion, suggesting that c-di-AMP controls not only cellular K^+ balance but also other metabolic and virulence pathways. SpeB induction in the Δ*dacA* mutant by K^+-specific ionophore treatment also supports the importance of cellular K^+ balance in SpeB production. However, the Δ*pde2* mutant does not revert its SpeB null phenotype when treated with ionophore, unlike the Δ*dacA* mutant, which suggests the underlying mechanism causing the SpeB null phenotype of the Δ*pde2* is different from the Δ*dacA* mutant.

We performed transposon mutagenesis in Δ*pde2* mutant to discover the potential genes controlling SpeB in *S. pyogenes*. I identified one of the genes from the *dlt* operon, *dlt*X, as a suppressor of the SpeB null phenotype of the Δ*pde2* mutant. The *dlt* operon consists of four to five genes *dlt(X)ABCD* in most Gram-positive bacteria and primarily incorporates D alanine into lipoteichoic acid. The in-frame deletion of *dltX* or insertional inactivation of *dltA* in the Δ*pde2* mutant restored SpeB expression. These mutations did not affect the growth in the lab media but showed increased sensitivity to polymyxin B, as previously reported. Since Dlt mutation changes cell surface charge and possibly causes cell envelope stress, I deleted the gene of the response regulator LiaR in LiaFSR that senses cell envelope stress. The Δ*pde2* Δ*liaR* mutant also produced SpeB but less than that of the Δ*pde2* Δ*dltX* mutant. qRT PCR showed that the cell wall stressor vancomycin did not significantly change the expression of the LiaFSR-regulated gene, *spxA2* in the Δ*pde2,* or Δ*pde2* Δ*dltX* mutant compared to the wild type or Δ*pde2* mutant. The transcriptional regulator SpxA2 might compete with the *speB* transcriptional activator RopB, but overexpression of *ropB* restored almost no SpeB in the Δ*pde2* mutant. My results suggest that the Dlt system and LiaFSR influence SpeB expression in the Δ*pde2* mutant through two separate

pathways; further investigation is required to understand how Pde2 and D-alanylation of teichoic acid are linked to SpeB expression in *S. pyogenes*. My findings provide insight into the c-di-AMP signaling pathway in GAS virulence regulation and pathogenesis, which could contribute to developing therapies targeting the c-di-AMP signaling pathway.

PREFACE

This Ph.D. work focuses on understanding the second messenger molecule c-di-AMP signaling pathway in the virulence regulation and pathogenesis in *Streptococcus pyogenes*. Group A Streptococcal (GAS) diseases have been known for centuries; still, it is considered a significant public health concern for both developed and developing countries. Approximately 18 million people suffer from severe GAS infections, and half a million die every year globally, indicating an urgent need for an alternative therapy for GAS treatment. The research done for this dissertation has shown that c-di-AMP regulates a significant virulence factor SpeB in *S. pyogenes*. The insight knowledge of this study may open new horizons in therapeutics for GAS treatment by targeting the c-di-AMP signaling pathway.

This research was conducted in Dr. Kyu Hong Cho's Laboratory at Indiana State University, Terre Haute, IN. As a graduate student, I was the leading investigator in this research and conceived the idea, collected data, performed laboratory experiments, and carried out data analysis under the supervision of Dr. Kyu Hong Cho at Indiana State University, IN.

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CHAPTER 1

General Introduction

Streptococcus pyogenes

Streptococcus pyogenes, or group A Streptococcus (GAS), is a major human pathogen responsible for several superficial, invasive, and autoimmune-related diseases. It is considered one of the top 10 infectious causes of human mortality (Carapetis et al., 2005; Walker et al., 2014). A survey estimated that over 18 million people suffer from severe GAS diseases, resulting in over half a million deaths annually (Carapetis et al., 2005). Although most GAS infections, such as pharyngitis and impetigo, are self-limiting, they can penetrate deeper tissues and produce severe, life-threatening diseases like necrotizing fasciitis, sepsis, and other conditions (Carapetis et al., 2005; Lamagni et al., 2005; Walker et al., 2014). In addition, autoimmune sequelae are more acute, and approximately 33 million people developed rheumatic heart disease (RHD) in 2015 globally (Watkins et al., 2017). The lack of effective vaccinations or medications and the emergence of new GAS strains with higher virulence properties are probably the leading causes of the increased global burden of GAS infections (Barnett et al., 2018; Carapetis et al., 2005). Therefore, understanding the GAS virulence regulation to specific disease processes is crucial to developing better treatment regimens.

The name '*Streptococcus pyogenes'* is derived from Greek words in which *Streptococcus* means a chain of berries, and *pyogenes* defines pus-forming. It is a

spherical or round shape, chain-forming bacteria that produces pus in most streptococcal infections (Alouf & Horaud, 1997; Evans, 1936). As a gram-positive bacteria, *S. pyogenes* has a thick cell wall with abundant peptidoglycan and teichoic acids, mostly lipoteichoic acids (LTA) in its cell wall (Neuhaus & Baddiley, 2003). According to the Lancefield classification, it belongs to group A *Streptococcus* (GAS) due to the presence of type A antigen on the cell surface, composed of N-acetylglucosamine attached to a rhamnose polymer backbone (Cunningham, 2000; Kanwal & Vaitla, 2022). Other important GAS properties are catalase-coagulase negative, oxidase negative, non-motile, and non-spore-forming bacteria. It grows best anaerobically at 37 degrees Celsius and produces white-greyish, moderate to small and smooth colonies on the agar plate (Kanwal & Vaitla, 2022). It is also classified as beta-hemolytic Streptococci due to its ability to cleave red blood cells and form a clear zone on the blood agar plate (Cunningham, 2000).

The common risk groups of GAS infections are children, immune-compromised or older people (Avire et al., 2021). It causes severe complications in infants and adults (Ralph & Carapetis, 2013). Additionally, GAS can appear as normal flora in the nasopharynx; approximately 5–15% of healthy people exhibit no symptoms (Oliver et al., 2018). It is highly contagious and can spread through skin contact, contact with infected objects, dust, inhaling air droplets, and nasal discharge. It can occasionally transmit through contaminated food (Cunningham, 2000; Kanwal & Vaitla, 2022). GAS infections are common in developing countries because of overcrowding, malnutrition, poor living conditions, and poor medical support (Avire et al., 2021; Cunningham, 2000).

Typically, GAS infections are mild infections of the throat (pharyngitis or strep throat) or skin (impetigo or skin shore) (Ibrahim et al., 2016; Kanwal & Vaitla, 2022). It also can cause

severe invasive diseases, including necrotizing fasciitis or ‘flesh-eating disease,’ cellulitis, bacteremia, and streptococcal toxic shock syndrome (STSS) by acquiring access to the blood or deeper tissue through cuts or abrasion of the skin (Cunningham, 2000; Kanwal & Vaitla, 2022). Besides, antibiotics such as penicillin, cephalosporin, or macrolides are frequently used to treat GAS infections. Though GAS is very sensitive to penicillin, its resistance to clindamycin or macrolides is increasing worldwide (Bryant et al., 2020). Moreover, lack of proper treatment or repeated GAS infections develops non-suppurative autoimmune sequelae, acute rheumatic fever (ARF), which can further damage the heart and cause severe rheumatic heart diseases (RHD) (Carapetis et al., 2005). The mortality rate of RHD is significantly high; approximately 319400 deaths occurred in 2015 globally (Watkins et al., 2017). Another important autoimmune disease caused by GAS is acute post-streptococcal glomerulonephritis (APSGN), a kidney disorder mediated by immune complex deposition in the glomerular basement membrane (Blyth et al., 2007; Shulman & Tanz, 2010).

GAS Virulence Factors and their Pathogenesis

The attachment of GAS to the host cell surface is the crucial primary step to establishing GAS infection. GAS adherence to host tissue follows two-step processes: the first is mediated by weak hydrophobic interaction, and the second utilizes the strong ligand-receptor irreversible adherence (Hasty et al., 1992). Strong attachment between the GAS ligand and the host receptor helps bacteria to survive by washing out mucous or salivary fluid flow. Additionally, GAS must compete with normal flora when adhering to the host tissue. Its successful colonization and penetration into deeper tissue mainly depend on its ability to overcome chemical, mechanical, or microbiological barriers at the entry site (Hasty et al., 1992). However, GAS can easily colonize the epithelial surface of the skin, throat, anus, or genital mucosa (Cunningham, 2000).

GAS expresses an array of cell wall-associated and secreted virulence factors essential for successful infection (Cunningham, 2000; Olsen et al., 2009). The cell surface adhesins, including the hyaluronic acid capsule (HA), pili, M proteins, and the *S. pyogenes* fibronectin-binding adhesin (SfbI), protein F contribute to adhesion and colonization to the host tissue (Rohde & Cleary, 2016). It has been reported that multiple adhesins on the bacterial cell surface aid in adhering host tissue intensely during infection (Hasty et al., 1992). Besides, GAS secretes several exotoxins extracellularly, which are often proteins in nature (Hynes & Sloan, 2016). Some are streptokinase, proteinase, esterase, the hemolysins SLO and SLS, CAMP factors, DNases, hyaluronidases, complement inhibitors, superoxide dismutase, and immunoglobulin degrading enzymes (Hynes & Sloan, 2016). However, the expression and function of different virulence factors may vary depending on the site of infection, the infection stages, or the genetic variation of different strains.

M Protein

One of the well-studied virulence factors of *S. pyogenes* is M protein, identified by Rebecca Lancefield in 1927 (Lancefield, 1928; Schwartz et al., 1990). The gene '*emm*' encodes M protein, an alpha-helical coiled-coil rod-shaped surface protein. It contains a hypervariable N terminal region and LPXTG motif-containing C terminal region (Lancefield, 1928). Since more than 200 types of M protein are identified in GAS, an individual can get repeated infections with different types of M strain (Metzgar & Zampolli, 2011). The versatile role of M protein in GAS infection includes resistance to phagocytosis, adherence to epidermal keratinocytes, microcolony formation, invasion of epithelial cells, and the cross-reactivity of anti-M protein antibodies with heart muscle (Cunningham, 2000; Metzgar & Zampolli, 2011; Oehmcke et al., 2010).

S. pyogenes may contribute to the development of human diseases through the antiphagocytic characteristics of the M protein. A previous report showed that the insertion of the *emm6* gene in an *emm* deficient strain restored its ability to escape phagocytosis (Perez-Casal et al., 1992). M protein binds to human immune factor H or H-like protein and inactivates the complement pathway by destroying C3-convertase, thus preventing opsonization by C3b, ultimately inhibiting phagocytosis (Horstmann et al., 1988; Johnsson et al., 1998; Kotarsky et al., 2001). Furthermore, the binding between M protein and fibrinogen contributes to developing resistance to phagocytosis (Carlsson et al., 2005). Besides, GAS M protein acts as a cross-reactive antigen that mimics host immune molecules and leads to development of acute rheumatic fever (Guilherme et al., 1995; Roberts et al., 2001). Antibodies against streptococcal M protein react with M protein and the myocardium, the middle thick layer of the heart wall (Cunningham et al., 1989; Cunningham et al., 1988).

Streptokinase

The virulence factor streptokinase is a single-chain protein consisting of 414 amino acids with three distinct domains (Wang et al., 1998). The critical function of streptokinase is to activate the host plasminogen, which is highly specific to human plasminogen (Boxrud & Bock, 2004; Marcum & Kline, 1983). Streptokinase cleaves plasminogen in plasma or extracellular fluids and converts it into enzymatically active plasmin. Activated plasmin can further degrade fibrin clots, connective tissue, extracellular matrix components, and adhesion proteins (McArthur et al., 2012; Wang et al., 1998). Moreover, plasmin activates metalloproteases, which cleave collagen structural components and cause severe tissue destruction (Plow et al., 1995). Thus, activated plasmin by streptokinase plays a crucial role in disseminating bacteria from the initial site of infection to other sterile parts of the body (Walker et al., 2005).

Pyrogenic Exotoxins

S. pyogenes secretes several pyrogenic exotoxins to establish infections. The exotoxins, SpeA and SpeC, are superantigens that activate T cells nonspecifically, leading to massive cytokine production (Llewelyn & Cohen, 2002). Another essential virulence factor SpeB is highly conserved in GAS strains (Chaussee et al., 1997). SpeB is a cysteine protease that cleaves numerous host serum proteins, including cytokines, chemokines, complement components, various immunoglobulins protease inhibitors, and its own surface proteins (Nelson et al., 2011). The unique ability of SpeB to degrade host proteins and its surface proteins may help GAS evade the host's defenses and invade deeper tissue (Rasmussen & Björck, 2002). Furthermore, extracellular secreted toxins contribute to severe GAS invasive diseases or toxigenic diseases, including necrotizing fasciitis, streptococcal toxic shock syndrome (STSS), or scarlet fever (Johansson et al., 2008; Spaulding et al., 2013). In addition, SpeB is highly expressed in autoimmune sequelae of acute post-streptococcal glomerulonephritis (APSGN) (Batsford et al., 2005). Since exotoxins are the main virulence components of GAS diseases, it is crucial to understand their virulence regulation in GAS pathogenesis.

Streptolysins

The two significant streptolysins produced by *S. pyogenes* are streptolysin S (SLS) and streptolysin O (SLO), which involve damage to a variety of human cells, including erythrocytes (Alouf, 1980; Molloy et al., 2011). SLO is a 540 amino acid long, oxygen-sensitive, thiol-activated cytolysin (Alouf, 1980). It binds to a monomer of the membrane's cholesterol that causes oligomerization and pore formation on the membrane leading to cell lysis (Bhakdi et al., 1985). SLO affects the cell membrane and facilitates the active transport of streptococcal NAD glycohydrolase into the host cells (Madden et al., 2001). The function of the NADase is to

hydrolysis β-NAD^+ into ADP-ribose or nicotinamide (Ghosh et al., 2010). SLO also plays a vital role in GAS pathogenesis. The previous report showed that SLO mutants appear to be attenuated for virulence (Ato et al., 2008). Additionally, it can induce an inflammatory response involving tissue destruction (Brosnahan & Schlievert, 2011).

SLS belongs to a group of proteins called thiazole/oxazole-modified microcins. (TOMM). It is a 2.7 kDa peptide that undergoes heavy post-translational modification, including several heterocyclisations (Molloy et al., 2011). SLS is oxygen-stable and damages numerous cell types, including erythrocytes, platelets, and leukocytes, by creating hydrophilic pores on the membrane (Ginsburg, 1999; Molloy et al., 2011). It is considered the causative agent of beta-hemolytic activity of *S. pyogenes* (Brosnahan & Schlievert, 2011). It has been reported that SLS and SpeB have a synergistic role in the mouse model in which mouse mortality is influenced by SLS, whereas local tissue injury is influenced more by SpeB (Hung et al., 2012).

Hyaluronidase, DNase and other Degrading Enzymes

GAS produces not only pyrogenic toxins but also secretes several degrading enzymes that facilitate to spread the infection. For example, the hyaluronidase enzyme cleaves hyaluronic acid, a vital constituent of human connective tissue (Meyer et al., 1940). GAS hyaluronidase can also degrade its hyaluronic acid capsule (Sandson et al., 1968).

Another well-characterized virulence factor is DNase. At least eight different DNases are identified in GAS including spnA, spdB, sda1, sda2, spd1, spd3, spd4, and sdn (Remmington & Turner, 2018). DNases are hypothesized to degrade the neutrophil extracellular trap (NETs), thus helping GAS evade the host's innate immune system (Buchanan et al., 2006). It also contributes to disseminating the infection by liquefying pus, similar to streptokinase (McCarty, 1948; Sherry & Goeller, 1950; Tillett et al., 1948; Zitvogel et al., 2015).

The streptococcal C5a peptidase (ScpA) is a serine protease that is thought to contribute to pathogenicity in the early stage of streptococcal diseases (Chen & Cleary, 1990). It contains a particular catalytic domain Ser-Asp-His that cleaves the complement component C5a. The degradation of C5a by ScpA inhibits the complement-mediated phagocytosis that allows bacteria to colonize better at the site of infections (Ji et al., 1996). Additionally, the C5a peptidase is believed to be a potential candidate for developing a streptococcal vaccine (Ji et al., 1997). Another serine protease, SpyCEP, interferes with bacterial clearance and promotes bacterial transmission to the neighborhood lymph node and systemic circulation (Kurupati et al., 2010). It inactivates neutrophil chemokines, mainly interleukin 8 (IL-8), thus inhibiting the recruitment of neutrophils to the infected area (Hynes & Sloan, 2016; Kurupati et al., 2010).

Virulence Related Transcriptional Regulators of GAS

S. pyogenes has shown a remarkable capacity to invade numerous tissue sites and produce diverse diseases in humans. Its ability to probe its environment and quickly change its gene expression accounts for its potential to infect various tissues. Thirteen two-component regulatory systems and at least 30 transcriptional regulators are currently known to control the expression of virulence factors in GAS (Kreikemeyer, McIver et al. 2003). These regulatory genes form a constantly changing network to detect and respond to environmental signals such as osmolality, temperature, pH, salt, and nutrition availability. Virulence-related regulators are primarily classified into three groups based on their modes of action: two-component signal transduction systems (TCSs), transcriptional activators/repressors, and non-coding RNAs (Vega, Malke et al. 2016). Some well-characterized transcription factors in GAS include CovR/S, RofA, and the RofA-like protein type master regulators, Rgg/RopB, carbohydrate catabolite regulators CcpA, LacD.1, and Mga; are described below.

Control of Virulence CovR/S

Control of virulence (CovR/S) or capsule synthesis regulator (CsrR/S) is a well-studied TCS in *S. pyogenes*. It consists of a periplasmic sensor domain of histidine kinase CovS and the DNA binding transcriptional regulator CovR (Miller et al., 2001). CovS senses the environmental signal and then transfers the signal to CovR, which regulates the expression by directly binding to the promoter region of virulence gene regulons (Horstmann et al., 2022; Miller et al., 2001). Since it has been shown to affect the transcription of up to 15% (271 genes) of all chromosomal genes in GAS serotype M1, it is regarded as a master regulator (Federle et al., 1999; Graham et al., 2002). CovR primarily functions as a transcriptional repressor and modulates the expression of many virulence factors, including streptolysin S (SagA), cysteine protease (SpeB), NAD glycohydrolase (Nga), streptolysin O (Slo), IL-8 degrading protease (SpyCEP), streptokinase (Ska), and streptococcal fibronectin protein (Fba) (Bernish & van de Rijn, 1999; Darmstadt et al., 2000; Graham et al., 2002; Heath et al., 1999; Levin & Wessels, 1998). It has been shown that CovR is highly conserved among beta-hemolytic streptococci, and homologs of CovR are found in various human pathogens, including *Staphylococcus aureus* (Khara et al., 2018; Liang et al., 2005). Furthermore, mutation in the *covS* gene results in CovRS inactivation, which shields GAS from phagocytosis and promotes severe invasive infection (Walker et al., 2007).(Sumby et al., 2006).

RofA and the RofA-like Protein Type Regulators (RALPs)

RALPs are essential to control the interaction between *S. pyogenes* and its host cells, avoid host cell damage, and regulate virulence factors during stationary phase growth (Beckert et al., 2001; Podbielski, Woischnik, Leonard, et al., 1999). Four members of the RofA-like protein have been found in *S. pyogenes,* including RofA, Nra, Ralp3, and RivR (Granok et al., 2000).

RALPs are involved in regulating the microbial surface components such as fibronectin-binding proteins F (SfbI) and F2, and collagen binding protein (Cpa) (Patti et al., 1994). In addition, several virulence factors, including streptolysin S, cysteine protease SpeB, superantigen SpeA and the virulence regulator, Mga, are directly controlled by RALP (Beckert et al., 2001; Kreikemeyer et al., 2002; Podbielski, Woischnik, Leonard, et al., 1999). RofA has been best characterized in *S pyogenes* and identified as a positive transcriptional activator of the *prtF* gene encoding fibronectin-binding protein F in a serotype M6 strain (Fogg & Caparon, 1997). Although RofA was first believed to be a positive regulator, multiple studies showed that RofA negatively regulates the transcription of streptolysin S (*sagA)*, SpeB protease *(speB)*, and SpeA superantigen *(speA*) (Beckert et al., 2001; Fogg & Caparon, 1997). Nra is another well-studied RALP that was found in serotypes M3, M18, and M49 (Kratovac et al., 2007). Nra was discovered in the serotype M49 GAS strain as a negative regulator for the genes encoding the proteins Cpa, a collagen-binding protein, and PrtF2, a second fibronectin-binding protein (Podbielski, Woischnik, Leonard, et al., 1999). Additionally, numerous virulence genes, including *speB, speA*, *sagA*, and *mga*, were also suppressed by Nra. It is highly expressed in the early stationary phase and does not seem to respond to changing atmospheric conditions (Podbielski, Woischnik, Leonard, et al., 1999). The other two members such as RALP-3 and RALP-4, were discovered by homology comparison within the serotype M1 genome sequence (Kreikemeyer et al., 2002; Vega et al., 2016). Ralp3 controls extracellular protein factor (Epf), Streptolysin S and cysteine protease SpeB while downregulating two operons involved in metabolism, including the lac operon and the fru operon (Kreikemeyer et al., 2007; Podbielski, Woischnik, Leonard, et al., 1999; Siemens et al., 2012). Ralp4 or RivR was discovered and classified as a RALP according to its 29% and 32% identity at amino acid level with RofA and

Nra, respectively. RivR is an essential regulator for growth and virulence in a CovR-deficient GAS strain. It has been shown that RivR improves transcriptional activation by Mga in vitro and boosts expression of the Mga regulon in vivo (Roberts & Scott, 2007).

Rgg/RopB

The transcriptional regulator RopB is a DNA binding protein that primarily regulates virulence-related genes in a growth phase-dependent manner in *S. pyogenes* (Lyon et al., 1998). It belongs to the Rgg family, and Rgg-like proteins are commonly found in other gram-positive bacteria, including *S. pneumoniae, S. agalactiae, S. mutans, S. oralis, S. sanguis,* and *Listeria monocytogenes* (Chaussee et al., 1999; Glaser et al., 2001; Lyon et al., 1998; Neely et al., 2003). RopB was first identified as an activator of a significant virulence factor SpeB. It is closely located to *speB* and influences *speB* transcription during the stationary phase (Chaussee et al., 1999; Lyon et al., 1998). It also controls the expression of several other proteins, including lysozyme, autolysin, ClpB heat shock protein, and streptodornase (Chaussee et al., 2001). In addition, Rgg/RopB affects the expression of amino acid metabolic genes during the stationary phase (Chaussee et al., 2004; Chaussee et al., 2003). Several two-component systems, such as CsrRS/CovRS, FasBCAX and Ihk/Irr, are also activated by RopB. In contrast, it negatively affects *mga* transcription at the stationary phase (Chaussee et al., 2002; Kreikemeyer et al., 2003).

The Multiple Virulence Gene Regulator (Mga)

Mga is one of the most critical virulence regulators in almost all *S. pyogenes* strains (Hollingshead et al., 1994; McIver et al., 1995). It is a 500 amino acid long, DNA binding protein with a molecular weight of approximately 62 kDa (Spanier et al., 1984). Mga was first discovered in *S. pyogenes* through spontaneous minor deletions at a location directly upstream of

the *emm* gene encoding M protein (Spanier et al., 1984). It contains two conserved helix-turn-helix (HTH) domains, HTH-3 (residues 53-72) and HTH-4 (residues 107-126), located close to the N-terminus, which are necessary for DNA binding and transcription activation for all identified genes (McIver & Myles, 2002; Vahling & McIver, 2006). Mga is defined as a 'stand-alone' regulator in GAS that controls virulence-related genes and allows bacteria to adapt to the host environment (Hondorp et al., 2012). It controls more than 10% of the GAS genome during exponential growth (Ribardo & McIver, 2006). Mga controls the M protein, an important virulence factor that helps bacteria resist phagocytosis (Hollingshead et al., 1994; Hondorp & McIver, 2007). It also stimulates the transcription of a core group of virulence genes, including M-like proteins (*arp*), cysteine protease (*speB*), C5a peptidase (*scpA*), collagen-like protein (*scl1*, *sclA*), fibronectin-binding proteins (*fba*, *sof*), and the secreted inhibitor of complement (*sic*) (Hondorp & McIver, 2007). Additionally, it appears to be involved in repress operons that code for proteins essential for the transport and metabolism of sugar molecules (Ribardo & McIver, 2006).

CcpA and LacD1

The catabolite control protein A (CcpA) is a master regulator which influences both virulence and carbon catabolite repression in *S. pyogenes* (Paluscio et al., 2018). It belongs to the family of transcription factors known as LacI/GalR, mainly present in Gram-positive bacteria (Deutscher et al., 2006). CcpA controls the transcription of about 20% of GAS's entire genome, including many virulence factors (Kietzman & Caparon, 2010). Moreover, it acts as a carbohydrate-dependent transcriptional repressor that binds to DNA at a palindromic consensus sequence in the promoter regions of the catabolite-responsive element (cre) site (Deutscher et al., 2006). The previous report showed that the histidine-containing protein (HPr) phosphorylated at

Ser46 (HPrSer46P) significantly increases CcpA affinity for cre sites (Aung-Hilbrich et al., 2002). Multiple virulence-related genes such as *sag* operon, *rivR*, *speB*, *mac*, and *spd3* have been shown to regulate by CcpA (Shelburne et al., 2008). Furthermore, CovR and CcpA co-regulate several virulence genes, such as *nga*, *slo*, *spyCEP*, *sagA*, *sdaD2*, *endoS*, *fba*, and *speB*; however, their expression is not affected directly by each other (Shelburne et al., 2010).

Another carbohydrate-dependent regulator, LacD1, involves carbon catabolic repression in GAS. It is an aldolase enzyme involved in lactose and galactose metabolism (J. A. Loughman & M. G. Caparon, 2006). It has been reported that LacD.1 is active concurrently with CcpA's carbon catabolite repression (CCR) of virulence-related genes. They can regulate up to 15% of the GAS genome in response to glucose and affect the expression of virulence genes, including Streptolysin S (*sagA*) and lactose oxidase (*lctO)* (Kietzman & Caparon, 2010, 2011). However, the virulence factor SpeB is also influenced by the co-regulation of CcpA and LacD.1. The previous studies showed that SpeB is enhanced by CcpA but inhibited by LacD.1 both in vitro and in vivo (Kietzman & Caparon, 2011).

Host Response to GAS

The host's first line of defense against GAS infections includes the physical barrier of the skin or mucosal epithelium and competition with the host's normal flora (Valderrama & Nizet, 2018). GAS's capacity to overcome those mechanical or microbiological barriers at the entry point determines its ability to colonize and penetrate deeper tissue. However, the host uses several protective mechanisms such as coughing, sneezing, epiglottal reflexes, and mucociliary movement to prevent the GAS transmission from the superficial epithelial layer to another part of the body (Hasty et al., 1992). The innate immune system becomes a vital component of the body's defense against invasive infection once GAS has established itself in the host and is

prepared to penetrate the epithelial barrier (Sarantis & Grinstein, 2012). Professional phagocytic cells like macrophages, dendritic cells, monocytes, and granulocytic cell types like neutrophils and mast cells are the essential front-line regulators of innate immunity (Sarantis & Grinstein, 2012). The phagocytic process is activated upon recognition of a ligand by cell surface receptors (Pauwels et al., 2017). The early immune cells (neutrophils, macrophages, and dendritic cells) express a class of receptors on the cell surface called pattern recognition receptors (PRRs) (Takeda et al., 2003). PRRs recognize multiple pathogen-associated molecular patterns (PAMPs), such as lipoteichoic acid, peptidoglycan, lipopolysaccharide, or unmethylated bacterial CpG DNA. The interaction between PAMPs and PRRs initiates signaling cascades that result in the production of cytokines and chemokines (Takeda et al., 2003). However, a specific type of PRR, a Toll-like receptor (LTR), can be stimulated by PAMPs in GAS (Valderrama & Nizet, 2018). It is well established that MyD88, the primary signaling adaptor protein of TLRs, is required to begin an adequate inflammatory response to GAS infection (Joosten et al., 2003; Loof et al., 2010; Loof et al., 2008).

In order to manage GAS infections, neutrophils produce reactive oxygen species (ROS), antimicrobial peptides, and neutrophil extracellular traps (NETs). The induction of extensive neutrophil extracellular traps (NETs) by GAS help the host to prevent the dissemination of GAS infection (Buchanan et al., 2006; Sumby et al., 2005). Additionally, macrophages and dendritic cells (DCs) are crucial for controlling GAS diseases. Numerous cytokines and chemokines, most importantly IL-1 and CXCL1, are secreted by macrophages and DCs in response to GAS, which will recruit additional neutrophils to the site of infection. (Harder et al., 2009; Kurupati et al., 2010). Moreover, all three complement pathways: classical, alternative, and mannose-binding lectin, can be activated by GAS (Merle et al., 2015; Reis et al., 2019). The deposition of the C3b

complement component on the surface of the GAS facilitates complement-mediated opsonization by phagocytic cells (Pangburn et al., 1977). Furthermore, two anaphylatoxins, C3a and C5a, are released by complement activation, acting as a chemotactic factor that attracts neutrophils to the site of infection. In addition, the membrane attack complex (MAC) formed by the combination of C5b and C7-9 ruptures the GAS membrane and causes cell apoptosis (Pandey et al., 2020).

GAS Therapeutic Treatment

Antibiotic therapy is recommended to treat mild and severe GAS infections (Dajani et al., 1995; Shulman et al., 2012b). Penicillin-based antibiotics like ampicillin and amoxicillin are excellent options for treating GAS infections due to the high sensitivity of *S. pyogenes* (Gerber, 1996; Kaplan et al., 1999; Shulman et al., 2012a). Alternatively, macrolides such as erythromycin, azithromycin, clarithromycin, or clindamycin are prescribed for patients allergic to penicillin (Choby, 2009; Kim, 2015). Moreover, early treatment with antibiotics is essential to control the GAS infection. An oral regimen of antibiotics is advised for roughly ten days in pharyngitis and 5-7 days in cellulitis (Gerber et al., 1989). Ineffective treatment or not completion of the antibiotic course can develop post-infectious sequelae, including acute rheumatic fever, acute post-streptococcal glomerulonephritis (Walker et al., 2014).

Besides, patients with severe life-threatening invasive infections like STSS and necrotizing fasciitis require early identification, accurate diagnosis, and aggressive treatment. The surgical debridement of infected tissue and the combination of broad-spectrum antibiotics in IV form is recommended to treat such GAS invasive diseases (Young et al., 2006). Furthermore, intravenous immunoglobulin IgG is an alternative option to treat STSS. Though there are no licensed vaccines for GAS treatment, M protein is a potential target due to its ability to activate bactericidal antibodies in human serum (Gerber et al., 1989). However, more than 200 M protein

serotypes and their ability to react with the heart muscle limit the potential of M protein as a vaccine candidate (Steer et al., 2009). Other vaccine candidates in pre-clinical trials include pyrogenic exotoxin SpeA and SpeC, streptococcal C5a peptidase, streptolysin O, streptococcal cysteine protease SpeB, fibronectin-binding protein SfbI, *S. pyogenes* cell envelope proteinase, arginine deiminase, and serum opacity factor (Rivera-Hernandez et al., 2019; Uchiyama et al., 2015; van Sorge et al., 2014; Wang et al., 2020; Wang et al., 1998). The recent discovery of second messenger molecules and their significant role in physiology and virulence is well established in gram-positive pathogenic bacteria, including *S. pyogenes* (Corrigan, Abbott, Burhenne, Kaever, & Gründling, 2011). Understanding the signaling pathway of second messenger molecules in virulence regulation and pathogenesis would contribute to developing an alternative GAS treatment targeting their signaling pathways.

Bacterial Nucleotide-Based Second Messenger

Bacterial second messengers are signaling molecules that transmit signals from cell-surface receptors to effector proteins. Bacteria use second messengers to sense, respond, and adapt to constantly changing environmental situations (Hengge et al., 2016; Kalia et al., 2013; Pesavento & Hengge, 2009). Some mono- or dinucleotides are utilized by bacteria as a second messenger to adapt to environmental changes, such as temperature, nutrients, oxidative stress, and pH (Commichau et al., 2015; Gomelsky, 2011). Pathogens experience this phenomenon because they are continually exposed to different conditions in their hosts. However, when the initial signal is complex for the target proteins to recognize, bacteria utilize second messengers to carry information to the targets, often proteins or RNA molecules, and speed up signal transduction (Commichau et al., 2019).

Some well-characterized second messengers are cyclic adenosine phosphate (cAMP), guanosine tetraphosphate or pentaphosphate ((p)ppGpp, cyclic di-guanosine monophosphate (c-di-GMP), cyclic guanosine monophosphate-adenosine monophosphate (cGAMP), and cyclic di-adenosine monophosphate (c-di-AMP) (Corrigan & Gründling, 2013). Among them, the best-studied second messenger cAMP is produced in bacteria, archaea, fungi, and eukaryotes. It is involved in nitrogen consumption, virulence regulation and carbon catabolic repression in bacteria (Feucht & Saier, 1980; Görke & Stülke, 2008; Green et al., 2014; McDonough & Rodriguez, 2011). (p)ppGpp acts as an alarmone that activates when bacteria starve for nutrients, especially in the stationary phase (Gaca et al., 2015; Steinchen & Bange, 2016). ppGpp mediates stringent response by reducing metabolic activities such as transcription, replication, GTP synthesis, and ribosome assembly (Ross et al., 2016; Steinchen & Bange, 2016). Another second messenger, cyclic di-GMP (c-di-GMP), is a global regulator regulating various cellular activities that support surface adaptation, biofilm formation, cell cycle progression, and virulence (Jenal et al., 2017). A newly discovered second messenger, cGAMP, has been found in bacteria and eukaryotes. 3′, 3′-cGAMP was first discovered in bacteria, and later, 2′, 3′-cGAMP was found in mammalian cells (Davies et al., 2012; Hornung et al., 2014). The eukaryotic 2′, 3′-cGAMP is synthesized by the host enzyme cGAS (cyclic GMP AMP synthase), which is activated by bacterial DNA in the host cytosol. Moreover, it plays a crucial role in the innate immune response through the activation of the stimulator of interferon (IFN) genes (STING) (Devaux et al., 2018; Woodward et al., 2010). Bacterial 3′, 3′ -cGAMP was shown to regulate chemotaxis and anaerobic respiration in *Vibrio cholerae* and *Geobacter metallireducens*, respectively (Davies et al., 2012; Nelson et al., 2015).

Cyclic di-adenosine monophosphate (c-di-AMP) is the most recent member of the second messenger family and is primarily found in Gram-positive and Gram-negative bacteria (Witte et al., 2008). Though c-di-AMP has been shown to be involved in diverse cellular activities in bacteria, including cell wall maintenance, potassium and osmotic homeostasis, antibiotic susceptibility, DNA damage repair, metabolism, virulence, genetic competence, sporulation and biofilm formation (Banerjee et al., 2010a; Bejerano-Sagie et al., 2006; Corrigan, Abbott, Burhenne, Kaever, & Grundling, 2011; Griffiths & O'Neill, 2012a; Oppenheimer-Shaanan et al., 2011; Pozzi et al.; Zhang et al., 2013), the detailed mechanism of controlling cellular processes and virulence are still not well understood.

Synthesis and Degradation of Cyclic di-Adenosine Monophosphate (c-di-AMP)

c-di-AMP is a small molecule that signals in response to environmental stimuli by interacting allosterically with proteins and riboswitches. c-di-AMP is ubiquitous in gram-positive bacteria, including human pathogens *Staphylococcus aureus*, *Streptococcus pyogenes*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Streptococcus spp.,* and a few Gram-negative bacteria, such as *Chlamydia trachomatis* and *Borrelia burgdorferi* (Andrade et al., 2016; Barker et al., 2013; Corrigan, Abbott, Burhenne, Kaever, & Gründling, 2011; Gándara & Alonso, 2015; Kamegaya et al., 2011; Pesavento & Hengge, 2009; Woodward et al., 2010). It is synthesized from two molecules of ATP or ADP by diadenylate cyclase (DAC) and degraded by phosphodiesterases (PDE) (Commichau et al., 2019). Five cyclases have been identified: DisA, CdaA or DacA, CdaS, CdaM, and CdaZ (Stülke & Krüger, 2020). They all contain the conserved diadenylate cyclase (DAC domain) in combination with other protein domains that regulate the activity of the active domain (Commichau et al., 2015; Gundlach et al., 2015). Most bacteria only produce one kind of diadenylate cyclase, DacA or DisA, although certain bacteria produce

numerous DACs. For instance, the spore-forming bacterium *B. subtilis* encodes the enzymes DisA, DacA, and CdaS, while *Clostridium* species produce DisA and DacA (Fahmi et al., 2017; Pathania et al., 2021). Furthermore, CdaM and CdaZ are identified in the highly genome-reduced bacterium *Mycoplasma pneumoniae* and the haloarchaeon *Haloferax volcanii*, respectively (Blötz et al., 2017; Braun et al., 2019). The most prevalent c-di-AMP synthase DacA is found in many bacteria, including gram-positive pathogens *S. pyogenes*, *S. pneumoniae*, *S. aureus*, and *L. monocytogenes* (Bai et al., 2014; Kamegaya et al., 2011; Woodward et al., 2010).

At least four different types of c-di-AMP degrading enzymes (PDEs), GDpP, PgpP, DhhP, and CdnP, have been identified that have either a DHH-DHHA1 (aspartate-histidine-histidine) or HD (histidine-aspartate) catalytic domain (Y. Bai et al., 2013; Blötz et al., 2017; Fahmi et al., 2017). PDEs degrade c-di-AMP into the linear form of phosphoadenyl adenosine (pApA), which can then be cleaved into two molecules of AMP (Y. Bai et al., 2013; Manikandan et al., 2014). Most c-di-AMP-producing bacteria have been found to carry GdpP and GdpP homologs, which contain the catalytic DHH/DHHA1 domain. This domain in GdpP cleaves the phosphodiester bonds of c-di-AMP and converts them into pApA (Rao et al., 2010a). A histidine-aspartate catalytic domain (HD) in PgpH's C-terminus binds to c-di-AMP with excellent specificity and breaks it down into 5'-pApA (Huynh et al., 2015a). Another phosphodiesterase, Pde2, a cytoplasmic protein, primarily hydrolyzes linear pApA to AMP (Y. Bai et al., 2013). Compared to the wild type, *pde2* gene deletion causes a rapid increase in intracellular pApA levels. So, the balance of intracellular pApA and c-di-AMP levels, essential for bacterial growth and survival, is thus maintained in part by Pde2 (Y. Bai et al., 2013; Bowman et al., 2016). A newly discovered phosphodiesterase CdnP in *S. agalactiae* is an extracellular cell wall-anchored protein that hydrolyzes c-di-AMP via AMP to adenosine

(Andrade et al., 2016; Firon et al., 2014). It has been suggested that CdnP helps *S. agalactiae* become more virulent by reducing the host innate immune system activated by c-di-AMP (Andrade et al., 2016).

The Role of c-di-AMP in Bacterial Physiology

The newly discovered second messenger, c-di-AMP, plays a significant role in physiology and virulence in firmicutes. c-di-AMP is essential for growth in rich media for several firmicutes, including *B. subtilis*, *L. monocytogenes*, *S. aureus* and *S. pneumoniae* (Jan Gundlach et al., 2017; Whiteley et al., 2017; Zeden et al., 2018). Moreover, excessive or drastically reduced amounts of the nucleotide appear harmful to the bacteria (Gundlach et al., 2015; Mehne et al., 2013). A prior study demonstrated that the expression of a highly active diadenylate cyclase interfered with cell division, and the absence of both phosphodiesterases accelerated the formation of suppressor mutations that blocked c-di-AMP (Gundlach et al., 2015; Mehne et al., 2013). Moreover, the accumulation of c-di-AMP causes many bacteria to become more sensitive to stress, notably at higher salt concentrations (Y. Bai et al., 2013). Therefore, c-di-AMP homeostasis is required in bacteria for its proper growth and fitness (Y. Bai et al., 2013; Chaudhuri et al., 2009; Corrigan et al., 2015; Song et al., 2005; Woodward et al., 2010).

Although c-di-AMP participates in many physiological processes, one of its primary functions is to regulate potassium homeostasis (J. Gundlach, C. Herzberg, V. Kaever, et al., 2017). It has been evident that the availability of potassium affects the amounts of c-di-AMP within cells. In *B. subtilis*, a strain lacking c-di-AMP can survive in environments with extremely low potassium levels. However, if the potassium supply is increased, the strain acquires suppressor mutations that aid potassium export (J. Gundlach, C. Herzberg, V. Kaever, et al., 2017; Pham et al., 2018). Many bacteria control the expression of potassium importers or

exporters by c-di-AMP. Several potassium transporters that bind c-di-AMP, including the RCK C domain-containing potassium transporter, KimA, KupA, and KupB, are regulated by c-di-AMP (Tascón et al., 2020). One of the most notable findings in c-di-AMP research was that c-di-AMP attaches to the ydaO riboswitch in *B. subtilis* and regulates potassium uptake. The interaction between c-di-AMP and ydaO causes a conformational change of the riboswitch that inhibits transcription elongation following transcriptional termination (J. Gundlach, C. Herzberg, V. Kaever, et al., 2017; Nelson et al., 2013). The potassium exporters are also regulated by c-di-AMP, similar to potassium importers. This phenomenon is observed in *S. aureus* potassium/proton antiporter CpaA, which is triggered by the binding of c-di-AMP to its RCK C domain (Chin et al., 2015).

Additionally, c-di-AMP is essential in regulating central nitrogen and carbon metabolism. Pyruvate carboxylase is a crucial c-di-AMP target in *L. monocytogenes*. When c-di-AMP binds to the enzyme, pyruvate carboxylase activity is allosterically inhibited, impairing the carbon flow into the tricarboxylic acid (TCA) cycle (Sureka et al., 2014). Furthermore, DarR, a c-di-AMP binding transcriptional factor, is vital for *M. smegmatis* to maintain membrane lipid homeostasis via controlling the expression of the genes involved in fatty acid metabolism (Zhang et al., 2013). Besides mutation in c-di-AMP degrading enzymes, phosphodiesterases increase intracellular c-di-AMP concentration, causing resistance to acid stress and beta-lactam antibiotics (Corrigan & Gründling, 2013; Rao et al., 2010a). In addition to its activities in cell wall homeostasis in *S. aureus* and *B. subtilis*, c-di-AMP also senses the integrity of DNA in *B. subtilis* (Corrigan, Abbott, Burhenne, Kaever, & Gründling, 2011; Oppenheimer-Shaanan et al., 2011; Pozzi et al., 2012). Recently, virulence regulation by c-di-AMP has been observed in *S.*

pyogenes. One of the significant virulence factors in GAS, the cysteine protease SpeB is controlled by c-di-AMP via the KtrAB potassium transporter in vitro (Faozia et al., 2021).

In general, c-di-AMP involves osmoregulation, DNA repair mechanisms, maintenance of cell wall homeostasis, fatty acid synthesis, virulence regulation, biofilm formation, and type I interferon response (Corrigan & Gründling, 2013; Faozia et al., 2021; Woodward et al., 2010). While c-di-AMP in bacteria is involved in a wide range of crucial functions, it is yet unknown what molecular processes control them. To fully understand the regulating mechanism, additional in-depth investigation is required to characterize the effector molecules in the c-di-AMP signaling pathway.

c-di-AMP Signaling Pathway in *S. pyogenes*

A major human-specific bacterial pathogen, *S. pyogenes*, generates c-di-AMP as a signaling molecule similar to other gram-positive bacteria, such as *S. aureus*, *B. subtilis*, *L. monocytogenes*, and other *Streptococcus. Spp*. (Andrade et al., 2016; Corrigan, Abbott, Burhenne, Kaever, & Gründling, 2011; Gándara & Alonso, 2015; Woodward et al., 2010). *S. pyogenes* produces only one type of c-di-AMP synthesizing enzyme, diadenylate cyclase (DacA), a membrane-associated enzyme. DacA catalyzes the synthesis of a single molecule of c-di-AMP from two molecules of ATP or ADP through a condensation reaction (Fahmi et al., 2019; Faozia et al., 2021). *S. pyogenes* produces two phosphodiesterases that are involved in c-di-AMP degradation, membrane-associated GdpP and cytosolic Pde2 (Fahmi et al., 2019). The phosphodiesterase GdpP cleaves c-di-AMP into the linear form of phosphoadenyl adenosine (pApA). Then Pde2 further degrades pApA to convert it into AMP (Fahmi et al., 2019; Faozia et al., 2021). c-di-AMP appears to bind to its target proteins or RNA molecules as a second messenger molecule, controlling cellular functions and virulence in *S. pyogenes.*

Aims of the Project

The present study investigated the c-di-AMP signaling pathway in SpeB virulence regulation and pathogenesis in *S. pyogenes*. The cysteine protease, SpeB, a significant virulence factor, plays a critical role in the pathogenesis of severe invasive diseases caused by *S. pyogenes* (Louie et al., 1998). Previously, our lab found that c-di-AMP has a significant role in maintaining *S. pyogenes* virulence. A high level of c-di-AMP containing *S. pyogenes* mutant strain Δ*gdpP* showed impaired SpeB production, decreased sensitivity to beta-lactam antibiotics and reduced virulence in a murine subcutaneous tissue model (Cho & Kang, 2013). However, it was not known how c-di-AMP controls SpeB in *S. pyogenes*. To decipher the underlying molecular mechanism of the c-di-AMP signaling pathway in SpeB regulation, I pursued the following specific aims:

1. Investigate the role of c-di-AMP in SpeB biogenesis and virulence in *S. pyogenes*
2. Determine how K^+ transporters KtrAB links c-di-AMP and SpeB biogenesis in *S. pyogenes*
3. Investigate how does Δ*pde2* mutant regulate SpeB virulence in *S. pyogenes*

CHAPTER 2

Cyclic-di-AMP Regulates SpeB Biogenesis and Virulence in *Streptococcus pyogenes*

Abstract

The second messenger, c-di-AMP in *S. pyogenes*, is synthesized by a diadenylate cyclase DacA and degraded by phosphodiesterases (PDEs) GdpP and Pde2. In this study, the biogenesis of a well-characterized virulence factor SpeB has been investigated in the gene deletion mutant strains, Δ*dacA* and Δ*pde2*. Different methods such as protease detection plate assay, western blot and quantitative PCR (reverse transcriptase real-time PCR) were performed to detect the production of SpeB. Both Δ*dacA* and Δ*pde2* mutant strains did not show any protease activity in the protease detection plate assay, and no SpeB was detected in the Western blot assay, either. Though Δ*dacA* and Δ*pde2* are involved in the opposite reaction in c-di-AMP biogenesis, their phenotype regarding SpeB production was almost identical. I performed a complementation test for mutant strains, Δ*dacA* and Δ*pde2*. Gibson Assembly or FastCloning method was used to clone the *dacA* and *pde2* genes into the pLZ12-Km shuttle vector. The resulting constructs were transferred into the mutant strains Δ*dacA* and Δ*pde2*, respectively. As expected, both complemented strains showed restored SpeB activity similar to the wild-type control. However, the growth of Δ*pde2* was not restored in Δ*pde2* (pPde2). Another major virulence determinant, the M protein of *S. pyogenes,* was also investigated by quantitative PCR analysis. Both Δ*dacA* and Δ*pde2* produced the same amount of M protein transcript as the wild

type, indicating that c-di-AMP does not regulate M protein biogenesis at the transcriptional level. I examined the virulence of the mutants with a murine subcutaneous infection model. Both mutant strains showed no lesion in the mouse tissue. My results show that c-di-AMP is essential in *S. pyogenes* pathogenesis, at least regulating SpeB biogenesis.

Introduction

Streptococcus pyogenes (GAS) is a prevalent gram-positive bacterial pathogen that causes many human diseases, from mild superficial throat or skin infection to severe life-threatening diseases. The most common infection caused by GAS is pharyngitis or strep throat, which accounts for 15-30% of cases in children and 5-15% in adults (Carapetis et al., 2005; Walker et al., 2014). It also causes impetigo, a superficial skin infection that frequently occurs in children, especially in tropical countries. Necrotizing fasciitis, sepsis, meningitis, cellulitis, and streptococcal toxic shock syndrome are some severe GAS invasive diseases that are relatively uncommon yet have a high fatality rate (Carapetis et al., 2005; Walker et al., 2014). Additionally, ineffective therapy for strep throat can result in more severe illnesses or post-infection sequelae such as post-streptococcal glomerulonephritis, acute rheumatic fever, and rheumatic heart disease (RHD) (Carapetis et al., 2005; Watkins et al., 2017). GAS is still a significant cause of high mortality and morbidity in underdeveloped countries. More than half a million people died from severe streptococcal diseases in 2005, and RHD alone resulted in more than 320,000 fatalities (Carapetis et al., 2005; Watkins et al., 2017).

S. pyogenes must be able to sense the distinct environmental signals from infection sites and adapt to the host tissues by controlling various cellular functions, including the biogenesis of virulence factors. Thus, a detailed understanding of the signaling pathway by which cellular activities, including the biogenesis mechanism of cell components and virulence factors, are

regulated will provide insights into the initial colonization, successive invasion, and spread of streptococcal infections.

Cyclic nucleotides that act as second messenger molecules play critical roles in signaling pathways that sense environmental changes such as stress, temperature, nutrition, and pH in both prokaryotes and eukaryotes (Hengge et al., 2016; Kalia et al., 2013; Pesavento & Hengge, 2009). As second messengers, these cyclic nucleotides transmit the signals to effector molecules (Hengge et al., 2016; Huynh et al., 2016). Cyclic di-adenosine monophosphate (c-di-AMP) is a new addition to the growing list of second messenger nucleotides. It has been identified in Gram-positive bacteria, including *Listeria monocytogenes*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Streptococcus* spp., and in a few Gram-negative bacteria such as *Chlamydia trachomatis* and *Borrelia burgdorferi* (Andrade et al., 2016; Barker et al., 2013; Corrigan, Abbott, Burhenne, Kaever, & Gründling, 2011; Gándara & Alonso, 2015; Hengge et al., 2016; Kamegaya et al., 2011; Pham et al., 2016; Woodward et al., 2010). c-di-AMP has been implicated in diverse cellular processes in bacteria, and its role in each bacterium appears to be distinctive (Fahmi et al., 2017). Its role has been discovered in fatty acid synthesis in *Mycobacterium smegmatis* (Zhang et al., 2013), in the growth of *S. aureus* under low K^+ conditions (Corrigan, Abbott, Burhenne, Kaever, & Gründling, 2011), in the sensing of DNA integrity in *B. subtilis* (Bejerano-Sagie et al., 2006; Oppenheimer-Shaanan et al., 2011) and in cell wall homeostasis in *S. aureus* and *B. subtilis* (Banerjee et al., 2010a; Corrigan, Abbott, Burhenne, Kaever, & Grundling, 2011; Griffiths & O'Neill, 2012a; Pozzi et al.). Though c-di-AMP has been shown to play a critical role in many pathogenic bacteria, neither its environmental stimuli nor the mechanisms controlling cellular processes and virulence are well understood (Corrigan et al., 2013; Pham et al., 2016).

c-di-AMP is synthesized from ATP or ADP by cyclase domain-containing proteins known as diadenylate cyclases (DACs). DAC enzymes catalyze the synthesis of a single molecule of c-di-AMP from two molecules of ATP or ADP through a condensation reaction (Commichau et al., 2015; Corrigan & Gründling, 2013; Huynh & Woodward, 2016; Kamegaya et al., 2011; Pesavento & Hengge, 2009). Some bacteria, such as *B. subtilis* and *Clostridium spp*. produce multiple DAC enzymes—however, most microorganisms produce only one DAC enzyme. *S. pyogenes* produces one DAC enzyme, DacA, which is the most common DAC as it is found in a wide variety of bacteria, including notable human pathogens such as *S. aureus, S. pneumoniae*, and *L. monocytogenes* (Corrigan et al., 2013; Rosenberg et al., 2015).

The c-di-AMP phosphodiesterases (PDEs) degrade c-di-AMP, converting it into the linear form of phosphoadenyl adenosine (pApA), which can then be further degraded into two molecules of AMP (Y. L. Bai et al., 2013; Manikandan et al., 2014). Three PDEs have been discovered to degrade c-di-AMP: GdpP, Pde2, and PgpH (Huynh et al., 2015b; Rao et al., 2010b). The presence of each class of PDEs varies by bacterial species, but most bacteria produce two c-di-AMP PDEs. *Streptococcus* and *Staphylococcus* species possess GdpP and Pde2, and other bacteria such as *L. monocytogenes* produce GdpP and PgpH (Huynh et al., 2015b).

Previously, we studied one of the PDEs, GdpP, in *S. pyogenes* (Cho & Kang, 2013). In the *gdpP* in-frame deletion strain, Δ*gdpP* produced less amount of virulence factor SpeB at a post-transcriptional level, indicating that c-di-AMP has an important role in SpeB biogenesis. Also, the mutant strain showed virulence lower than the wild type.

SpeB is a cysteine protease secreted at the stationary phase. It accounts for over 90% of the total secreted proteins in many strains, including our lab strain, HSC5

(Podbielski, Woischnik, Kreikemeyer, et al., 1999). SpeB damages the host directly by destroying host proteins such as fibronectin and vitronectin (Kapur, Topouzis, et al., 1993) or indirectly by activating host matrix metalloproteases (Burns et al., 1996). SpeB also degrades host immune factors such as immunoglobulins (Collin & Olsen, 2001; Collin et al., 2002; Eriksson & Norgren, 2003), C3b (Terao et al., 2008) and plasminogen (Cole et al., 2006) and activates immune-modulating host molecules such as kinins (Herwald et al., 1996) and IL-ß (Kapur, Majesky, et al., 1993). SpeB liberates streptococcal cell surface virulence factors such as M protein, protein F, and C5a peptidase (Rasmussen & Björck, 2002). The release of these surface proteins disturbs the host immune system (Rasmussen & Björck, 2002). SpeB is initially translated as a preproprotein (43 kDa) containing a signal sequence and a pro region. During secretion, the signal sequence is cleaved off, and SpeB is secreted as proSpeB zymogen (~40 kDa), which is inactive. After being secreted, the zymogen is folded and processed to the active protease (28 kDa) by cleavages in the pro region, mainly through autocatalysis (Cho & Kang, 2013).

In addition to GdpP, *S. pyogenes* produces two other enzymes involved in c-di-AMP biogenesis: the c-di-AMP synthase DacA (Spy_1036) and the other phosphodiesterase Pde2 (Spy_0720). Here, I studied the enzymes involved in c-di-AMP biogenesis by analyzing their gene deletion strains, Δ*dacA* and Δ*pde2,* to decipher the role of c-di-AMP in virulence and pathogenesis in *S. pyogenes.*

Results

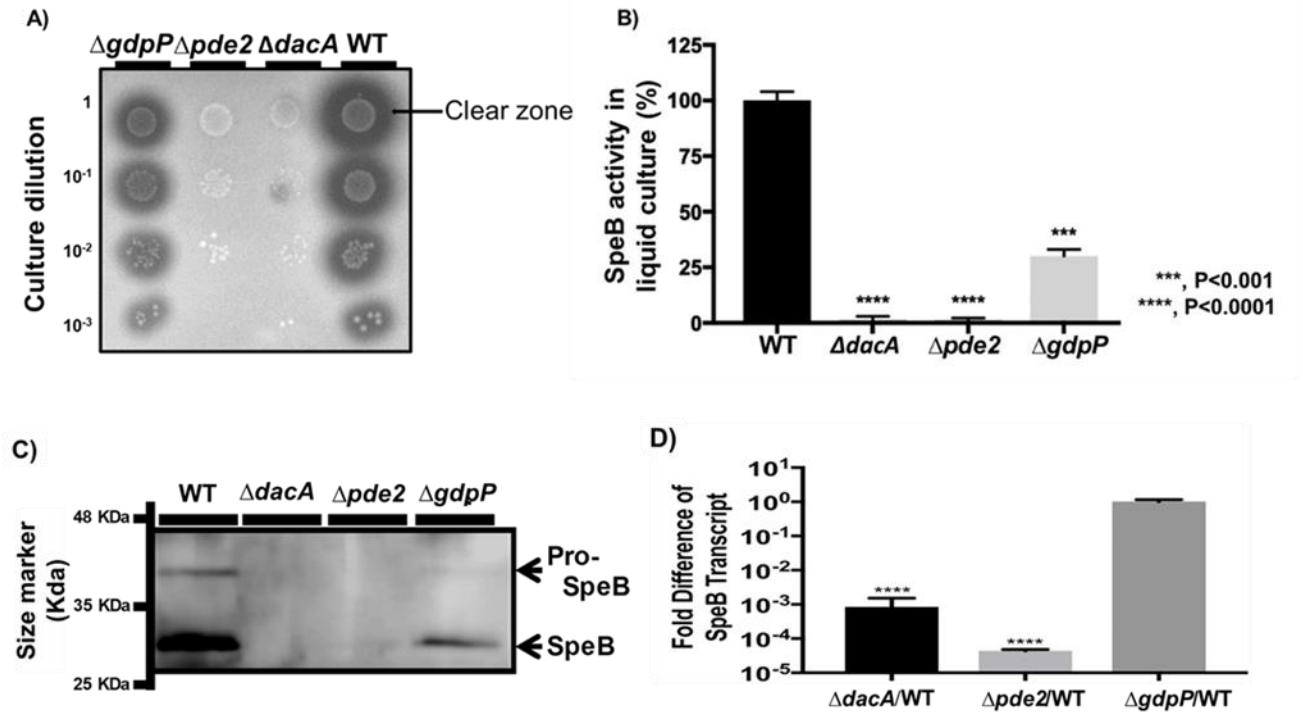
C-di-AMP Influences the Production of a Major Virulence Factor SpeB in *S. pyogenes*

All of the mutants with deletion of a gene involved in c-di-AMP synthesis or degradation presented a defect in the production of the secreted protease SpeB (Fig. 2.1A). As we reported

previously, the production of SpeB by *ΔgdpP* was reduced relative to that of the wild type. *ΔdacA* and *Δpde2* showed a much more severe defect than *ΔgdpP* in SpeB production (Cho & Kang, 2013). Both the *ΔdacA* and *Δpde2* strains showed no protease activity on protease indicator plates (Fig. 2.1A) and in liquid culture (Fig. 2.1B) and did not produce detectable zymogen or processed SpeB as examined by Western blotting (Fig. 2.1C). Interestingly, even though *dacA* and *pde2* have opposing activities of c-di-AMP synthesis and degradation, respectively, their phenotypes in relation to SpeB production were very similar.

Figure 2.1.

ΔdacA and Δpde2 produce no SpeB due to a transcriptional defect.



Note. (A) Protease indicator plates showing an absence of SpeB activity from *ΔdacA* and *Δpde2*. Strains were grown overnight and spotted (2 µl) on 2% skim milk agar plates after serial dilution. Protease activity forms a clear zone around spotted cells. SpeB is the major protease secreted by *S. pyogenes* HSC5, as a *ΔspeB* strain produces no zone of clearance (Port et al., 2014). *ΔdacA*

and *Δpde2* show no SpeB activity, and *ΔgdpP* shows reduced SpeB activity. Each strain name is shown above the picture, and the dilution degree of cultures is indicated at the left side of the picture. (B) Comparison of SpeB activity in the culture supernatants of the mutants. The same culture supernatants for the Western blotting were used to measure relative SpeB activity using fluorescein isothiocyanate (FITC)-casein. (C) Western blot showing no detectable zymogen or processed SpeB protein produced by *ΔdacA* and *Δpde2*. Culture supernatants collected from stationary-phase cultures grown in C medium were used for Western blotting. Anti-SpeB-antibodies are polyclonal rabbit antibodies generated against purified SpeB. Secreted 40-kDa pro-SpeB (the upper band on the Western blot) becomes active 28-kDa SpeB (the lower band) through processing in the culture. Each strain name is shown above the blot, and protein marker sizes are presented on the left side. (D) Disruption of *dacA* or *pde2* results in a severe defect in *speB* transcription. The relative abundance of the *speB* transcript during stationary-phase growth in mutant strains was determined using qRT-PCR and compared to that of the wild type. Each column represents the *speB* transcript abundance in a mutant relative to the wild type. The figure shows the means and standard deviations from three independent experiments. Asterisks indicate the significance of differences (***, $P < 0.001$; ****, $P < 0.0001$) between a mutant and the wild type as calculated by one-way ANOVA followed by Dunnett's multiple-comparison test. The following strains were tested: wild type (WT), *dacA* deletion mutant (*ΔdacA*), *pde2* deletion mutant (*Δpde2*), and *gdpP* deletion mutant (*ΔgdpP*).

The *ΔdacA* and *Δpde2* Mutants Have a Transcriptional Defect in *speB* Expression

The *speB* transcript abundance in the mutants relative to that in the wild type was determined through quantitative reverse transcriptase PCR (qRT-PCR). Both *ΔdacA* and *Δpde2* expressed 10^3 to 10^4 less *speB* transcript than the wild type (Fig. 2.1D), indicating that the SpeB

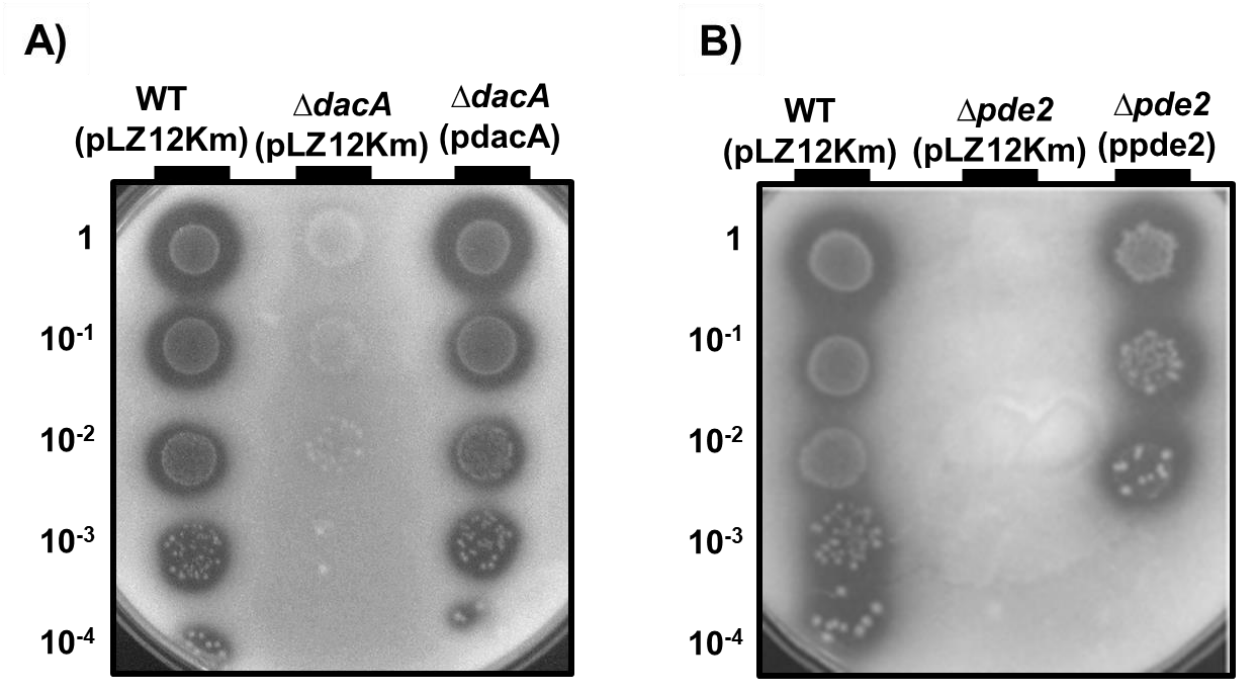
expression defect caused by *dacA* and *pde2* deletion occurs at the transcriptional level. The reduction of *speB* transcript in *Δpde2* was more extreme than that in *ΔdacA*. In contrast, the *speB* transcript level in *ΔgdpP* was equivalent to that in the wild type (Fig. 2.1D) even though *ΔgdpP* produced less SpeB in the Western blot assay (Fig. 2.1C). This indicates that the defect in SpeB production caused by the deletion of *gdpP* occurs at a posttranscriptional level, as reported previously (Cho & Kang, 2013).

Complementation of *dacA* and *pde2* Restores SpeB Activity

For a complementation assay, I introduced the *dacA* and *pde2* genes into each corresponding strain, *ΔdacA* or *Δpde2*, using a multicopy streptococcal expression plasmid, pABG5, that can express a gene under the control of the *rofA* promoter (Granok et al., 2000). When the SpeB activities of the complemented strains, *ΔdacA* (pDacA) and *Δpde2* (pPde2), were measured using protease indicator agar plates, both strains showed restored SpeB activity similar to that of the wild-type control (Fig. 2.2A and 2.2B). However, the growth of *Δpde2* was not restored in *Δpde2* (pPde2) (Fig. 2.2B). The complementation of *gdpP* also restores SpeB activity, as was shown in our previous report (Cho & Kang, 2013).

Figure 2.2.

Complementation of dacA and pde2 restores the SpeB activity.



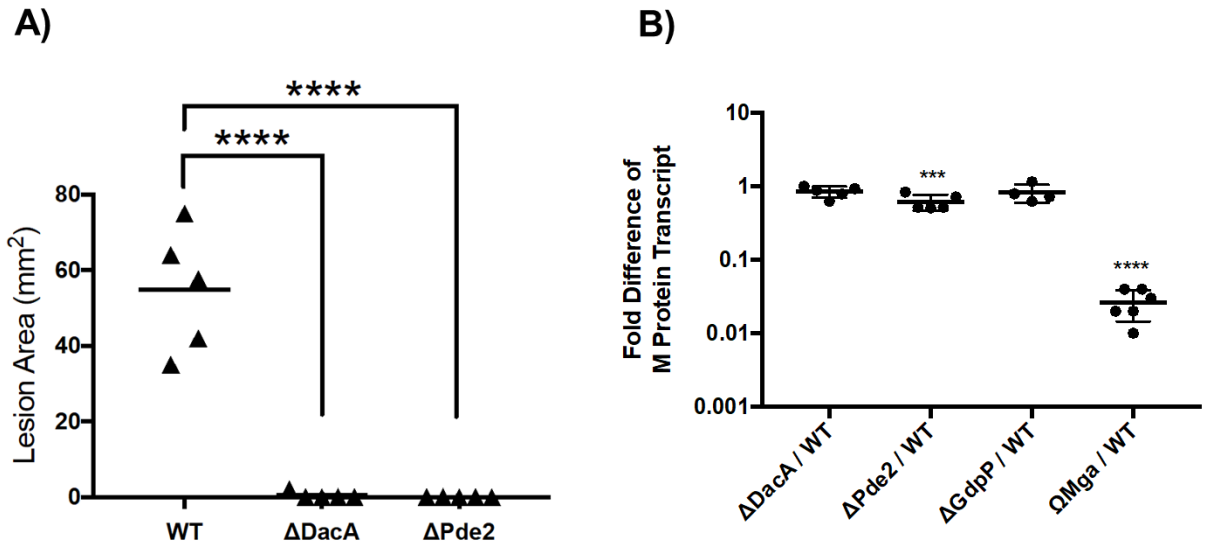
Note. The *dacA* and *pde2* genes were expressed ectopically in *ΔdacA* or *Δpde2* using the expression vector pABG5 to complement the deleted genes. The plasmid pABG5, which is a derivative of pLZ12Km, is a multicopy plasmid in *S. pyogenes*. The resulting plasmids, pDacA and pPde2, were transformed into *ΔdacA* or *Δpde2* to generate *ΔdacA* (pDacA) and *Δpde2* (pPde2), and the SpeB activities of the complemented strains were determined using protease indicator agar plates. *ΔdacA* (pDacA) showed growth and SpeB activity similar to those of the wild-type controls (A). On the other hand, *Δpde2* (pPde2) showed fully restored SpeB activity but not growth rate (B). The following strains were tested: wild-type, WT; *dacA*-complemented strain, *ΔdacA* (pDacA); and *pde2*-complemented strain, *Δpde2* (pPde2).

The Virulence of Δ*dacA* and Δ*pde2* is Highly Attenuated in a Murine Model of Soft Tissue Infection

The ability of the *ΔdacA* and *Δpde2* mutants to cause disease in soft tissue was evaluated using a murine subcutaneous infection model. The sizes of lesions caused by strains were measured three days post-infection when ulcer formation was maximal. In this infection model, *ΔdacA* and *Δpde2* did not produce any detectable lesions (Fig. 2.3A), while the wild type produced significant lesions, demonstrating that the virulence of *ΔdacA* and *Δpde2* is severely attenuated. We have previously shown that *gdpP* deletion attenuates *S. pyogenes* virulence to 50% in the same infection model (Cho & Kang, 2013). M protein, a cell surface-anchored adhesin and antiphagocytic factor that influences virulence in animal models can occasionally become lost during the process of in-frame deletion (Cho et al., 2013). When the amounts of M protein transcript in mutants were quantitated through qRT-PCR, they were comparable to that in the wild type, indicating that the M protein expression ability of the strains used in this study was not impaired (Fig. 2.3B).

Figure 2.3.

The deletion of dacA or pde2 attenuates the virulence of S. pyogenes.



Note. (A) The ability of the *ΔdacA* and *Δpde2* mutants to cause lesions in a murine subcutaneous infection model is shown. Virulence was evaluated based on of the area of the lesion produced at the time when lesion formation was maximal (3 days post-infection). The triangles represent lesion sizes in mice caused by the injection of the wild type, *ΔdacA*, or *Δpde2*. The solid bars indicate the mean values of the ulcer sizes. The asterisks above each bracket indicate the significance of differences (****, P 0.0001) between each mutant and the wild type as calculated by the Mann-Whitney U test statistic. The following strains were tested: wild type (WT), *dacA* deletion mutant (Δ*DacA*), and *pde2* deletion mutant (*Δpde2*). (B) The M protein gene (*emm*) transcript amount in mutant strains relative to that in the wild type was determined with real-time RT-PCR. As a negative control, *Ωmga*, a mutant with a *mga* gene disruption, was used. *mga* is the major positive transcriptional regulator for *emm* expression. Asterisks indicate the significance of differences (***, P < 0.001; ****, P < 0.0001) between the mutant and the wild type as calculated by one-way ANOVA followed by Dunnett's multiple-comparison test. The

following strains were tested: wild type (WT), *dacA* deletion mutant (*ΔdacA*), *pde2* deletion mutant (Δ*pde2*), *gdpP* deletion mutant (Δ*gdpP*), and *mga* disruption mutant (*Ωmga)*.

Discussion

This study demonstrates that all the enzymes identified in *S. pyogenes* involved in c-di-AMP synthesis and degradation influence SpeB production. The deletion of *dacA* and *pde2* almost completely abolished SpeB production, while *gdpP* deletion reduced it. The deletion of *gdpP* causes SpeB reduction at a posttranscriptional level, which agrees with our previous study (Cho & Kang, 2013). In contrast, the defect of SpeB production caused by *dacA* or *pde2* deletion is caused at the transcriptional level. Interestingly, although *dacA* and *pde2* are involved in opposing reactions (synthesis and degradation of c-di-AMP, respectively), their null mutants presented similar phenotypes with respect to SpeB production. This result may indicate that an optimum cellular concentration of c-di-AMP is required for proper SpeB production. Since c-di-AMP regulates the expression of *speB* at the transcriptional level, it is highly plausible that a *speB* transcriptional regulator mediates in this control.

c-di-AMP plays a role in biofilm formation in *S. pyogenes.* The *ΔdacA* mutant produced almost no biofilm, while *Δpde2* formed 1.4 times more biofilm than the wild type (Fahmi et al., 2019). It has been shown that M protein plays a crucial role in the initial attachment of *S. pyogenes* to abiotic surfaces during biofilm formation (Cho & Caparon, 2005). The wild type and the mutants tested in this study expressed almost the same amount of M protein transcript (Fig. 2.3B), indicating that c-di-AMP controls the production of a factor or factors other than M protein that influence biofilm formation in *S. pyogenes.*

The data presented here demonstrate that c-di-AMP regulates the expression of the virulence factor SpeB, indicating that c-di-AMP likely plays a crucial role in the virulence of *S.*

pyogenes (Fig. 2.1). Indeed, the virulence of *ΔdacA* and *Δpde2* was almost completely attenuated in a mouse model of subcutaneous infection (Fig. 2.3A). Since misregulation of c-di-AMP abolishes virulence, its signaling pathway may be an excellent target for developing antivirulence drugs (Maura et al., 2016). To develop antivirulence drugs, it is imperative to understand how c-di-AMP controls bacterial physiology and virulence. This study paves the way to unveil further the roles of c-di-AMP signaling networks in the pathogenesis of *S. pyogenes.*

Materials and Methods

Bacterial Strains and Media

S. pyogenes HSC5 (*emm* genotype 14) (Hanski et al., 1992; Port et al., 2013) was employed for all experiments, including strain construction. SF370 locus numbers (SPy_####) are used as references for genes in HSC5 (Ferretti et al., 2001). Molecular cloning experiments utilized *Escherichia coli* DH5 α TOP10 (Invitrogen), cultured in Luria-Bertani broth. Routine culture of *S. pyogenes* employed Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (Difco) (THY medium) with incubation at 37°C in sealed tubes without agitation. Unless otherwise indicated, C medium (J. A. Loughman & M. Caparon, 2006) was used to grow *S. pyogenes* for SpeB activity assay, Western blotting, and RNA preparation for real-time PCR. To produce solid media, Bacto agar (Difco) was added to a final concentration of 1.4% (wt/vol). Since *S. pyogenes* is an aerotolerant anaerobe that grows better under anaerobic conditions, cultures on solid media were incubated under the anaerobic condition produced by a commercial product (GasPak; catalog no. 260678 [BBL]). When appropriate, antibiotics were added to the media at the following concentrations if they are not specified: kanamycin, 50 μg/ml for *E. coli* and 500 μg/ml for *S. pyogenes*; and erythromycin, 500 μg/ml for *E. coli* and 1 μg/ml for *S. pyogenes.*

Manipulation of DNA

Plasmid DNA was isolated via standard techniques and used to transform *S. pyogenes* or *E. coli* as described previously (Caparon et al., 1991). Restriction endonucleases, ligases, and polymerases were used according to the manufacturers' recommendations. Chromosomal DNA was purified from *S.pyogenes* by using a standard kit (Wizard genomic DNA kit [Promega] or GenElute bacterial genomic DNA kit [Sigma]). When required, DNA fragments were purified using the Mini Elute gel extraction kit (Qiagen) following agarose gel electrophoresis.

Strain Construction

The construction of the mutants Δ*dacA*, Δ*pde2*, *ΔgdpP*, *Ωemm,* and *Ωmga* has been described elsewhere (Cho & Caparon, 2005; Cho & Kang, 2013; Fahmi et al., 2019). The *emm* gene encodes M protein, and *mga* encodes Mga, a positive transcriptional regulator for M protein expression. *Ωemm* and *Ωmga* have a gene disruption of *emm* or *mga*, respectively, due to a plasmid insertion into the genes through homologous recombination, so they do not express M protein.

Complementation strains were generated using the streptococcal multicopy plasmid pABG5 (Granok et al., 2000). The *dacA* or *pde2* gene was amplified for the complementation by PCR and inserted into pABG5, which can express a gene under the control of the *rofA* promoter. The insertion was performed by the fast cloning method or Gibson assembly (Gibson, 2011; Li et al., 2011). The plasmids pDacA and pPde2 were transferred into the corresponding strains to make the complemented strains Δ*dacA* (pDacA) and Δ*pde2* (pPde2).

qRT-PCR

RNA isolation from *S. pyogenes* cultures and quantitative reverse transcriptase PCR (qRT-PCR) was conducted as described elsewhere (Cho & Kang, 2013). The primers for qRT-

PCR are listed in Table 2.1. The gyrase A subunit gene (*gyrA*) was used as the internal reference gene to normalize the expression level of a specific transcript between samples (Kang et al., 2010). The reported data represent the means and standard errors from three independent assays performed on different days with a new RNA sample.

Analysis of SpeB Expression

The measurement of SpeB expression was performed using a protease indicator medium, Western blot analysis, and SpeB activity assay as described previously (Cho & Caparon, 2008; Port et al., 2014).

Table 2.1.

Primers used.

Name[a]	Sequence[b]
Mutagenic Primers[c]	
To create pDacA	
5pABG5FC 3pABG5 FC	cgaccaagagagccataaacacc cattttctctcctctcgaattcagttcc
5DacA-pABG5FC	gaattcgagaggagagaaaATGAATAATTTATCTAGTATCGATATTAAA TTTTTATTAAG
3DacA-pABG5 FC	ttatggctctcttggtcgCGTTTCATTTAGATTTCCCTCCTAGAATTTTC
To create pPde2	
5pABG5Gibson 3pABG5Gibson	taacaaagtgcaggggccca acacttagaaagccaaataagtatttgataagtgtattctcc
5Pde2-pABG5Gibson	tacttatttggctttctaagtgtAAAAAGAAAGATTAAAGCATGATAACAAC TTTTG
3Pde2-pABG5Gibson	cccctgcactttgttaCTAAATCTCTTGGCAAACAGCGATG
Analysis primers[d]	
RTspeB-F RTspeB-R	TGTCGGTAAAGTAGGCGGAC GAGCTGAAGGGTTTAGTGCG

RTemm-F	TTCAGACGCAAGCCGTAAG
RTemm-R	TCTAAATCACGGCGAAGACC
RTgyrA-F	AACAACTCAAACAGGTCGGG
RTgyrA-R	CTCCTTCACGGCTAGATTC

Note. [a]Primers are categorized as forward (F) or reverse (R) relative to the direction of a transcript. Forward primers anneal to the noncoding template strand, while reverse primers anneal to the coding strand. [b]Sequences are shown from 5' to 3'. Uppercase sequences anneal to the HSC5 chromosome, and lowercase sequences anneal to plasmid sequences. Underscores indicate junctions between contiguous DNA regions. Restriction enzyme sites are underlined. The 6 His tag sequence is in bold. [c]Mutagenesis primers were used for PCRs to amplify DNA segments used to construct plasmids for gene deletion. [d]Analysis primers were used in regular PCR to confirm (CF) gene deletion or in qRT-PCR (RT) to measure the level of gene transcription.

Murine Subcutaneous Infection

The ability of *S. pyogenes* strains to cause disease in soft tissue was evaluated using 6- to 8-week-old SKH1 hairless mice (Charles River Labs) as described previously (Cho & Kang, 2013). Each mouse was subcutaneously injected with approximately 1×10^7 CFU in a 100- µl volume into the right flank. The area of the lesion that formed was documented every 24 h by digital photography, and the lesion area was calculated from the digital record using ImageJ (NIH). Any differences in the areas of lesions between experimental groups were tested for significance by the Mann-Whitney U test. This study was carried out in accordance with the guidelines in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. This animal study was approved by the Institutional Animal Care and Use Committee (IACUC) (protocol # 993331-2) of Indiana State University (ISU). All mice were anesthetized

with isoflurane when the lesion sizes were measured and were euthanized by carbon dioxide asphyxiation at the end of the experiment.

Statistical Testing

All statistical tests were performed using GraphPad Prism (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

CHAPTER 3

c-di-AMP-Regulated K^+ Importer KtrAB Affects SpeB Expression and Pathogenesis in *S. pyogenes*

Abstract

Potassium (K^+) plays a vital role in bacterial metabolism and physiology, such as maintaining turgor pressure and activating certain cellular enzymes. From our previous study, it is evident that c-di-AMP regulates a significant virulence factor SpeB in *S. pyogenes*, but how SpeB is regulated by c-di-AMP is not clear. Here, I report a role for K^+ transport in c-di-AMP-mediated SpeB expression. The deletion of *ktrB* in the Δ*dacA* mutant restores SpeB expression. KtrB is a subunit of the K^+ transport system KtrAB that forms a putative high-affinity K^+ importer. KtrB forms a membrane K^+ channel, and KtrA acts as a cytosolic gating protein that controls the transport capacity of the system by binding ligands including c-di-AMP. SpeB induction in the Δ*dacA* mutant by K^+ specific ionophore treatment also supports the importance of cellular K^+ balance in SpeB production. However, the null pathogenicity of the Δ*dacA* mutant in a murine subcutaneous infection model is not restored by *ktrB* deletion, suggesting that c-di-AMP controls not only cellular K^+ balance but also other metabolic and/or virulence pathways. The deletion of other putative K^+ importer genes, *kup* and *kimA*, does not phenocopy the deletion of *ktrB* regarding SpeB induction in the Δ*dacA* mutant, suggesting that KtrAB is the primary K^+ importer that is responsible for controlling cellular K^+ levels under laboratory growth conditions.

Introduction

Most organisms produce cyclic nucleotide second messengers that regulate cellular activities by binding to effector molecules such as proteins or RNAs. Cyclic nucleotide-involved signaling pathways sense environmental changes, such as temperature, nutrition, pH, and other stressors, and transmit the signals to effector molecules (Hengge et al., 2016; Huynh et al., 2016; Kalia et al., 2013; Pesavento & Hengge, 2009). Many bacteria have been shown to produce several different cyclic dinucleotides, such as c-di-AMP (cyclic di-AMP), c-di-GMP (cyclic di-GMP), and cGAMP (cyclic GMP-AMP). c-di-AMP, produced by many bacteria and archaea (Devaux et al., 2018), is involved in controlling diverse cellular processes, such as fatty acid biosynthesis (Zhang et al., 2013), DNA integrity detection (Bejerano-Sagie et al., 2006; Mehne et al., 2013; Oppenheimer-Shaanan et al., 2011), and cell wall homeostasis (Banerjee et al., 2010b; Corrigan, Abbott, Burhenne, Kaever, & Gründling, 2011; Griffiths & O'Neill, 2012b; Pozzi et al., 2012), in a bacterium-specific manner. However, more than a decade of research has shown that a major role of c-di-AMP in most bacteria is in maintaining proper turgor pressure by controlling the activity of ion and/or osmolyte transporters (Commichau & Stülke, 2018; Pham et al., 2018; Zeden et al., 2018). Many important pathogens, including *Streptococcus* species (*S. pyogenes*, *S. agalactiae*, and *S. mutans*), *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Listeria monocytogenes*, appear to export c-di-AMP into the environment. When these pathogens are internalized into their host cells, the secreted c-di-AMP is detected by the host cell's STING (STimulator of INterferon Gene) (Devaux et al., 2018; Woodward et al., 2010). STING also senses 2 ´ 3 ´-cGAMP (2 ´ 3 ´cyclic GMP AMP), another cyclic dinucleotide produced by the host enzyme cGAS (cyclic GMP AMP synthase) that is activated by bacterial DNA in the host cytosol (Devaux et al., 2018; Woodward et al., 2010). When sensing these c-

dinucleotides, STING activates type I interferon production. Thus, c-di-AMP regulates not only bacterial but also host cellular processes during infection. However, the detailed role of c-di-AMP during infection is largely unknown. *S. pyogenes*, also known as group A Streptococcus (GAS), is a Gram-positive pathogen that generally causes noninvasive diseases, such as strep throat and impetigo.

However, these superficial infections sometimes develop serious diseases, such as rheumatic heart disease, streptococcal toxic shock syndrome, post streptococcal glomerulonephritis, and necrotizing fasciitis. A minimum global burden by *S. pyogenes* infection is estimated at over 18 million cases of severe diseases, resulting in over half a million annual deaths (Fahmi et al., 2019). Despite the dire consequences of this pathogen, commercial vaccines are not yet available.

My recent study has revealed that c-di-AMP regulates virulence of *S. pyogenes* (Fahmi et al., 2019). When *dacA*, the only c-di-AMP synthase gene in the *S. pyogenes* chromosome, is deleted, the Δ*dacA* mutant strain reduced SpeB expression. Here, I show that the null mutation of a high-affinity K^+ channel protein KtrB in the Δ*dacA* mutant reverts SpeB activity of Δ*dacA* mutant, suggesting that the SpeB null phenotype of Δ*dacA* results from KtrAB malfunction caused by the absence of c-di-AMP. However, the deletion of *ktrB* in the Δ*dacA* mutant was unable to restore virulence in a murine model, suggesting that c-di-AMP also plays roles in cell physiology and/or virulence other than K^+ transport in *S. pyogenes*.

Results

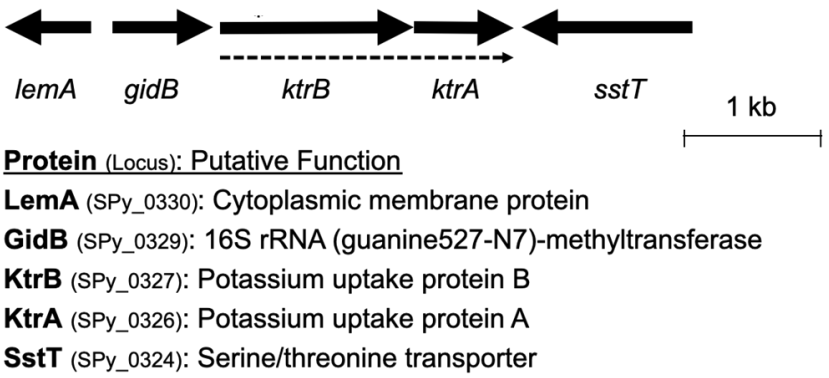
Mutation of the K^+ Importer Subunit *ktrB* Restores the SpeB Expression of Δ*dacA* Mutant

I have previously reported that the deletion of c-di-AMP synthase gene, *dacA*, abolishes the ability of *S. pyogenes* to produce the secreted cysteine protease SpeB (Fahmi et al., 2019).

Unexpectedly, however, one of the 18 Δ*dacA* strains created by the *dacA* in-frame deletion process showed SpeB+. Whole-genome sequencing of this mutant revealed a single base pair deletion in *ktrB* (Spy_0327 based on the SF370 reference; L897_01540 in HSC5). KtrB is a subunit of the KtrAB system, known to be a high-affinity K^+ importer (J. Gundlach, C. Herzberg, D. Hertel, et al., 2017; J. Gundlach, C. Herzberg, V. Kaever, et al., 2017; Rocha et al., 2019). KtrB is a membrane-integrated protein that forms a K^+ channel and interacts with the cytosolic protein KtrA (Spy_0326; L897_01535 in HSC5) that controls the transport activity of KtrB (Vieira-Pires et al., 2013). KtrA orthologs, KtrA in *S. aureus*, CabP in *S. pneumoniae*, and CapPA in *S. mutans*, are known c-di-AMP-binding proteins (Peng et al., 2016; Vieira-Pires et al., 2013). In *S. pyogenes*, *ktrA* and *ktrB* form a bicistronic operon in the order of KtrBA (Fig. 3.1).

Figure 3.1.

ktrB genomic context.

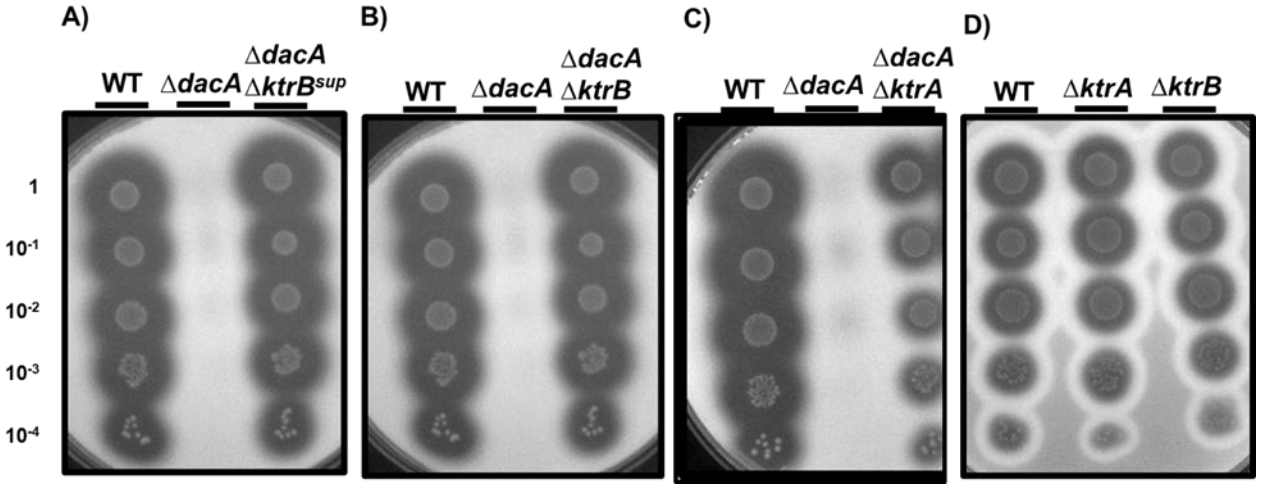


Note. Each arrow indicates an individual open reading frame and its orientation. *ktrB* is coexpressed with the downstream gene *ktrA*, which is shown with a dotted arrow under the *ktrB* and *ktrA* genes. The predicted proteins encoded by these open reading frames and their putative functions are shown below the gene organization.

To confirm that *ktrB* is responsible for the suppression of the SpeB phenotype observed in the Δ*dacA* Δ*ktrB*^{sup} strain, an in-frame deletion of *ktrB* was generated in the Δ*dacA* mutant, and the SpeB activity of the Δ*dacA* Δ*ktrB* mutant was examined. As expected, the Δ*dacA* Δ*ktrB* mutant produced SpeB, similar to the Δ*dacA* Δ*ktrB*^{sup} mutant and the wild type (Fig. 3.2B). To further confirm the role of the KtrAB system in regulating c-di-AMP-mediated SpeB regulation, the *ktrA* gene was subsequently deleted in the Δ*dacA* mutant, and the resulting Δ*dacA* Δ*ktrA* mutant also produced SpeB, although to a lesser extent than the wild type (Fig. 3.2C). SpeB activity was examined in Δ*ktrA* and Δ*ktrB* single-gene deletion mutants, and their SpeB activity was similar to that of the wild type (Fig. 3.2D). Quantitative reverse transcription-PCR (qRT-PCR) analysis measuring *speB* transcript levels in the cells demonstrated that the SpeB activity change in the Δ*dacA* Δ*ktrB* and Δ*dacA* Δ*ktrA* mutants occurs at the transcriptional level (Fig. 3.3).

Figure 3.2.

Deletion of ktrB restores the SpeB activity of Δ*dacA mutant.*

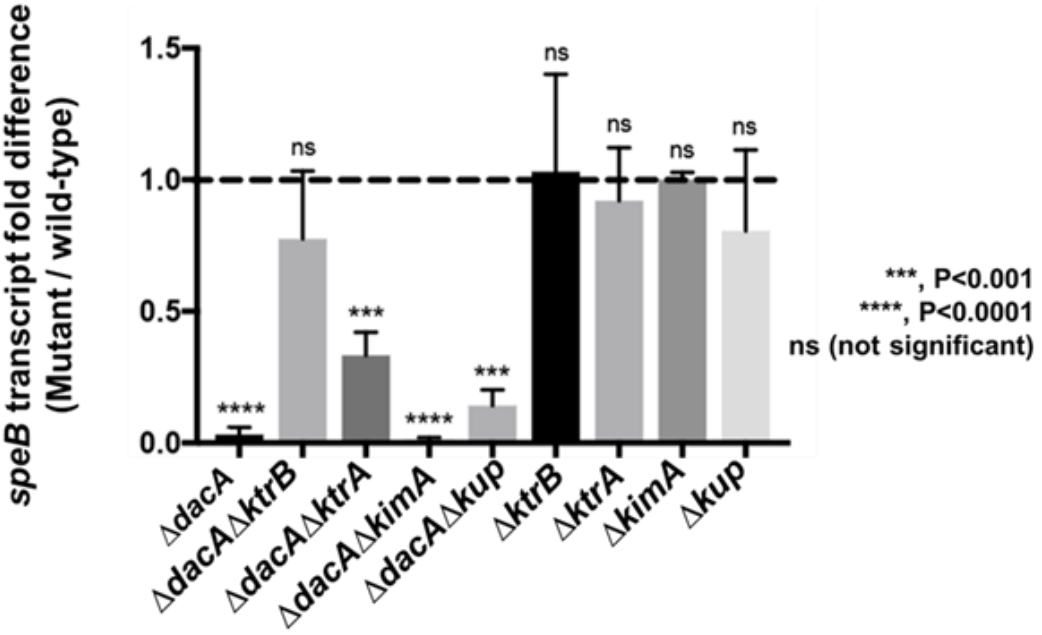


Note. The activity of the secreted protease SpeB is shown on protease indicator plates. Strains were grown overnight and spotted (2µl) onto protease indicator agar plates after serial dilution.

Protease activity displays a clear zone around the spotted cells after incubation. Strain names are shown above the images, and dilution degrees of the spotted cultures are indicated at the left side of the images. Plates were incubated for 24 h.

Figure 3.3.

SpeB activity variations of the mutants were caused at the transcriptional level.



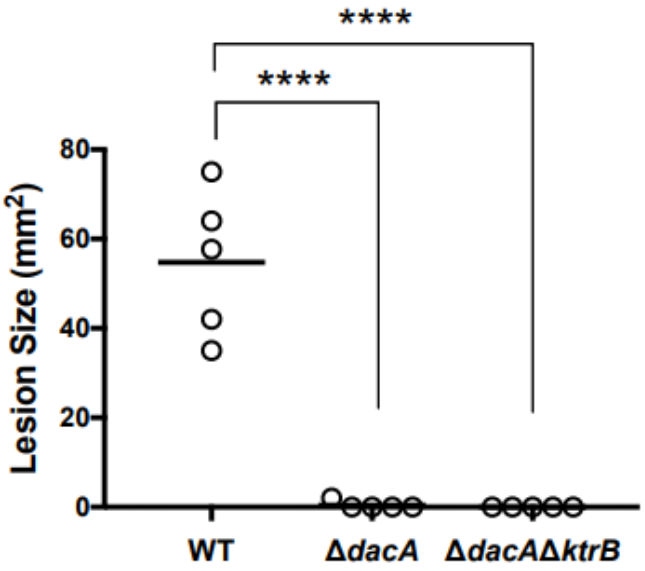
Note. The relative abundance of the *speB* transcript during the stationary-phase growth in mutants was determined using qRT-PCR and compared to that of the wild type. Each column represents the *speB* transcript abundance in a mutant relative to that in the wild type. Shown are the means and standard deviations from three independent experiments. Asterisks indicate the significance of the difference between a mutant and the wild type, as calculated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$).

Mutation of *ktrB* does not Restore the Attenuated Virulence of the Δ*dacA* Mutant

The Δ*dacA* mutant displays no virulence in a murine subcutaneous infection model, as seen by the absence of a lesion (Fahmi et al., 2019). The virulence of the Δ*dacA* Δ*ktrB* mutant was examined using the same soft-tissue infection model, and, surprisingly, none of the mice injected with the Δ*dacA* Δ*ktrB* mutant developed lesions (Fig. 3.4). Thus, even though SpeB-negative phenotype of the Δ*dacA* mutant were reverted to those of the wild type by *ktrB* mutation (Fig. 3.2B), the virulence loss of the Δ*dacA* mutant was not reverted by the same mutation.

Figure 3.4.

Deletion of ktrB does not revert the severely attenuated virulence of the ΔdacA mutant.



Note. The ability of wild-type HSC5 (A), Δ*dacA* (B), and Δ*dacA* Δ*ktrB* (C) strains to cause lesions in a murine subcutaneous infection model is shown. The virulence of each strain was evaluated by measuring the area of the lesion formed on day 3 postinfection when lesion sizes were maximal. Each circle represents the size of a lesion formed by an injection of the wild-type,

Δ*dacA*, or Δ*dacA* Δ*ktrB* strain. Each solid bar indicates the mean value of ulcer sizes. The asterisk marks above each bracket indicate the significance of difference (****, $P < 0.0001$) between each mutant and the wild type as calculated by the Mann-Whitney U test statistic.

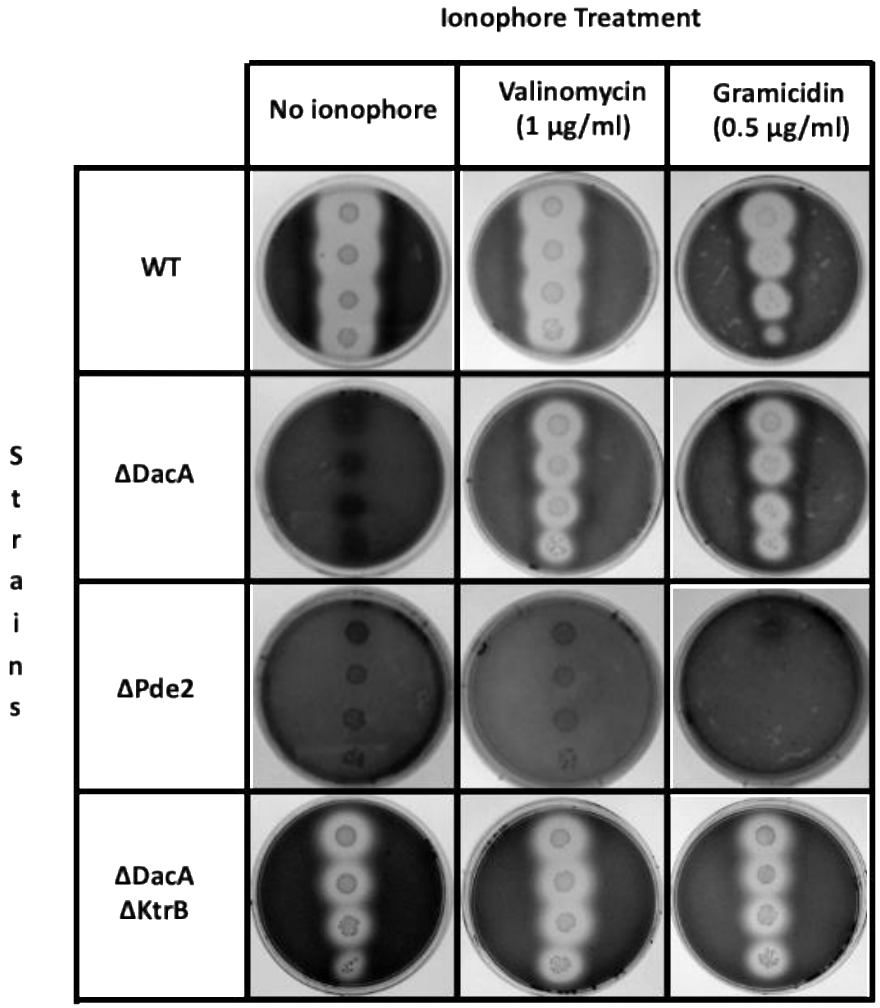
Valinomycin and Gramicidin Ionophore Treatment Mimics the Effect of *ktrB* Mutation on the Restoration of SpeB Activity of the Δ*dacA* Mutant

Ionophores are small hydrophobic lipid-soluble molecules that transport ions across cell membranes. Because ionophores are not coupled to energy sources, they only transport ions down their electrochemical gradient and are used as antibiotics due to their ability to collapse ion gradients across cellular membranes. Since the deletion of *ktrB* restores SpeB production in the Δ*dacA* mutant, I examined the effect of the ionophores that disturb cellular K^+ balance, such as valinomycin and gramicidin, on SpeB production (David & Rajasekaran, 2015). Valinomycin is a carrier ionophore that transports K^+ across the membrane. Gramicidin is a channel-forming ionophore that transports small inorganic monovalent cations, such as K^+ and Na^+, across the membrane (David & Rajasekaran, 2015). Since both of these ionophores have growth inhibition effects, sub-growth-inhibitory concentrations of these ionophores were determined by monitoring *S. pyogenes* growth (OD_{600}) at different concentrations. A concentration of 1µg/ml valinomycin or 0.5µg/ml gramicidin has little to no effect on the growth of wild-type HSC5 in liquid culture. When tested on solid media, these conditions also minimally affected the growth and SpeB activity of the wild type (Fig 3.5). When the Δ*dacA* mutant was treated with the ionophores, it regained the ability to produce SpeB (Fig. 3.5), mimicking the effect of *ktrB* mutation on SpeB activity of the Δ*dacA* mutant (Fig. 3.2). When the Δ*dacA* Δ*ktrB* mutant was treated with ionophores, it did not significantly change SpeB activity (Fig. 3.5). GdpP and Pde2 are two c-di-AMP phosphodiesterases identified in *S. pyogenes* (Fahmi et al., 2019). Similar to

the Δ*dacA* mutant, the Δ*pde2* mutant does not produce SpeB (Fahmi et al., 2019). However, unlike the Δ*dacA* mutant, the Δ*pde2* mutant did not show SpeB activity when treated with the ionophores (Fig. 3.5). This result indicates that the underlying mechanism causing the SpeB null phenotype of the Δ*pde2* mutant is different from that of the Δ*dacA* mutant.

Figure 3.5.

Ionophores specific to K^+ (valinomycin) and monovalent cations (gramicidin) restore SpeB activity of the ΔdacA mutant to the wild-type level.



Note. The effect of ionophores on SpeB activity of *S. pyogenes* strains was observed. Overnight cultures of *S. pyogenes* strains were serially diluted with THY medium and spotted onto protease

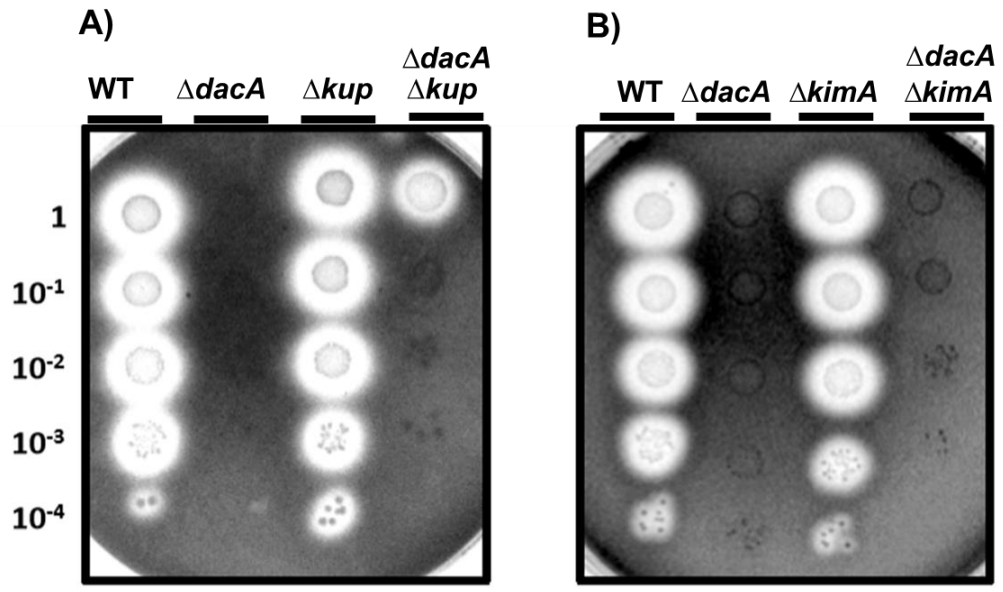
indicator plates containing no ionophore, valinomycin (1µg/ ml), or gramicidin (0.5µg/ml). Culture dilution degrees were 10^1, 10^2, 10^3, and 10^4 from the top.

Deletion of Putative K^+ Importer Gene *kup* or *kimA* does not Phenocopy *ktrB* Deletion

The *S. pyogenes* genome encodes homologs of at least two additional K^+ importers other than KtrAB, which include a Kup homolog (SPy_1414 in SF370, L897_05730 in HSC5) and a KimA homolog (SPy_2088 in SF370, L897_08830 in HSC5). In-frame deletion mutants of each of these K^+ transporter homologs were generated in the Δ*dacA* mutant, and SpeB activity of the resulting Δ*dacA* Δ*kup* and Δ*dacA* Δ*kimA* mutants was examined. The Δ*dacA* Δ*kup* mutant showed very low SpeB activity on a protease agar plate following extended incubation (Fig. 3.6).

Figure 3.6.

Deletion of a putative K^+ importer gene, kup or kimA, does not phenocopy ktrB deletion in the ΔdacA mutant.



Note. Unlike the deletion of *ktrB*, the deletion of another K^+ importer gene, *kup* or *kimA*, did not restore the SpeB activity of the Δ*dacA* mutant to the wild-type level. Strain names are shown

above the pictures, and the dilution degrees of the cultures are indicated at the left side of the images. The protease indicator plates were incubated for 48 h in this experiment (most strains developed zones of clearance in 24 h, but the Δ*dacA* Δ*kup* strain took 48 h to become visible).

Similarly, the Δ*dacA* Δ*kimA* mutant did not exhibit SpeB activity. These results indicate that the role of KimA and Kup in the Δ*dacA* mutant under the in vitro growth conditions was not as crucial as that of KtrAB. SpeB activity of Δ*kimA* and Δ*kup* single-gene deletion mutants was comparable to that of the wild type (Fig. 3.6). qRT-PCR analysis measuring the *speB* transcript levels in the cells demonstrate that the SpeB activity change in these mutants occurred at the transcriptional level (Fig. 3.3).

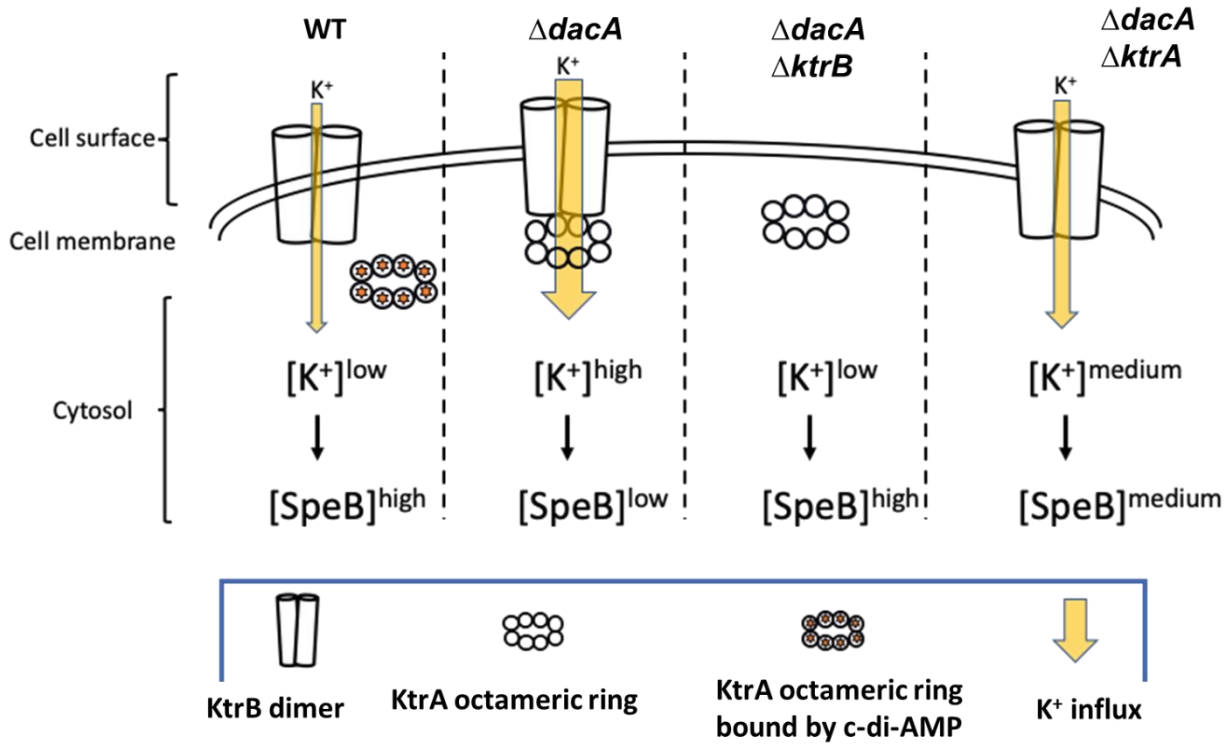
Discussion

The potassium ion (K^+) is the most abundant intracellular cation and has a primary role in turgor compensation and osmotic adjustment (Bossemeyer et al., 1989; Epstein, 2003). Thus, controlling cellular K^+ levels through K^+ transporters is crucial for the survival of cells in diverse osmotic environments. It has been shown in Gram-positive bacteria that c-di-AMP inhibits transporters that maintain turgor pressure and osmotic adjustment, such as potassium transporters and/or glycine-betaine transporters (Commichau & Stülke, 2018; Gundlach et al., 2019; Pham et al., 2018; Quintana et al., 2019; Zeden et al., 2018). For example, c-di-AMP inhibits the activity of the multiple potassium transporters, the KtrAB system, the KtrCD system, and KimA in *B. subtilis* and the KupA and KupB potassium transporters in *Lactococcus lactis* (Gundlach et al., 2019; Quintana et al., 2019). In this study, KtrB, the channel component of the K^+ importer KrtAB system, was identified as a suppressor of the SpeB null phenotype of the Δ*dacA* mutant, suggesting that the control of cellular K^+ concentration connects c-di-AMP signal transduction and SpeB production. KtrAB is a member of the Trk/Ktr/HKT K^+ transporter superfamily that

consists of uniporters and symporters (K^+/Na^+ or K^+/H^+) (Szollosi et al., 2016). KtrAB appears to be a high-affinity K^+ importer, as the Km value of the KtrAB system in *B. subtilis* for K^+ is in the micromolar range (J. Gundlach, C. Herzberg, D. Hertel, et al., 2017; J. Gundlach, C. Herzberg, V. Kaever, et al., 2017; Rocha et al., 2019). The KtrAB system is composed of a homodimeric KtrB membrane protein complex and a cytosolic KtrA octameric ring (Vieira-Pires et al., 2013). Each KtrB subunit forms a potassium channel, so deletion of *ktrB* abolishes the K^+ transport ability of the KtrAB system. KtrA is a gating component regulating KtrB activity (Vieira-Pires et al., 2013). KtrA contains regulator of conductance of potassium (RCK) domains at the amino terminus (RCK_N) and carboxy terminus (RCK_C). ATP and ADP bind to the RCK_N domain. When ATP binds to the domain, the K^+ transport ability of KtrAB increases, but when ADP binds, the ability decreases. It has been shown that KtrA orthologs in *S. aureus* (KtrA), *S. pneumoniae* (CabP), and *S. mutans* (CapPA) are c-di-AMP-binding proteins (Bai et al., 2014; Corrigan et al., 2013; Peng et al., 2016). c-di-AMP binds to the RCK_C domain of KtrA and inhibits the K^+ transport ability of the KtrAB system (Corrigan et al., 2013). KtrA bound by c-di-AMP does not interact with KtrB in *S. pneumoniae* (Bai et al., 2014). KtrB without KtrA has almost half of its full K^+ transport activity in *B. subtilis* (Szollosi et al., 2016). A model for the interaction of c-di-AMP and K^+ and SpeB expression is shown in Fig. 3.7.

Figure 3.7.

Model explaining the relationship between the activity of the KtrAB system and SpeB production in S. pyogenes.



Note. Interactions between c-di-AMP and the KtrAB system and their effect on SpeB expression in the wild type (WT), Δ*dacA*, Δ*dacA* Δ*ktrB*, and Δ*dacA* Δ*ktrA* strains are shown.

As the deletion of *dacA* abolishes the ability of *S. pyogenes* to synthesize c-di-AMP (Fahmi et al., 2019), the KtrA octameric ring in the absence of c-di-AMP likely interacts with the KrtB dimer, thereby promoting K^+ import (Fig. 3.7, Δ*dacA* mutant) (Bai et al., 2014). Alternative K^+ transporters and glycine-betaine transporters may import additional K^+ and glycine-betaine in the absence of c-di-AMP (Commichau & Stülke, 2018; Pham et al., 2018; Zeden et al., 2018). This increased cellular K^+ concentration may trigger repression of SpeB expression via a decrease in *speB* transcription through a currently undefined mechanism. Removal of the K^+

channel KtrB in a c-di-AMP-devoid strain likely leads to a decrease in intracellular K^+ and subsequent restoration of SpeB expression via an increase in *speB* transcription (Fig. 3.7, Δ*dacA* Δ*ktrB* mutant). This model is further supported through exposure to ionophores, as a sublethal treatment of the Δ*dacA* mutant with valinomycin or gramicidin presumably leads to an efflux of K^+ from the cells and restoration of SpeB production in the Δ*dacA* mutant (Fig. 6). However, ionophore treatment of the Δ*dacA* Δ*ktrB* mutant has no effect on SpeB production, because the ΔdacA Δ*ktrB* mutant presumably already has low cellular K^+ (Fig. 6). The Δ*dacA* Δ*ktrA* mutant appears to import more K^+ than the wild type, since SpeB activity of the strain is less than that of the wild type (Fig. 3.7, Δ*dacA* Δ*ktrA* mutant). Lower K^+ import than that of the wild type does not appear to influence SpeB expression, because all of the single K^+ transporter gene deletion mutants, Δ*ktrA*, Δ*ktrB*, Δ*kup*, and Δ*kimA*, showed the same SpeB activity as the wild type (Fig. 3.2, 3.3, and 3.7). Three putative K^+ importers were identified in the *S. pyogenes* genome that could be involved in maintaining intracellular K^+ concentration. These importers are the KtrAB, Kup, and KimA systems. *S. pyogenes* Kup is an *Escherichia coli* Kup ortholog with 31.7% amino acid identity. *E. coli* Kup is a low-affinity K^+ importer and is believed to be the major K^+ importer system under acidic conditions (Bossemeyer et al., 1989; Epstein, 2003; Trchounian & Kobayashi, 2000). In *L. lactis*, the Kup transporters are c-di-AMP binding proteins, and their potassium transport activities are inhibited upon binding to c-di-AMP (Quintana et al., 2019). In 2017, Gundlach et al. revealed that membrane protein YdaO in *B. subtilis* is a high-affinity K^+ importer, so they renamed the importer KimA (K^+ importer A) (J. Gundlach, C. Herzberg, V. Kaever, et al., 2017). KimA in *B. subtilis* is inhibited by c-di-AMP at both the transcriptional and protein activity levels (Gundlach et al., 2019). A recent structural study revealed that *B. subtilis* KimA is a K^+/ H^+ symporter and also a member of the Kup family (Tascón et al., 2020). *S.*

pyogenes encodes a homolog of KimA, Spy_2088, with 20% amino acid identity. In-frame deletion of *ktrA*, *ktrB*, *kimA*, or *kup* in the Δ*dacA* mutant revealed that only the deletion of *ktrB* restored SpeB production equal to the wild-type level (Fig. 3.2). This suggests that the KtrAB system is the main K^+ importer maintaining cellular K^+ concentration under the in vitro growth conditions. Kup and KimA may have functions under different growth conditions, such as lower or higher osmotic or pH conditions than those tested in this study. Even though *ktrB* deletion in the Δ*dacA* mutant reverted many defective phenotypes of the Δ*dacA* mutant, the *ktrB* deletion did not revert the loss of virulence of the Δ*dacA* mutant, indicating that other virulence traits regulated by c-di-AMP beyond SpeB production and resistance to stressors cannot be recovered by *ktrB* deletion alone. Thus, c-di-AMP in *S. pyogenes* appears to regulate multiple cellular pathways in addition to K^+ transport.

Materials and Methods

Bacterial Strains and Media

S. pyogenes HSC5 (emm genotype 14) (Hanski et al., 1992; Port et al., 2013) was employed for all experiments, including strain construction. SF370 locus numbers (SPy_####) are used as references for genes in HSC5 (Ferretti et al., 2001). Molecular cloning experiments utilized *Escherichia coli* DH5α or TOP10 (Invitrogen), which was cultured in Luria-Bertani broth. The routine culture of *S. pyogenes* employed Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (Difco) (THY medium), and cells were grown at 37°C in sealed tubes without agitation. Unless otherwise indicated, C medium (Lyon et al., 1998) was used to grow *S. pyogenes* for SpeB activity assay and RNA preparation for real-time qRT-PCR. Bacto agar (1.4%, wt/vol; Difco) was added to make solid media. Cultures on solid media were incubated under the anaerobic condition created by a commercial product (GasPak; catalog no. 260678;

BBL). When appropriate, antibiotics were added to the media at the following concentrations if they are not specified: kanamycin, 50µg/ml for *E. coli* and 500µg/ml for *S. pyogenes*; erythromycin, 500µg/ml for *E. coli* and 1µg/ml for *S. pyogenes*.

Manipulation of DNA

Plasmid DNA was isolated via a commercial kit (Gene Elute plasmid miniprep kit; Sigma) and used to transform *S. pyogenes* or *E. coli* as described previously (Caparon et al., 1991). Enzymes for DNA cloning and PCR were used according to the recommendations of the manufacturers. Chromosomal DNA was purified from *S. pyogenes* by using a standard kit (Wizard genomic DNA purification kit [Promega] or GenElute bacterial genomic DNA kit [Sigma]).

Strain Construction

In-frame deletion mutations on chromosomal loci were generated by employing the shuttle vector with a temperature-sensitive replication origin, pJRS233 (Cho & Kang, 2013; King et al., 2000). Briefly, PCR products immediately upstream and downstream of the deletion target gene were generated. The primers used to create the PCR products are listed in Table 2. These two PCR products were inserted into pJRS233 using the Gibson assembly (New England BioLabs) to create a deletion allele. A plasmid with a deletion allele, *p*Δ*krtB*, *p*Δ*ktrA*, *p*Δ*kimA*, or *p*Δ*kup*, was used to replace each target gene by the gene deletion method that employs the temperature-sensitive replication origin, as described previously (Cho & Kang, 2013; King et al., 2000). The fidelity of all molecular constructs and gene deletions was confirmed by PCR and/or DNA sequencing (www.Psomagen.com).

SpeB Activity Measurement using Protease Indicator Plates

Strains were grown overnight in THY medium. The overnight cultures were serially diluted with fresh THY medium, and the diluted cells (2µl) were spotted onto protease indicator agar plates (C medium agar plates containing 2% skim milk). The protease indicator plates then were incubated anaerobically at 37°C for 24 h (or 48 h for extended incubation), and SpeB activity, which displays a clear zone around the spotted cells, was observed.

Table 3.1.

Primers used.

Name[a]	Sequence[b]	Remarks
Mutagenic Primers[c]		
To create pΔKtrB		
5KtrBifVector 3KtrBifVector	CATCCTTG-GACGTTGTAAAACGACGGCCAG GTGATTTGAAG-GCACATCCCCCTTTCGCC	For vector amplification (6000 bps)
5KtrBifF1 3KtrBifF1	GGGATGTGC-CTTCAAATCACTAGTGGAATGGAACAC CATGTTTTCTCCAATATCTCCTAC-TATAATAACAAAAATAAGTTAAAAAAGCTAAG	For upstream fragment amplification (721 bps)
5KtrBifF2 3KtrBifF2	GTAGGAGATATTGGAGAAAACATG-TTAAAACGTAAAACTGTCGG GTTTTACAACGTC-CAAGGATGTTTTTTTATTATTTAAGATAGCCAAGATAATC	For downstream fragment amplification (725 bps)
To create pΔKtrA		
5KtrAifVector 3KtrAifVector	CTACTAAAAC-CCCAGTCACGACGTTGTAAAACGACG GAAAAGTTGCC-CGCCTTGCAGCACATCC	For vector amplification (6020 bps)
5KtrAifF1 3KtrAifF1	GCAAGGCG-GGCAACTTTTCGTAAATTATCCAATCAGTCTC CAATGATAGTATTCGGTTTTCTCCTTAACCCACTAGAATATCAGTAG	For upstream fragment amplification (720 bps)

5KtrAifF2 3KtrAifF2	GTTAAGGAGAAAACCGAATACTATCATTGTGGCCATCGC CGTGACTGGG-GTTTTAGTAGGAACAATGCTTTTTGTCGC	For downstream fragment amplification (722 bps)
To create pΔKimA		
5p7INT- FC2 3p7INT- FC2	CCTGTGTGAAATTGTTATCCGCTC GTCGTGACTGGGAAAACCCTGG	For vector amplification (5799 bps)
5KimAup 3KimAup	GGGTTTTCCCAGTCACGAC- AACTGACTTTACAGTGACTATTAGCAACCT ATAGTTTTTTCTCAATAATGCTCTC- CTTTTTGTTTGCAAATTGC	For upstream fragment amplification (1032 bps)
5KimAdown 3KimAdown	GAGAGCATTATTGAGAAAAAACTAT- GACAAGAGTGATTAATTTAGATGGC GCGGATAACAATTTCACACAGG- CCGTGGAAGTTACCTCCTGAAATAAC	For downstream fragment amplification (1034 bps)
To create pΔKup		
5p7INT- FC2 3p7INT- FC2	CCTGTGTGAAATTGTTATCCGCTC GTCGTGACTGGGAAAACCCTGG	For vector amplification (5799 bps)
5Kupup-2 3Kupup-2	GGGTTTTCCCAGTCACGAC- CTACCTCTCAATGATTGAGAACTTAACGAAAAAAC CTCAATGATTTTTCTTATACTCCTC- CTAATTTTTTAAAAATTATAACAAAATAACTG	For upstream fragment amplification (734 bps)
5Kupdown-2 3Kupdown-2	GAGGAGTATAAGAAAAATCATTGAG- ATGATAAGTCTCAATGATTTTTCTTTTC GCGGATAACAATTTCACACAGG- TCCCCCTTACTTTAAAAGTCACGAAAGTTCTAAG	For downstream fragment amplification (732 bps)

Note. [a]Primers are categorized as 5′ or 3′ based on the direction of a transcript. Primers whose names start with 5′ (forward primers) anneal to the non-coding template strand, while those with 3′ (reverse primers) anneal to the coding strand.

[b]Sequence is shown 5' to 3'. Hyphens (-) indicate junctions between contiguous DNA regions. [c]Mutagenesis primers were used for PCR reactions to amplify DNA segments used to construct plasmids for gene deletion.

Murine Subcutaneous Infection

The ability of *S. pyogenes* strains to cause disease in soft tissues was evaluated using 6-8 week old SKH1 hairless mice (Charles River Labs) as described previously (Cho & Kang, 2013). Briefly, each mouse was subcutaneously injected with approximately 1 x 10^7 CFU of *S. pyogenes* in a 100 µl volume into the right flank. The area of the lesion that formed was measured every 24 hrs by digital photography. Any differences in the areas of lesions between experimental groups were tested for significance by the Mann–Whitney U-test. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. This animal study was approved by the Institutional Animal Care and Use Committee (IACUC) (protocol # 993331-2) of Indiana State University (ISU).

The Effect of Ionophores on SpeB Expression

S. pyogenes strains were grown overnight in THY media. The cultures were 10-fold serially diluted with THY media and 2µl of each dilution was spotted on the protease indicator plates containing 1 µg/ml valinomycin (Sigma, V0627) or 0.5 µg/ml gramicidin (sigma, G5002) (Stock solution: 10mg/ml valinomycin in DMSO and 10mg/ml gramicidin in ethanol). The plates were incubated at 37˚C anaerobically for one or two days, and the clear zones by SpeB activity around the spotted cultures were observed.

qRT-PCR

Real time qRT-PCR was conducted as described elsewhere (Cho & Kang, 2013). The primers for qRT-PCR are listed in Table 3.1. The gyrase A subunit gene (*gyrA*) was used as the internal reference gene to normalize the expression level of a specific transcript between samples

(Kang et al., 2010). The reported data represent the means and standard errors from three independent assays, each performed on a different day with a new RNA sample.

Statistical Testing

All statistical tests were performed using GraphPad Prism. Each statistical test applied to the experiments was described in the figure legends.

CHAPTER 4

The Mutation of the Dlt System Conferring D-Alanylation to Teichoic Acids Suppresses the SpeB Null Phenotype of the Δ*pde2* Mutant of *Streptococcus pyogenes*

Abstract

The newly discovered second messenger molecule, c-di-AMP, plays a critical role in pathogenesis and virulence in *S. pyogenes*. Previously, I reported that deleting the c-di-AMP phosphodiesterase gene, *pde2*, in *S. pyogenes* severely suppresses SpeB transcription. To gain insight into the mechanism of SpeB regulation in the Δ*pde2* mutant, we performed transposon mutagenesis in the Δ*pde2* mutant. I identified one of the genes from the *dlt* operon, *dlt*X, as a suppressor of the SpeB null phenotype of the Δ*pde2* mutant. The *dlt* operon consists of four to five genes *dlt(X)ABCD* in most Gram-positive bacteria and primarily incorporates D-alanine into lipoteichoic acid. The in-frame deletion of *dltX* or insertional inactivation of *dltA* in the Δ*pde2* mutant restored SpeB expression. These mutations did not affect the growth in lab media but showed increased negative cell surface charge and enhanced sensitivity to polymyxin B, as previously reported. As Dlt mutation changes cell surface charge and possibly causes cell envelope stress, I deleted the gene of the response regulator *liaR* in LiaFSR that senses and responds to cell envelope stress. The Δ*pde2* Δ*liaR* mutant also produced SpeB but less than that

of the Δ*pde2* Δ*dltX* mutant. Gene expression study using qRT-PCR showed that the cell wall stressor vancomycin did not significantly change the expression of a LiaFSR-regulated gene, *spxA2* in the Δ*pde2,* or Δ*pde2* Δ*dltX* mutant compared to that of the wild type or the Δ*pde2* mutant, respectively. SpxA2 might compete with the *speB* transcriptional activator RopB, but overexpression of *ropB* restored almost no SpeB in the Δ*pde2* mutant. My results suggest that the Dlt system and LiaFSR influence SpeB expression in the Δ*pde2* mutant through two separate regulatory pathways; further investigation is required to understand how Pde2 and D-alanylation of teichoic acid are linked to SpeB expression in *S. pyogenes*.

Introduction

Bacteria and archaea utilize second messenger cyclic nucleotides to sense and respond to changes in their environment, including pH, temperature, nutrients, and other stressors, to adapt to new environments (Hengge et al., 2016; Huynh et al., 2016; Kalia et al., 2013; Pesavento & Hengge, 2009). The cyclic nucleotides act as signaling molecules that relay the signals by interacting with their target proteins or riboswitches in response to external or internal stimuli (Commichau et al., 2019). Typically, specific mono- or dinucleotides are used as a second messenger molecule, for example, cyclic adenosine phosphate (cAMP), guanosine tetraphosphate or pentaphosphate ((p)ppGpp), cyclic di-guanosine monophosphate (c-di-GMP), cyclic guanosine monophosphate-adenosine monophosphate (cGAMP), and cyclic di-adenosine monophosphate (c-di-AMP) (Corrigan & Gründling, 2013).

c-di-AMP is a recently discovered second messenger molecule. It is only produced by prokaryotes. It is primarily found in most Gram-positive bacteria, including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Streptococcus spp.*, and many Gram-negative bacteria, including *Chlamydia trachomatis* and

Borrelia burgdorferi (Andrade et al., 2016; Barker et al., 2013; Corrigan, Abbott, Burhenne, Kaever, & Gründling, 2011; Gándara & Alonso, 2015; Kamegaya et al., 2011; Pesavento & Hengge, 2009; Witte et al., 2008; Woodward et al., 2010). c-di-AMP involves in osmoregulation, DNA repair mechanisms, maintenance of cell wall homeostasis, fatty acid synthesis, virulence regulation, biofilm formation, and/or type I interferon response (Corrigan & Gründling, 2013; Faozia et al., 2021; Woodward et al., 2010). While a wide range of crucial cellular processes or virulence is regulated by c-di-AMP, the detailed mechanism of controlling these functions is still not well understood. The model organism used in this study, *S. pyogenes* or Group A Streptococcus (GAS), is an obligate human pathogen that causes diverse diseases from mild superficial infections to severe invasive toxigenic or post-streptococcal autoimmune sequelae (Walker et al., 2014).

GAS is still a major public health concern in developed and developing countries. Around 700 million people worldwide suffer from pharyngitis, which can lead to severe diseases. Lack of inadequate treatment or repeated GAS infections can develop non-suppurative autoimmune sequelae, acute rheumatic fever (ARF), which can further damage the heart and cause rheumatic heart diseases (RHD) (Carapetis et al., 2005). The mortality rate of RHD is significantly high; approximately 319,400 deaths occurred in 2015 globally (Watkins et al., 2017). *S. pyogenes* expresses an array of cell wall-associated and secreted virulence factors essential to cause various GAS diseases. Thus, understanding GAS virulence regulation mechanisms is crucial to combat GAS infections (Cunningham, 2000; Olsen et al., 2009). Though GAS diseases have been known for centuries, an effective vaccine is still unavailable. In *S. pyogenes*, c-di-AMP regulates cellular activities and virulence factors in which the mechanisms are mostly unknown. Previously, I reported that misregulation of c-di-AMP

homeostasis by the deletion of c-di-AMP synthase gene *dacA* or degradation encoding gene *pde2* abolish the transcription of a significant virulence factor, SpeB (Fahmi et al., 2019; Faozia et al., 2021). SepB null phenotype of the Δ*dacA* mutant is mediated via the potassium transporter KtrAB. However, the mechanism of how the SpeB null phenotype of the Δ*pde2* mutant is caused is not known (Faozia et al., 2021). Using transposon mutagenesis, I identified *dltX*, the first gene in the *dlt* operon, as a suppressor of the SpeB null phenotype of the Δ*pde2* mutant. The primary function of the *dlt* operon is to incorporate D-alanine ester into teichoic acids resulting in an increased positive surface charge and resistance against the host's cationic antimicrobial peptides (CAMPs) (Andreas Peschel et al., 1999). Multiple studies showed that the inactivation of the *dlt* operon becomes more sensitive to CAMPs (R. Kamar et al., 2017; Andreas Peschel et al., 1999). Since the *dlt* operon modulates the cell surface charge, the mutation in the *dlt* operon might alter the cell envelope stress. Gram-positive bacteria encode a highly conserved cell envelope stress response regulator LiaFSR. The three-component system LiaFSR senses and responds to cell envelope stress induced by CAMPs (Lin et al., 2020).

In this study, I investigated how the Dlt and LiaFSR systems are involved in SpeB regulation in the Δ*pde2* mutant. My data reveal that the Dlt and LiaFSR systems regulate SpeB in the Δ*pde2* mutant through two separate regulatory mechanisms.

Results

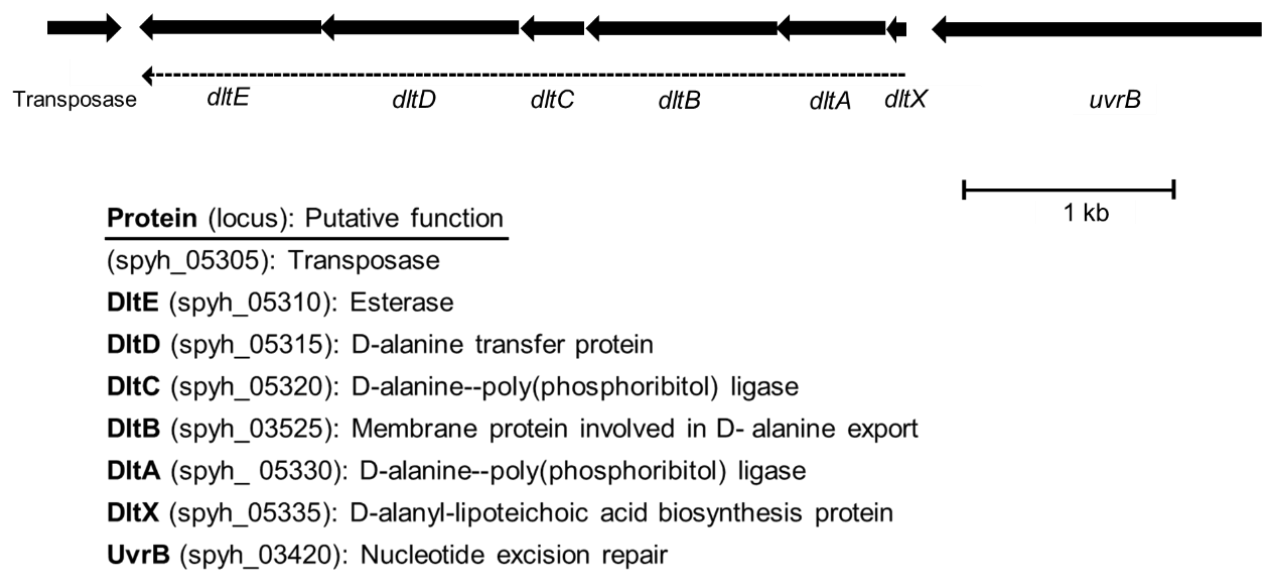
The Dlt System Influences SpeB Expression in the Δ*pde2* Mutant

My previous studies showed that the deletion of a c-di-AMP phosphodiesterase gene *pde2* abolishes SpeB expression in *S. pyogenes* (Fahmi et al., 2019). We, therefore, performed transposon mutagenesis in the Δ*pde2* mutant background to discover potential genes involved in the regulation of SpeB in the Δ*pde2* mutant. I identified the *dltX* gene as a suppressor of the

SpeB null phenotype of the Δ*pde2* mutant. The insertion of the transposon in the *dltX* gene or its promoter region restored SpeB in the Δ*pde2* background, and the SpeB activity level was a little bit less than that of the wild type. The *dlt* operon is highly conserved in gram-positive bacteria, encoding several gene products that incorporate D-alanine ester into teichoic acid (Neuhaus & Baddiley, 2003; Andreas Peschel et al., 1999). In *S. pyogenes*, the *dlt* operon is comprised of six genes, *dltXABCDE,* in which the first gene, *dltX*, encodes a membrane-associated small protein consisting of 47 amino acids (Fig. 4.1). To confirm that *dltX* is responsible for suppressing the SpeB phenotype observed in the Δ*pde2* Δ*dltX* ^{sup} strain, I constructed an in-frame deletion mutant Δ*pde2* Δ*dltX* and examined its protease activity. As expected, the double mutant Δ*pde2* Δ*dltX* restored SpeB, equivalent to that of the suppressor mutant Δ*pde2* Δ*dltX* ^{sup} (Fig. 4.2A, 4.2B). I also checked the *dltA* transcript level in the in-frame deletion mutant Δ*pde2,* Δ*dltX*, and Δ*pde2* Δ*dltX* compared to the wild-type. The deletion of the *dltX* gene in the wild-type or Δ*pde2* background did not significantly change the *dltA* transcript relative to the wild-type (Fig 4.3). To investigate the role of other genes in the *dlt* operon, I disrupted *dlt*A in the Δ*pde2* background. The resulting Δ*pde2* Ω*dlt*A mutant (which has a polar effect on downstream *dlt* genes) also expressed the same level of SpeB activity as the Δ*pde2* Δ*dltX* mutant (Fig. 4.2B), suggesting that the Dlt system controls SpeB activity in the Δ*pde2* mutant. Δ*dltX* and Δ*dlt*A mutants were tested for SpeB activity and showed SpeB activity similar to that of the wild type (Fig. 4.2C).

Figure 4.1.

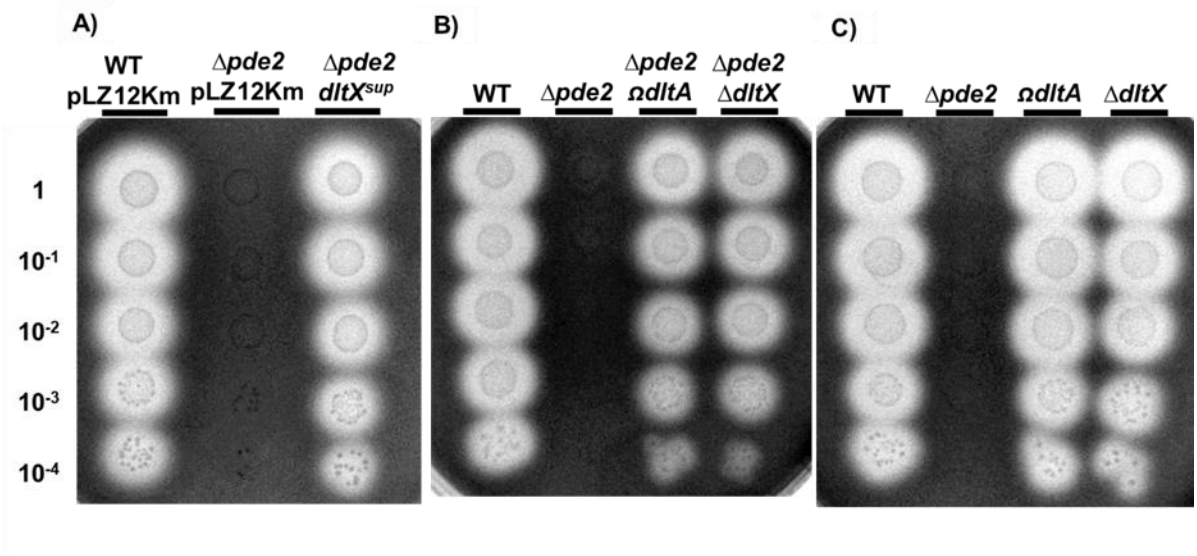
Genetic organization of dlt operon in S. pyogenes.



Note. dlt operon consists of *dltX, dltA, dltB, dltC, dltD,* and *dltE*. Each arrow indicates an individual open reading frame and its orientation. *dltX* is co-expressed with the downstream genes in the *dlt* operon, which is shown with a dotted arrow under the *dlt* genes. The predicted proteins encoded by these open reading frames and their putative functions are shown below the gene organization.

Figure 4.2.

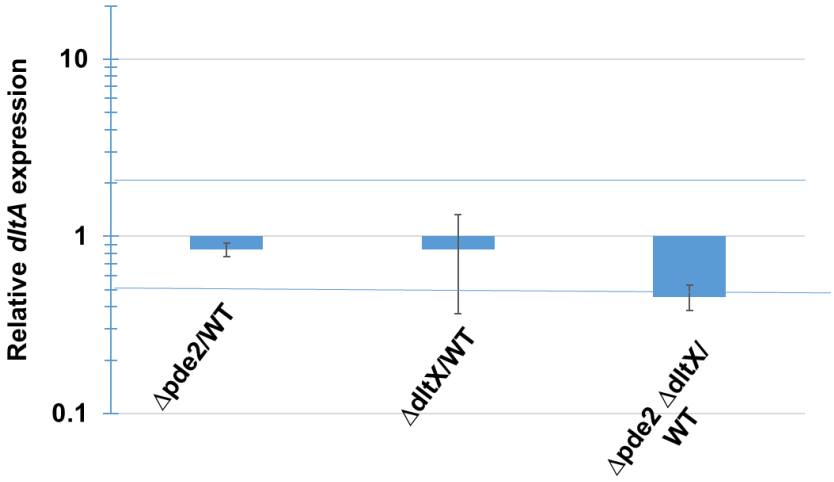
Inactivation of the dltX or dltA gene restores the SpeB activity of the Δpde2 mutant.



Note. The activity of the secreted protease SpeB is shown on protease indicator plates. Strains were grown overnight and spotted (2µl) onto protease indicator agar plates after serial dilution. Protease activity displays a clear zone around the spotted cells after incubation. Strain names are shown above the images, and dilution degrees of the spotted cultures are indicated on the left side of the images. Plates were incubated at 37˚C for 24 h. The pLZ12Km plasmid was used as a control for kanamycin addition to the plates. pLZ12Km has the same kanamycin resistance gene the transposon used for the suppressor screening.

Figure 4.3.

Transcription of dltA did not change in the Δpde2 and ΔdltX mutants compared to the wild type.



Note. The relative abundance of the *dltA* transcript during mid-exponential-phase growth was determined using qRT-PCR. The *dltA* transcript was measured in the Δ*pde2*, Δ*dltX*, and Δ*pde2* Δ*dltX* mutants compared to the wild type. The figure shows the means and standard deviations. Data are derived from three independent cultures assayed in duplicate. The following strains were tested: wild type (HSC5), *pde2* deletion mutant (*Δpde2*), *dltX* deletion mutant (*ΔdltX*), and *dltX* deletion mutant in the *Δpde2* background (*Δpde2 ΔdltX*).

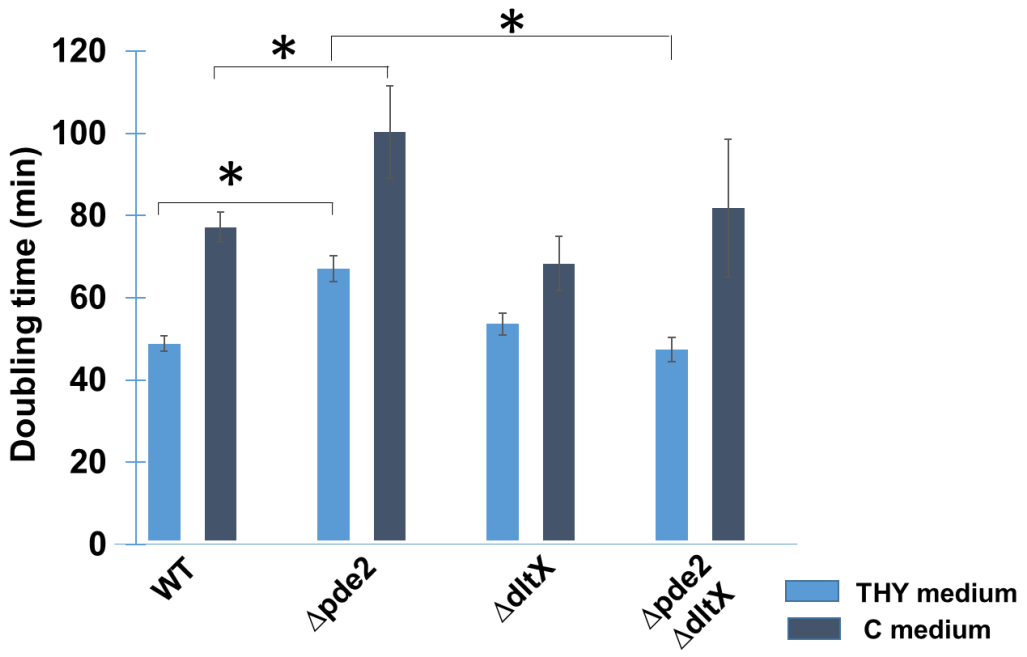
Loss of the *dltX* Gene does not Affect in Vitro Growth of *S. pyogenes*

I measured the generation time of the Δ*dltX* mutants in Todd-Hewitt medium with 0.2% yeast extract (THY medium) (protein and carbohydrate-rich) and C medium (protein-rich, carbohydrate-poor) (Fahmi et al., 2019). The Δ*dltX* mutant showed a growth pattern similar to the wild type. As previously reported, the Δ*pde2* mutant displayed a growth defect in both media, and its generation time increased to ~ 1.5 times those of the wild type (Fahmi et al., 2019). However, the doubling time of the double mutant Δ*pde2* Δ*dltX* was restored to those of the wild-

type levels (Fig. 4.4). This result suggests the deletion of the *dltX* gene in the Δ*pde2* background contributes to the recovery of the growth defect of the Δ*pde2* mutant.

Figure 4.4.

The ΔdltX mutant shows a growth pattern similar to the wild type, and the deletion of dltX in the Δpde2 background recovers the growth defect of the Δpde2 mutant.



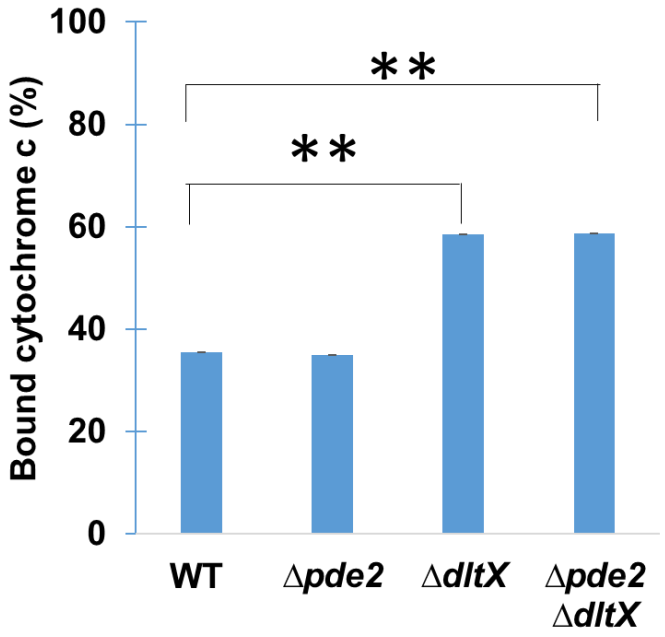
Note. The doubling times of the mutants, *Δpde2, ΔdltX*, and *Δpde2 ΔdltX*, grown in THY or C medium were measured. The doubling times of *ΔdltX* mutant were not significantly different from those of the wild type in THY medium tested. In contrast, Δ*pde2* showed a longer doubling time than that of the wild type, as reported previously. The data are the means and standard errors of the means derived from two independent experiments. The following strains were tested: wild type (WT), *pde2* deletion mutant (*Δpde2*), *dltX* deletion mutant (Δ*dltX*), and *pde2 dltX* double deletion mutant (*Δpde2 ΔdltX*). (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; two-tailed t-test).

Deletion of the *dltX* Gene Increases the Negative Surface Charge of *S. pyogenes*

It was previously reported that defects in the D-alanylation of lipoteichoic acids (LTA) significantly alter the surface charge of GAS (Kristian et al., 2005). To determine the role of DltX in the D-alanylation of LTAs, wild type, Δ*pde2*, Δ*dltX*, and Δ*pde2* Δ*dltX* mutants were incubated with the cationic protein cytochrome c. Mutants lacking the *dltX* gene, Δ*dltX and* Δ*pde2* Δ*dltX* bound more cytochrome c than the wild-type strain (Fig. 4.5), demonstrating an increase in net negative surface charge in the Δ*dltX* mutants. However, the Δ*pde2* mutant bound almost the same amount of cytochrome c compared to the wild type (Fig. 4.5).

Figure 4.5.

ΔdltX and Δpde2 ΔdltX show increased negative surface charge.



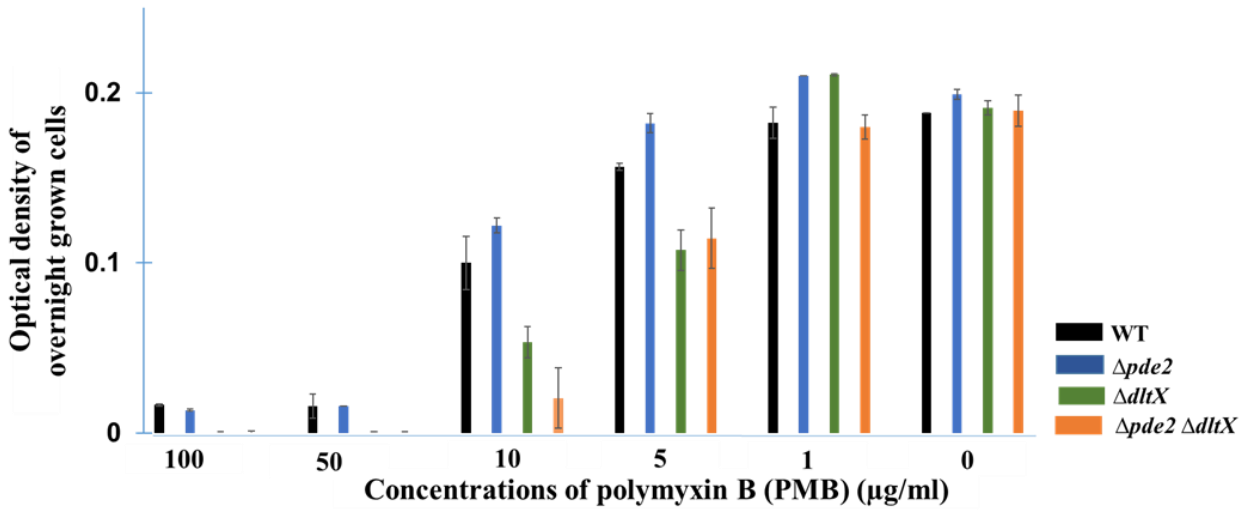
Note. Cells were treated with the cationic protein cytochrome c as described in materials and methods. The data are the means and standard errors of the means derived from three independent experiments. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; two-tailed t-test).

The *dltX* Gene is Essential for Resistance to Cationic Antimicrobial Peptides Polymyxin B

Since D-alanylation by the *dlt* operon lowers the attraction of cationic antimicrobial peptides (CAMPs) by reducing negative surface charge (Kristian et al., 2005; A. Peschel et al., 1999), I tested if the *dltX* gene is essential for resistance to CAMPs. The inhibitory concentration of polymyxin B (PMB) was measured for the wild-type, *Δpde2,* and Δ*dltX* mutants. The strains were grown in THY medium overnight in the presence of various concentrations of PMB (Fig 4.6). The Δ*pde2* mutant showed similar sensitivity to the wild-type strain, but the Δ*dltX* mutants were more susceptible to PMB. The Δ*dltX* and Δ*pde2* Δ*dltX* mutants showed almost no growth in both media with 10 µg/ml of PMB (Fig 4.6), suggesting that *dltX* has a direct role in developing resistance against PMB in *S. pyogenes*.

Figure 4.6.

ΔdltX and Δpde2 ΔdltX show increased sensitivity to polymyxin B.



Note. Cells were grown in THY medium in the presence of different concentrations of PMB (100, 50, 10, 5, and 1 µg/ml). The inhibitory concentration of PMB for *ΔdltX* and *Δpde2 ΔdltX* were significantly reduced compared to the wild type or the Δ*pde2* mutant. The data are the

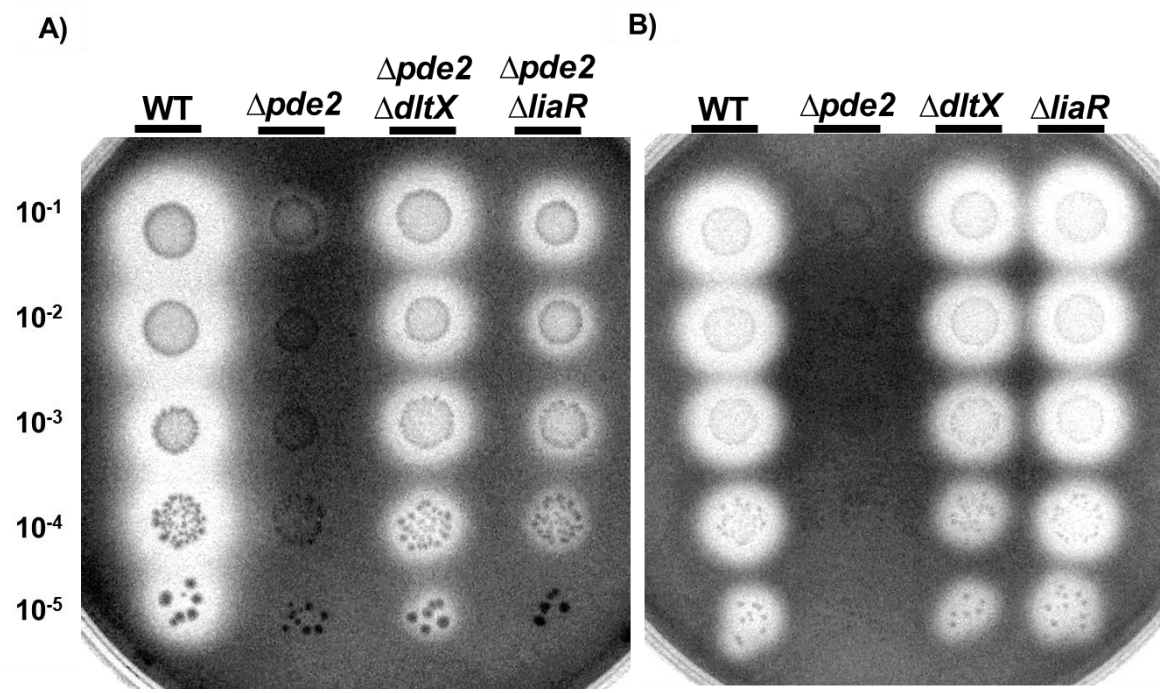
means and standard errors of the means derived from three independent experiments. The following strains were tested: wild type (WT), *pde2* deletion mutant (*Δpde2*), *dltX* deletion mutant (Δ*dltX*), and *pde2 dltX* double deletion mutant (*Δpde2 ΔdltX*).

Three-Component System LiaFSR Influences SpeB at Least Partially in the Δ*pde2* Mutant

LiaFSR gene regulatory system is well-known in Gram-positive bacteria, including *S. pyogenes* (Lin et al., 2020). It is a three-component system composed of LiaS (sensor kinase), LiaF (a membrane-bound repressor protein), and LiaR (a response regulator) (Lin et al., 2020). The critical function of LiaFSR is to sense and respond to the cell envelope stress induced by cationic antimicrobial peptides (Arias et al., 2011; Lin et al., 2020). In *S. pyogenes*, LiaFSR regulates SpeB expression as elevated *speB* transcript was observed in a *liaR*-deficient strain (Sanson et al., 2021). I postulated that Dlt mutation changes cell surface charge and possibly alters cell envelope stress. Hence, I utilized the cell envelope stress regulator LiaFSR system and examined its role in SpeB regulation in the Δ*pde2* mutant. I deleted the response regulator *liaR* in the Δ*pde2* background and measured SpeB activity. The double mutant Δ*pde2* Δ*liaR* restored SpeB expression but less than the wild type or Δ*pde2* Δ*dltX* mutant (Fig. 4.7A), suggesting that LiaFSR influences SpeB expression at least partially in the Δ*pde2* mutant. The single mutant Δ*liaR* showed similar SpeB activity compared to the wild type or Δ*dltX* mutant (Fig. 4.7B).

Figure. 4.7.

The deletion of liaR- partially restored the SpeB activity of the Δpde2 mutant.



Note. The clear zones on the protease indicator plates display the activity of the SpeB protease secreted by *S. pyogenes* strains. The deletion of *liaR* in the Δ*pde2* strain restored SpeB but less than that of Δ*pde2* Δ*dltX*. Strain names are shown above the pictures, and the dilution degrees of the overnight cultures are indicated on the left side of the pictures. The wild type (WT), *pde2* deletion mutant *(*Δ*pde2), dltX* deletion mutant (Δ*dltX*), *liaR* deletion mutant *(*Δ*liaR), pde2* and *dltX* double deletion mutant *(*Δ*pde2* Δ*dltX),* and *pde2* and *liaR* double deletion mutant *(*Δ*pde2* Δ*liaR)* were used for this study.

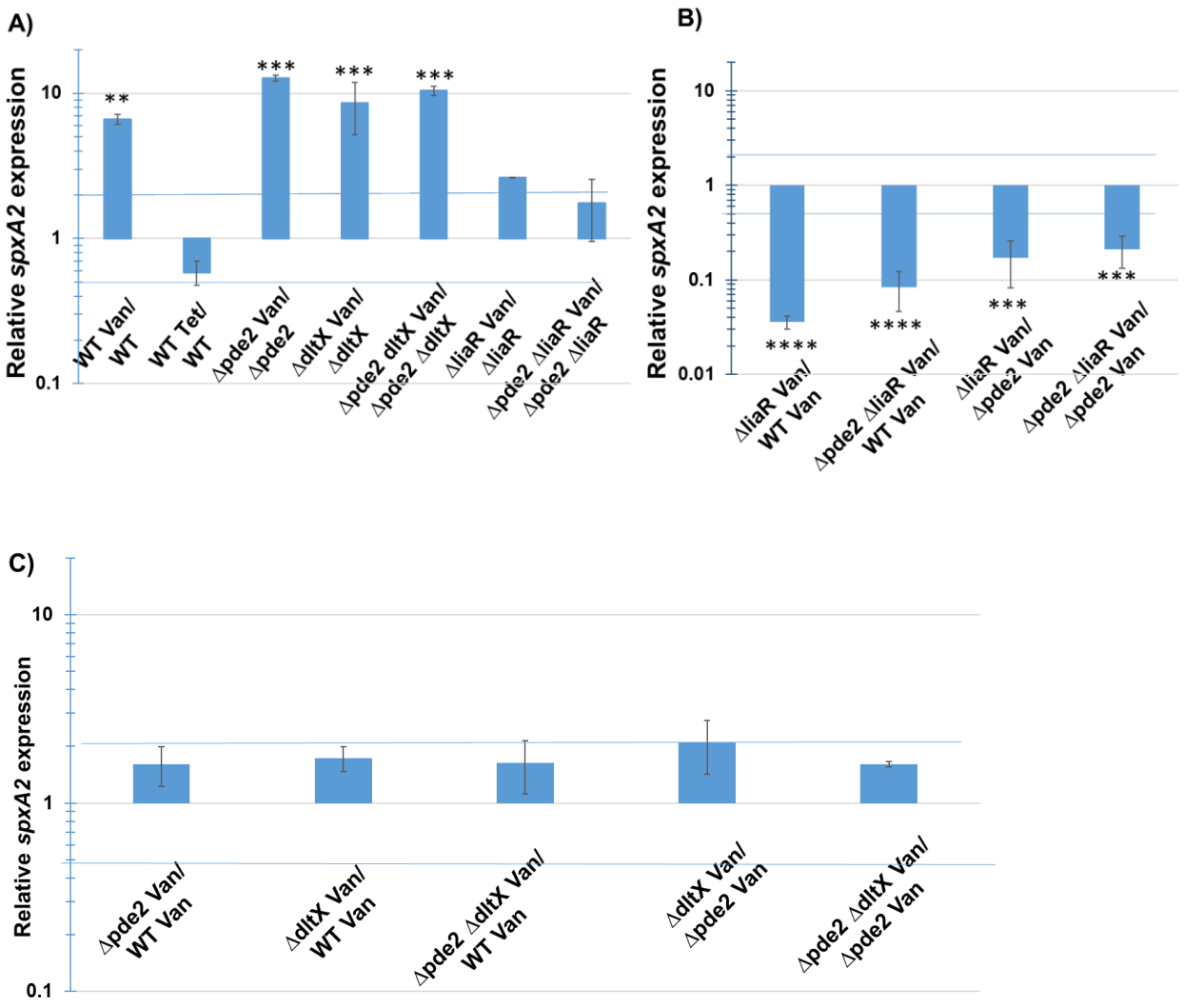
LiaFSR-Regulated Gene *spxA2* does not Influence the SpeB Activity in the Δ*pde2,* Δ*dltX,* or Δ*pde2* Δ*dltX* Mutant at the Transcriptional Level

The RNA polymerase binding protein SpxA is a transcriptional regulator commonly found in firmicutes (Lin et al., 2020; Port et al., 2017). *S. pyogenes* encodes two highly similar

SpxA paralogs, SpxA1 and SpxA2 (Port et al., 2017). SpxA2 has been observed to respond to cell envelope stresses, and SpxA1 is associated with oxidative stress regulation (Jonathon L Baker et al., 2020; Port et al., 2017). Multiple studies showed that *spxA2* transcription highly depends on the cell envelope stress regulator LiaFSR (Sanson et al., 2021; Suntharalingam et al., 2009). It is likely that *spxA2* is stimulated by LiaFSR and responds to membrane stressors in gram-positive bacteria, including *S. pyogenes* (J. L. Baker et al., 2020; Sanson et al., 2021). In addition, *spxA2* acts as an antiactivator that negatively regulates SpeB in *S. pyogenes* (Port et al., 2017). I also showed that SpeB is influenced by LiaFSR partially in the Δ*pde2* mutant (Fig 4.7A). Moreover, the Δ*dltX* mutant or the double mutant Δ*pde2* Δ*dltX* showed an increased negative surface charge compared to the wild type or the Δ*pde2* mutant (Fig 4.5). The defects in D-alanylation could modulate the cell envelope stress by changing the surface charge. I tested if SpeB restoration by Dlt mutation in the Δ*pde2* background is influenced by LiaFSR-regulated gene *spxA2.* I measured *spxA2* transcript level in the presence of cell wall stressor vancomycin by qRT- PCR. Vancomycin-treated cells dramatically enhanced the transcript level of *spxA2* in the wild-type, Δ*pde2*, and Δ*dltX* mutants compared to the untreated cells. However, vancomycin-treated Δ*liaR* mutants did not lead to increased *spxA2* transcript levels, as previously reported (Fig 4.8A) (J. L. Baker et al., 2020; Lin et al., 2020). As expected, the protein synthesis inhibitor tetracycline, used as a negative control, showed no change in the *spxA2* transcript. Moreover, the Δ*liaR* and the Δ*pde2* Δ*liaR* mutant displayed a reduced level of *spxA2* transcript than the wild-type or the Δ*pde2* mutant (Fig 4.8B). However, no significant change of *spxA2* was observed in the Δ*pde2*, Δ*dltX*, or Δ*pde2* Δ*dltX* mutant compared to the wild type or the Δ*pde2* mutant (Fig 4.8C).

Figure 4.8.

The deletion of dltX, pde2, or both does not change the expression of spxA2.



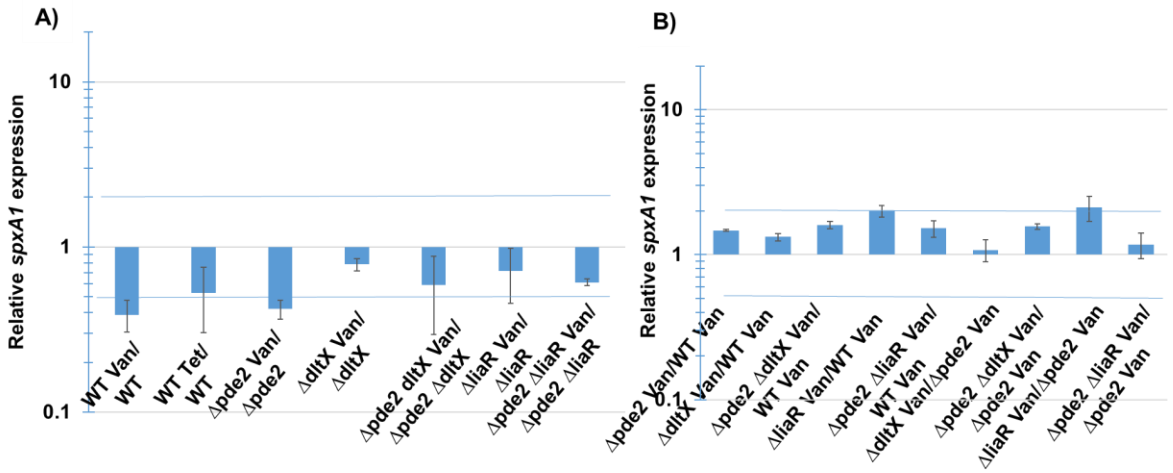
Note. The relative abundance of the *spxA2* transcript during mid-exponential-phase growth was determined using qRT-PCR. The effect of cell wall stressor vancomycin on the activation of the LiaFSR system was evaluated by measuring the transcription level of *spxA2*. (A) The transcript level of *spxA2* was quantitated in *S. pyogenes* strains treated with vancomycin and compared to the untreated cells. The *spxA2* transcript was measured in the Δ*liaR* mutants (B) and Δ*pde2* and Δ*dltX* mutants (C) and compared to the wild type or the Δ*pde2* mutant in the presence of

vancomycin. The figure shows the means and standard deviations. Data are derived from three independent cultures assayed in duplicate. The following strains were tested: wild type (HSC5), *pde2* deletion mutant (*Δpde2*), *dltX* deletion mutant (*ΔdltX*), *dltX* deletion mutant in the *Δpde2 background* (*Δpde2 ΔdltX*) and *liaR* deletion mutant (*ΔliaR*) and *liaR* deletion mutant in the *Δpde2* background (*Δpde2 ΔliaR*). Abbreviation: Van, Vancomycin; Tet, Tetracycline. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; two-tailed t-test).

I also measured the relative *spxA1* transcript level in the *Δpde2, ΔdltX,* and *ΔliaR* mutants in the presence of vancomycin. Unlike *spxA2*, transcription of *spxA1* did not increase in the presence of cell envelope stressor vancomycin (Fig 4.9 A). Moreover, *spxA1* transcript did not change in Δ*pde2*, Δ*dltX*, or Δ*liaR* mutants compared to the wild type or Δ*pde2* mutant (Fig 4.9 B). My data suggest that transcription of *spxA1* is not involved in cell envelope stress response regulated by LiaFSR. Though *spxA2* transcription is highly dependent on LiaFSR, the SpeB transcription of Δ*pde2* or Δ*dltX* mutant was not influenced by *spxA1* or *spxA2*.

Figure 4.9.

Transcription of spxA1 did not change in the Δpde2, ΔdltX, or ΔliaR mutants compared to the wild type or the Δpde2 mutant with vancomycin.



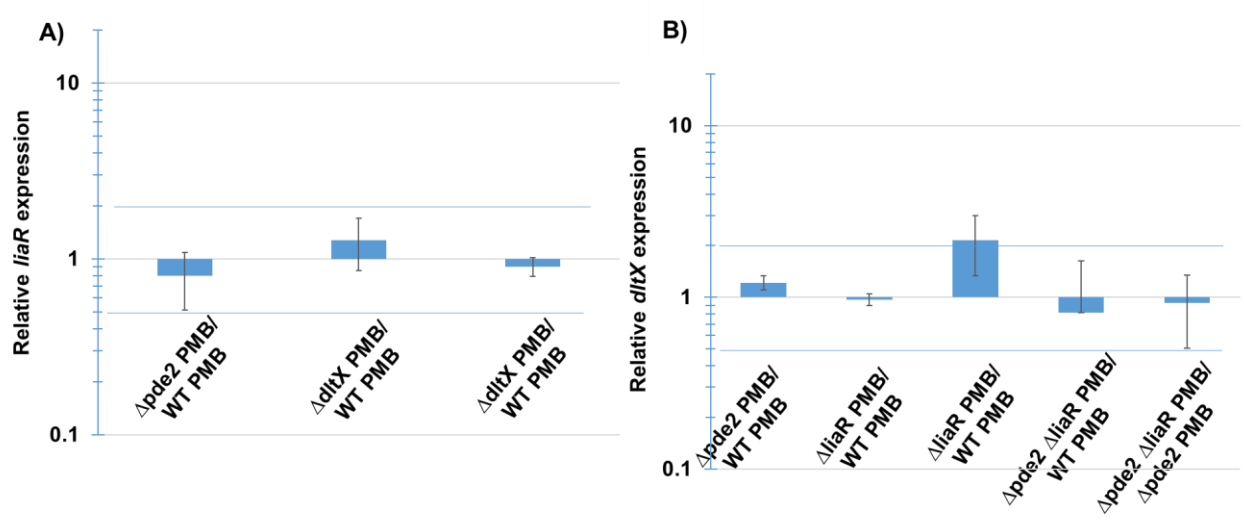
Note. The relative abundance of the *spxA1* transcript during mid-exponential-phase growth was determined using qRT-PCR. (A) The transcript level of *spxA1* was quantitated in *S. pyogenes* strains treated with vancomycin and compared to the untreated cells. (B) The *spxA1* transcript was measured in the Δ*pde2*, Δ*dltX*, and Δ*liaR* mutants compared to the wild type or the Δ*pde2* mutant in the presence of vancomycin. The figure shows the means and standard deviations. Data are derived from three independent cultures assayed in duplicate. The following strains were tested: wild type (HSC5), *pde2* deletion mutant (*Δpde2*), *dltX* deletion mutant (*ΔdltX*), *dltX* deletion mutant in the *Δpde2* background (*Δpde2 ΔdltX*) and *liaR* deletion mutant (*ΔliaR*) and *liaR* deletion mutant in the *Δpde2* background (*Δpde2 ΔliaR*). Abbreviation: Van, Vancomycin; Tet, Tetracycline.

The Dlt System and LiaFSR are not linked in the SpeB Regulation in the Δ*pde2* Mutant at the Transcriptional Level

The Dlt system and LiaFSR regulate SpeB in the Δ*pde2* mutant (Fig 4.2B, 4.7A). Therefore, I quantified the relative transcript level of *dltX* and *liaR* in the presence of cell envelope stressor polymyxin B to investigate if there is any link between the Dlt system and LiaFSR. My qRT PCR data showed that the transcript level of *liaR* did not change in the Δ*pde2* or Δ*dltX* mutant relative to the wild type or Δ*pde2* mutant, respectively (Fig 4.10A). Moreover, no significant change of the *dltX* transcript was observed in the Δ*pde2* or Δ*liaR* mutant compared to the wild type or Δ*pde2* mutant either (Fig 4.10B). My results suggested that the Dlt system and LiaFSR influence SpeB in the Δ*pde2* mutant through two separate regulatory pathways.

Figure 4.10.

Dlt and LiaFSR system influence SpeB in the Δpde2 mutant through two separate regulatory pathways.



Note. The relative abundance of the *liaR* and *dltX* transcripts during mid-exponential-phase growth was determined by qRT-PCR. (A) The transcript level of *liaR* was quantitated in *S. pyogenes* strains treated with polymyxin B and compared to the wild type (HSC5) or the Δ*pde2* mutant. (B) The *dltX* transcript was measured in the Δ*pde2* and Δ*liaR* mutants and compared to the wild type or the Δ*pde2* mutant in the presence of polymyxin B. The figure shows the means and standard deviations. Data are derived from two independent cultures assayed in duplicate. The following strains were tested: wild type (HSC5), *pde2* deletion mutant (*Δpde2*), *dltX* deletion mutant (*ΔdltX*), *liaR* deletion mutant (*ΔliaR*) and *liaR* deletion mutant in the *Δpde2* background (*Δpde2 ΔliaR*). Abbreviation: PMB, polymyxin B.

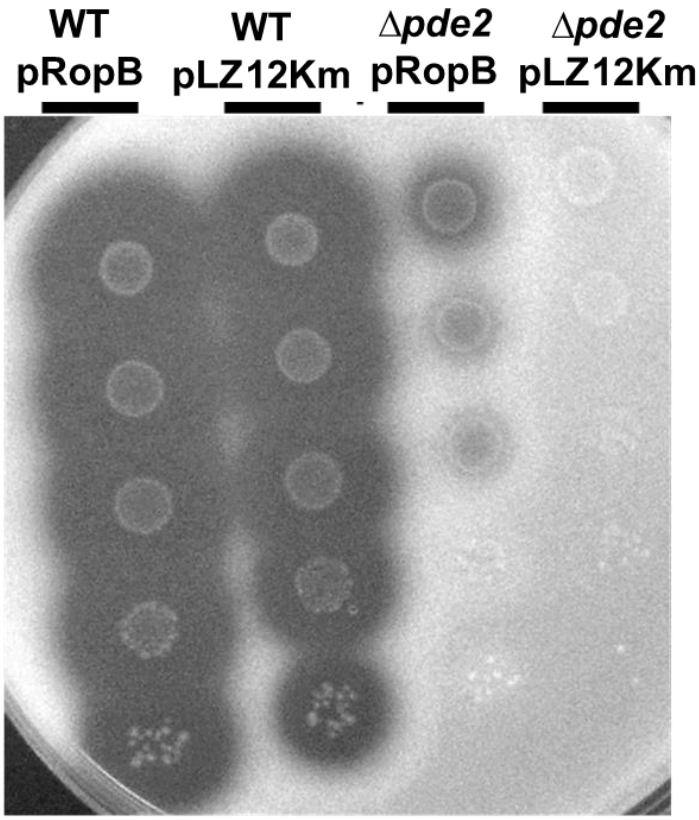
Overexpression of *ropB* does not Influence *speB* Expression in the Δ*pde2* Mutant

RopB is an Rgg-like transcriptional activator that initiates *speB* transcription by binding to its promoter region (Anbalagan et al., 2011; Lyon et al., 1998). A previous report proposed

that the RNA polymerase binding protein SpxA2 competes with RopB to recruit RNA polymerase to the *speB* promoter and inhibits the initiation of *speB* transcription (Port et al., 2017). I speculated that the overexpression of *ropB* in *S. pyogenes* suppresses *spxA2* and increases SpeB activity. The *ropB* gene was overexpressed from an ectopic promoter on a multicopy plasmid pABG5 in the wild type and the Δ*pde2* mutant. As expected, the strain WT (pRopB) expressed more SpeB than the strain with the empty vector (Fig 4.11). However, the overexpressed *ropB* showed little SpeB in the Δ*pde2* background, so the Δ*pde2* (pRopB) strain could not restore SpeB like the wild-type (Fig. 4.11). These results indicate that neither RopB nor SpxA2 are the main factors providing the SpeB phenotype of the Δ*pde2* mutant.

Figure 4.11.

The Δpde2 strain with overexpressed ropB, Δpde2 (pRopB), restored little SpeB activity.



Note. The *ropB* gene was inserted into the plasmid pABG5, a derivative of a multicopy plasmid pLZ12Km. The resulting plasmid, pRopB, was transformed into the wild type and the Δ*pde2* mutant to generate WT (pRopB) and Δ*pde2* (pRopB), respectively. The SpeB activities of those strains were determined using protease indicator agar plates. The following strains were tested: wild-type WT; the WT strain with overexpressed *ropB*, WT (pRopB); and the Δ*pde2* strain with overexpressed *ropB*, Δ*pde2* (pRopB).

Discussion

Previously, I reported that the significant virulence factor SpeB is regulated by a c-di-AMP phosphodiesterase *pde2* at the transcriptional level in *S. pyogenes* (Fahmi et al., 2019). In the present study, I investigated the underlying SpeB regulation mechanism in the Δ*pde2* mutant. I identified two novel regulators, the Dlt system and LiaFSR control SpeB expression of the Δ*pde2* mutant.

Nearly all gram-positive bacteria encode the conserved *dlt* operon, consisting of four to five genes *dlt(X)ABCD*, primarily involved in the incorporation of D-alanine ester into teichoic acids (TAs) (Eric Abachin et al., 2002; Boyd et al., 2000; Kristian et al., 2005; Andreas Peschel et al., 1999). Multiple studies reported that *dltA, dltB, dltC,* and *dltD* are the core genes responsible for the D-alanylation of teichoic acids (TAs) (Abi Khattar et al., 2009; Andreas Peschel et al., 1999). D-alanylation is entirely abolished when any of the *dlt* core genes (dltA to dltD) is inactivated in bacteria, including *S. aureus, S. pyogenes, S. pneumoniae, Enterococcus faecalis,* and Group B *Streptococcus* (Andreas Peschel et al., 1999). Recently, *dltX* has been discovered as an essential gene for D-alanylation in *B. thruingiensis* (Rita Kamar et al., 2017). In *S. pyogenes*, the *dlt* operon consists of six genes *dltXABCDE,* in which *dltX* encodes a small protein of 47 amino acids long. The mutation in the *dlt* operon showed pleiotropic effects,

including increased negative cell surface charge and increased sensitivity to CAMPs, enhanced autolysis, increased acid sensitivity as well as decreased biofilm formation, reduced adhesion to epithelial cells and expresses altered virulence properties in many gram-positive bacteria (Eric Abachin et al., 2002; Boyd et al., 2000; Koprivnjak et al., 2006; Kovács et al., 2006; Kristian et al., 2005; Andreas Peschel et al., 1999). According to Cox et al., the GAS *dltA* gene is essential for D-alanylation. The absence of *dltA* significantly lowers the expression of GAS virulence factors, including M protein and SIC (streptococcal complement inhibitor) protein (Cox et al., 2009). My findings demonstrate that the Dlt system controls the virulence factor SpeB in *S .pyogenes* Δ*pde2* mutant. Inactivation of *dltA* or *dltX* in the Δ*pde2* background restored SpeB, similar to that of the wild type (Fig 4.2B). The induction of SpeB in the Δ*pde2* mutant by Dlt mutation suggests that the SpeB null phenotype of the Δ*pde2* mutant results from impaired D-alanylation caused by increased c-di-AMP.

D-alanylation is a crucial bacterial defense mechanism against cationic antimicrobial peptides (CAMPs) (Rita Kamar et al., 2017). The gram-positive thick cell wall plays a significant role in shielding against the host's AMPs. More than 60% of the total mass of the cell wall is made up of negatively charged teichoic acids (TAs) (Neuhaus & Baddiley, 2003; Silhavy et al., 2010). TAs are commonly found as wall teichoic acids (WTAs) that are linked to peptidoglycan, or lipoteichoic acids (LTAs), anchored to the cytoplasmic membrane via their glycolipid moiety (Fischer, 1988; Fischer et al., 1990). The critical components of TAs are disaccharide anchors and phosphodiester-linked polyglycerol phosphate or polyribitol phosphate, which contribute to its net negative surface charge (Bera et al., 2007; Kojima et al., 1985). The positively charged AMPs (cathelicidins, defensin, etc.) are electrostatically attracted by the

negatively charged bacterial surface, which damages the bacterial cell membrane. Thus hosts defend themselves from severe invasive diseases (Yount & Yeaman, 2013).

Bacteria also modify their cell surface charge by incorporating positively charged residues to counteract CAMPs. This modification can be carried out by adding L-lysine to phosphatidylglycerol mediated by the *mprF* gene or incorporating D-alanine ester on free hydroxyls of the repeating sugar mediated by *dlt* operon as has been observed in several firmicutes (E. Abachin et al., 2002; Cox et al., 2009; Kristian et al., 2005; Andreas Peschel et al., 1999; Saar-Dover et al., 2012; Weidenmaier & Peschel, 2008). In Group B *Streptococcus*, D-alanylation appears to be the primary mechanism of CAMP resistance. D-alanine incorporation most likely modifies the conformation of LTAs, increasing cell wall density and decreasing CAMP penetration (Saar-Dover et al., 2012). *S. pyogenes* cell envelope is most abundant with lipoteichoic acids (LTAs). The loss of lipoteichoic acid D-alanylation by GAS *dltA* mutation showed an increased negative surface charge, enhanced susceptibility to AMP, lysozyme, and neutrophil killing, as well as decreased adhesion and invasion into the human pharyngeal epithelial cells (Kristian et al., 2005). However, the role of the *dltX* gene in D-analylation has not been studied in *S. pyogenes*.

Here, I found that the Δ*dltX* mutants have increased negative surface charge and are highly susceptible to cationic antimicrobial peptide polymyxin B (Fig 4.5, Fig 4.6). These findings suggest that the *dltX* gene deletion causes D-alanylation defects, which reduce positive surface charge and are more attracted to cationic polymyxin B. Similar results were observed in *B. thuringiensis*, where *dltX* is an essential gene for D-alanylation (R. Kamar et al., 2017). I also observed that the Δ*dltX* and Δ*pde2* Δ*dltX* mutants have similar growth rates to the wild type, but the growth defect by the Δ*pde2* mutant is recovered when the *dltX* gene is deleted in the Δ*pde2*

background (Fig 4.4). The disruption of the *dltX* gene contributes to repairing the growth defect of the Δ*pde2* mutant with a yet unidentified mechanism.

Since D-alanylation alters the cell surface charge, it could modulate the cell envelope stress in gram-positive bacteria. Prior work has shown that the *dlt* mutation in *B. subtilis* influences the LiaFSR three-component system that can sense and respond to cell envelope stress. The Δ*dltD* mutant in *B. subtilis* expresses increased LiaFSR activity compared to the parent strain, most likely because of its higher cell membrane stress induced by *dltD* mutation (Hyyryläinen et al., 2007; Lin et al., 2020; Mascher, 2006). In *S. pyogenes*, the sensor kinase (LiaS) and the repressor protein (LiaF) are colocalized in the cell membrane and are involved in microdomain (Exportal) formation. In 2020, Lin et al. reported that the disruption of GAS Exportal by CAMPs activates the LiaFSR system, and mutation in LiaFSR alters SpeB activity (Lin et al., 2020). I found that the response regulator LiaR influences SpeB expression partially in the Δ*pde2* mutant (Fig 4.7A). Unlike the Δ*pde2* Δ*dltX* mutant, the double mutant Δ*pde2* Δ*liaR* did not restore SpeB as much as that of the Δ*pde2* Δ*dltX* mutant. The LiaR regulator may not be entirely responsible for the complete restoration of SpeB in the Δ*pde2* mutant.

I further investigated if SpeB restoration in Δ*pde2* mutant by Dlt mutation is mediated by LiaFSR-regulated gene *spxA2*. SpxA2 is an RNA polymerase-binding protein primarily found in firmicutes (J. L. Baker et al., 2020; Nakano et al., 2003; Sanson et al., 2021). Several gram-positive bacteria have two highly similar *SpxA* paralogs, *spxA1* and *spxA2* (Nakano et al., 2005; Port et al., 2017). SpxA1 mainly responds to oxidative stress, and SpxA2 regulates numerous cellular activities such as cell division, cell wall homeostasis, fatty acid biosynthesis, virulence regulation, biofilm formation, as well as cell envelope stress regulation (Baker et al., 2014; J. L. Baker et al., 2020; Kajfasz et al., 2010; Kajfasz et al., 2015). SpxA1 *a*nd SpxA2 are found in *S.*

pyogenes and have an opposite effect on virulence regulation or stress response (Port et al., 2017). Δ*SpxA1* mutant was highly attenuated and enhanced polymyxin B (PMB) resistance, while Δ*spxA2* became hypervirulent and more sensitive to PMB (Port et al., 2017). Furthermore, GAS *spxA2* transcription is highly dependent on the LiaFSR system, similar to other firmicutes (J. L. Baker et al., 2020; Nakano et al., 2003; Sanson et al., 2021). My qRT–PCR data confirmed that LiaR is essential for the *spxA2* gene expression (Fig 4.8B). The *spxA2* transcript level was significantly reduced in the Δ*liaR* mutants compared to the wild type or the Δ*pde2* mutant in the presence of the cell wall stressor vancomycin (Fig 4.8B). However, the transcription of *spxA1* or *spxA2* did not change in the Δ*pde2*, Δ*dltX*, or Δ*pde2* Δ*dltX* mutant compared to the wild type or the Δ*pde2* mutant, respectively (Fig 4.8C. 4.9B). These findings indicate that SpeB restoration in Δ*pde2* by Dlt mutation is not influenced by LiaFSR-regulated gene *spxA2*. I observed no significant change in *liaR* transcript level in the Δ*pde2* or Δ*dltX* mutant compared to the wild type or the Δ*pde2* mutant. Moreover, the transcription of *dltX* did not alter in the Δ*pde2* or Δ*liaR* mutant relative to the wild type or the Δ*pde2* mutant (Fig 4.10A, 4.10B). The Dlt system and LiaFSR are probably not linked in the SpeB regulation in the Δ*pde2* mutant at the transcriptional level. In addition, the overexpression of *speB* transcriptional activator *ropB* in the Δ*pde2* mutant restored little SpeB (Fig 4.11). A prior study has proposed that SpxA2 competes with RopB to recruit RNA polymerases to the *speB* promoter region and negatively regulates SpeB (Port et al., 2017). However, overexpressed *ropB* could not restore SpeB in the Δ*pde2* mutant. This suggests that neither RopB nor SpxA2 is involved in the SpeB null phenotype of the Δ*pde2* mutant. The regulation of SpeB expression is highly complex, and *speB* transcription is regulated by a range of transcriptional factors (Carroll & Musser, 2011; Neely et al., 2003). Since RopB is not the sole

factor affecting *speB* transcription, it is necessary to look into other *speB* transcriptional factors to comprehend the connection among Pde2, Dlt system and LiaFSR in *S. pyogenes*.

Overall the results of this study illustrate that the *dltX* gene is required to modulate the cell surface charge by D-alanylation in *S. pyogenes*. SpeB expression of the Δ*pde2* mutant is influenced by the Dlt system and LiaFSR via two different regulatory mechanisms.

Materials and Methods

Bacterial Strains and Media

S. pyogenes HSC5 (emm genotype 14) (Hanski et al., 1992; Port et al., 2013) was employed for all experiments, including strain construction. SF370 locus numbers (SPy_####) are used as references for genes in HSC5 (Ferretti et al., 2001). Molecular cloning experiments utilized *Escherichia coli* DH5α or TOP10 (Invitrogen), which was cultured in Luria-Bertani broth. The routine culture of *S. pyogenes* employed Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (Difco) (THY medium), and cells were grown at 37°C in sealed tubes without agitation. Unless otherwise indicated, C medium (Lyon et al., 1998) was used to grow *S. pyogenes* for SpeB activity assay and RNA preparation for real-time qRT-PCR. Bacto agar (1.4%, wt/vol; Difco) was added to make solid media. Cultures on solid media were incubated under the anaerobic condition created by a commercial product (GasPak; catalog no. 260678; BBL). When appropriate, antibiotics were added to the media at the following concentrations if they are not specified: kanamycin, 50μg/ml for *E. coli* and 500μg/ml for *S. pyogenes*; erythromycin, 500μg/ml for *E. coli* and 1μg/ml for *S. pyogenes*: chloramphenicol, 10μg/ml for *E. coli* and 3μg/ml for *S. pyogenes.*

Manipulation of DNA

Plasmid DNA was isolated via a commercial kit (Gene Elute plasmid miniprep kit; Sigma) and used to transform *S. pyogenes* or *E. coli* as described previously (Caparon et al., 1991). Enzymes for DNA cloning and PCR were used according to the recommendations of the manufacturers. Chromosomal DNA was purified from *S. pyogenes* by using a standard kit (Wizard genomic DNA purification kit [Promega] or GenElute bacterial genomic DNA kit [Sigma]).

Strain Construction

Generation of the mutant's Δ*dltX*, *ΩdltA*, Δ*pde2* Δ*dltX*, Δ*pde2 ΩdltA*, Δ*liaR*, and Δ*pde2*Δ*liaR* has been described elsewhere (Cho & Caparon, 2005; Cho & Kang, 2013; Faozia et al., 2021). The *ΩdltA* or Δ*pde2 ΩdltA* mutant was constructed by insertional disruption of the *dltA* gene through single homologous recombination. An internal region of the *dltA* gene was amplified by PCR, then inserted into the suicide vector pSPC18 (Lyon et al., 2001). The resultant plasmid was used to transform in the wild type or the Δ*pde2* mutant.

Determination of Doubling Time

Strains were grown overnight in THY medium and diluted 1:100 into fresh THY or C medium. The optical density of the culture at 600 nm (OD_{600}) was measured every 30 min. The OD_{600} values in exponential-growth-phase cells, which show a linear increase over time (x) versus $\log_{10}$ OD_{600} (y), were used to calculate the time (minutes) needed for the cell density (OD_{600}) to double [doubling time (minutes) = $\log_{10}$ 2/ ($\Delta\log_{10}$ OD_{600}/minutes)]. The reported data represent the means and standard errors derived from two independent assays.

Susceptibility to Polymyxin B

The susceptibility of mutant strains to the cell membrane-targeting antibiotic polymyxin B was monitored. *S. pyogenes* cells grown in THY medium overnight were harvested and inoculated in fresh Thy medium containing polymyxin B (100, 50, 10, 5, 1, 0.5, and 0 µg/ml). Cells were then grown in a 96-well plate overnight at 37°C, and the OD_{600} of the overnight cultures (~18 h post-inoculation) was measured to determine the final cell density. This experiment was performed in triplicate.

Cytochrome c Binding Assay

Bacteria were grown to the early exponential phase in THY media. Then cells were incubated in DMEM chemically defined media overnight. Cells were collected and washed twice with morpholinoepropanesulfonic acid (MOPS) buffer (20 mM, pH 7). The final cell OD was adjusted to 3 in MOPS buffer and incubated with 0.2 mg/ml cytochrome c for 10 minutes at room temperature (Sigma-Aldrich, St. Louis, MO). As a control MOPS buffer, 0.2 mg/ml cytochrome c was incubated under the same conditions without bacteria. After 10 min, bacteria were removed by centrifugation, and the cytochrome c content of the supernatants was quantified photometrically at 530 nm.

qRT-PCR

Real-time qRT - PCR was conducted as described elsewhere (Cho & Kang, 2013). The primers for qRT - PCR are listed in Table 4.1. The gyrase A subunit gene (*gyrA*) was used as the internal reference gene to normalize the expression level of a specific transcript between samples (Kang et al., 2010). The reported data represent the means and standard errors from three independent assays performed on different days with a new RNA sample.

SpeB Activity Measurement using Protease Indicator Plates

Strains were grown overnight in THY medium. The overnight cultures were serially diluted with fresh THY medium, and the diluted cells (2µl) were spotted onto protease indicator agar plates (C medium agar plates containing 2% skim milk). The protease indicator plates were then incubated anaerobically at 37°C for 24 h (or 48 h for extended incubation), and SpeB activity, which displays a clear zone around the spotted cells, was observed.

Statistical Testing

Each statistical test applied to the experiments was described in the figure legends.

Table 4.1.

Primers used.

Name[a]	Sequence[b]	Remarks
Mutagenic Primers[c]		
To create pΔDltX		
FC3p7INT-2 FC5p7INT-2	gtcgtgactgggaaaaccctgg cctgtgtgaaattgttatccgctc	For vector amplification (4091 bps)
5dltX1000 3dltX1000	gggttttcccagtcacgacCGACTGGGCTACTTGATCCTGG gggttttcccagtcacgacCGACTGGGCTACTTGATCCTGG	For insert amplification (2185 bps)
To create pΩDltA		
5pUC18 3pUC18	cgggtaccgagctcgaattcg cctgcaggcatgcaagcttg	For vector amplification (4228 bps)
5KOdltA 3KOdltA	**cttgcatgcctgcagg**ccttgtctcactatcagagattgagtcag **cgagctcggtacccg**caccctgctcccctga	For insert amplification (692 bps)
To create pΔliaR		
FC3p7INT-2 FC5p7INT-2	gtcgtgactgggaaaaccctgg cctgtgtgaaattgttatccgctc	For vector amplification (4091 bps)

5liaR1000 3liaR1000	gggttttcccagtcacgacGGATAGGCGATGAAAAAACGTTAC TATGC gcggataacaatttcacacaggCATCATAGTACCCTTCTTTAGCC AAACC	For insert amplification (2691 bps)
Analysis primers[d]		
RTspxA1-F RTspxA1-R	ACAAGTCCATTAAGCCGTGATG AGGGCGACGAAGAAGACTTG	
RTspxA2-F RTspxA2-R	GAACTTAGGAAAAAGAACCGCTAACTAA CGCAATCGAGAGCTTTGGC	
RTdltX-F RTdltX-R	TCAAGAATGAGAGGAATTGCTG ACCAAAGAAATAGACCAGCAAC	
RTliaR-F RTliaR-R	CGTGAAGGGGTTGATTTGGC TAACCCTTCGCTCCTGCATC	
RTgyrA-F RTgyrA-R	AACAACTCAAACAGGTCGGG CTCCTTCACGGCTAGATTC	

Note. [a]Primers are categorized as 5′ or 3′ based on the direction of a transcript. Primers whose names start with 5′ (forward primers) anneal to the non-coding template strand, while those with 3′ (reverse primers) anneal to the coding strand. [b]Sequences are shown 5′ to 3′. Uppercase sequences anneal to the HSC5 chromosome, and lowercase sequences anneal to plasmid sequences. [c]Mutagenesis primers were used for PCR reactions to amplify DNA segments used to construct plasmids for gene deletion. [d]Analysis primers were used in regular PCR to confirm (CF) gene deletion or in qRT-PCR (RT) to measure the level of gene transcription.

CHAPTER 5

Conclusions

The research presented in this dissertation constitutes a three-part investigation conducted to understand further the c-di-AMP signaling pathway in regulating one of the major virulence factors, SpeB, in *S. pyogenes*. In the first study, I investigated whether c-di-AMP regulates SpeB at the transcriptional, post-transcriptional, or translational levels. I reported that c-di-AMP regulates SpeB at the transcriptional level. The mutant lacking c-di-AMP synthesizing enzyme Δ*dacA* reduces *speB* transcript a thousand times compared to the wild type. On the other hand, the deletion of c-di-AMP degradation encoding enzyme Pde2 displayed a more severe defect in *speB* transcription. Δ*pde2* showed ten thousand times less *speB* transcript than the wild type. It is interesting to note that despite *dacA* and *pde2* being involved in opposite processes (the synthesis and degradation of c-di-AMP, respectively), their null mutants showed similar phenotypes with regard to SpeB production. This finding might suggest that an optimum cellular concentration of c-di-AMP is necessary for adequate SpeB synthesis. In addition, I observed an almost total reduction of the virulence of *ΔdacA* and *Δpde2* mutant in a mouse subcutaneous infection model. These results support that c-di-AMP's vital role in *S. pyogenes* virulence and pathogenesis.

In the second study, I was interested in determining how potassium transporter KtrAB links c-di-AMP and SpeB biogenesis in *S. pyogenes.* I reported that c-di-AMP controls SpeB in the Δ*dacA* mutant at the transcriptional level via the KtrAB K^+ importer. I found that the SpeB

null phenotype of the Δ*dacA* mutant is caused by the change of intracellular K^+ concentration regulated by the KtrAB transporter *in vitro*. In addition, SpeB induction in the Δ*dacA* mutant by K^+-specific ionophore treatment also supports the importance of cellular K^+ balance in SpeB production. However, ionophore treatment did not change the SpeB null phenotype of the Δ*pde2* mutant, suggesting that intracellular K^+ is not involved in regulating SpeB in the Δ*pde2* mutant. This study revealed the role of c-di-AMP in virulence regulation via potassium transport in *S. pyogenes*.

My final study investigated the mechanism of SpeB virulence regulation in the *Δpde2* mutant deficient in c-di-AMP degradation. My results showed that the Dlt mutation in the Δ*pde2* mutant reverts the SpeB null phenotype of the Δ*pde2* mutant. It is likely that SpeB induction in the Δ*pde2* mutant results from the impaired D-alanylation of the *dlt* operon caused by elevated c-di-AMP. I also utilized the LiaFSR envelope stress regulator to examine if impaired D alanylation alters envelope stress by changing the net negative surface charge. I found that LiaFSR also influences SpeB at least partially in Δ*pde2* mutant. My findings demonstrated that the Dlt system and LiaFSR affect SpeB in the Δ*pde2* mutant, although not in the same pathway. Further research is necessary to fully comprehend how Pde2 and teichoic acid D-alanylation or Pde2 and LiaFSR system relate to SpeB expression in *S. pyogenes*. Comparative transcriptome analysis of the Δ*pde2* Δ*dltX* or Δ*pde2* Δ*liaR* mutant compared to the *Δpde2* mutant would contribute to unveiling the underlying SpeB regulation pathway by the Dlt system or LiaFSR system in the Δ*pde2* mutant. Moreover, transposon mutagenesis into the Δ*pde2* Δ*dltX* mutant could be an alternative approach to identify genes linked to Pde2 and teichoic acid D-alanylation in SpeB regulation in *S. pyogenes.*

In summary, this study addressed the role of c-di-AMP in virulence and pathogenesis in *S. pyogenes*. The insight knowledge will also lead to understanding the c-di-AMP signaling pathway in other c-di-AMP-producing bacteria. In addition, identifying unknown *speB* transcriptional factors under the influence of c-di-AMP would contribute to deciphering the c-di-AMP complex network in SpeB regulation. In a separate set of experiments (data not shown), transposon mutants in a Δ*dacA* mutant background were screened to identify SpeB+ mutants. I identified several target genes, including 16S methyltransferase, Cro/Cl family transcriptional regulator ComR, peptide-binding protein OppA, cysteine synthase, and iron ABC transporter substrate-binding protein whose disruption via transposon insertion led to SpeB+ phenotype in a Δ*dacA* mutant. Studying those additional genes could uncover novel signaling pathways of c-di-AMP regulating SpeB in *S. pyogenes*. Moreover, the discovery of anti-virulence drugs by targeting the c-di-AMP signaling pathway would open new horizons in therapeutics by better understanding the signaling pathway of c-di-AMP in GAS virulence and pathogenesis.

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