5-2021

The Impact of Oxygen Availability and Oxidative Stress on Regulation of Cyclic-dimeric-GMP in Listeria monocytogenes strain F2365

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The Impact of Oxygen Availability and Oxidative Stress on Regulation of Cyclic-dimeric-GMP in *Listeria monocytogenes* strain F2365

by

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A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of Honors Requirements

May 2021

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Abstract

Listeria monocytogenes is a gram-positive foodborne pathogen that results in the infectious disease listeriosis. The second messenger molecule cyclic-dimeric-GMP has been found to be responsible for the regulation of expression of many of its virulence factors with diguanylate cyclases and phosphodiesterases regulating the intracellular concentration of cyclic-di-GMP through synthesis and degradation, respectively. This study investigated the possibility that the availability of oxygen is the environmental signal crucial to the regulation of these enzymes. Prior research in our laboratory has demonstrated that the intracellular concentration of cyclic-di-GMP is elevated when exposed to anaerobic conditions. Due to this, it was hypothesized that diguanylate cyclases are upregulated and phosphodiesterases are downregulated in response to lack of oxygen availability. *Listeria monocytogenes* has three recognized diguanylate cyclases, *dgcA, dgcB,* and *dgcC,* and three recognized phosphodiesterases, *pdeB, pdeC,* and *pdeD*. This study analyzed the expression of these genes under aerobic and anaerobic conditions by qPCR. Results showed that two of the diguanylate cyclases were upregulated in anaerobic conditions in comparison to aerobic conditions, and one phosphodiesterase was upregulated under anaerobic conditions. This suggests that there is differential regulation of the diguanylate cyclases and phosphodiesterases in response to anaerobic conditions. This study also analyzed whether oxidative stress could pose as an environmental signal affecting the concentration of cyclic-di-GMP within the cell. To test this, hydrogen peroxide was added to aerobic cultures, and the concentration of cyclic-di-GMP was compared to the controls. Results indicated a significant increase in cyclic-di-GMP in cultures that were treated in comparison to untreated controls. The results of this research suggest that anaerobic conditions and reactive oxygen species likely play a pertinent role in the regulation of

expression of cyclic-di-GMP within the cell. Further research into the mechanisms involved within the cyclic-di-GMP signaling pathway is required to decipher the modes of regulation utilized by diguanylate cyclases and phosphodiesterases and the methods used by *Listeria* when faced with oxidative stress.

Keywords: *Listeria monocytogenes,* Cyclic-di-GMP, Phosphodiesterase, Diguanylate Cyclase, Oxidative Stress, Anaerobic, Hydrogen peroxide

Dedication

This thesis is dedicated to my mother, who has supplied me with all the tools I need to succeed in life and whose love and support follows me into any endeavor I take on. Additionally, this is dedicated to my roommate and friend Antara Sharma, who was a continuous source of encouragement and my most consistent confidante through frustrations.

Acknowledgements

Firstly, I would like to thank my advisor, Dr. Janet Donaldson, for being a wonderful mentor throughout this entire process. There were many obstacles that arose while conducting this research, and she worked with me each time to attempt to resolve the issues. Without the energy and assistance she offered and the knowledge she supplied me with, this project would have never been finished. I would also like to thank Dr. Donaldson's graduate assistant, Damayanti Chakravarty, who guided me through many parts of this experiment and was patient with me despite a bombardment of questions at each step. The majority of my practical knowledge of laboratory equipment and procedures I owe to her. In addition, thanks to the Honors College for being a constant support system throughout my entire college career and my journey into academic inquiry.

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List of Abbreviations

cyclic-di-GMP	Cyclic Dimeric Guanosine Monophosphate
Ct	Cycle threshold
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
dgc	Diguanylate Cyclase
pde	Phosphodiesterase
qPCR	Quantitative Polymerase Chain Reaction
InlA	Internalin A
InlB	Internalin B
PBS	Phosphate Buffered Saline

Chapter 1. Introduction

Listeria monocytogenes is a gram-positive bacterium and foodborne pathogen that is the causative agent of the food-borne disease listeriosis. This species can contaminate raw fruits and vegetables, meats, unpasteurized dairy products, and other unprocessed foods (1). The bacterium's ability to survive under a large range of temperature, pH, salt, and oxygen concentrations makes it of particular concern in food production plants. *Listeria* biofilms may persist for years within these plants, causing consistent recontamination of products and subsequent illness (1, 2).

Listeriosis is particularly lethal in those with weakened immune systems, such as the elderly, infants, pregnant women, and immunocompromised individuals (2). It is estimated that out of all deaths due to food-borne illnesses in the United States each year, *L. monocytogenes* is responsible for about 19% and has an estimated 20-30% mortality among those that contract listeriosis despite antibiotic treatment (3). Listeriosis is characterized by fever, aches, nausea and diarrhea, with more severe cases leading to sepsis, meningitis, encephalitis or death (4). Previous outbreaks of listeriosis include a multi-state outbreak in cantaloupes in 2011, which resulted in 33 deaths and 147 confirmed cases, making it one of the most severe documented outbreaks of food-borne disease (5). Due to the potential severity of this illness and the danger it poses to vulnerable populations, it is crucial to research its pathogenesis with the aim of developing more effective preventative measures.

In order to better treat infections caused by *L. monocytogenes*, it is pertinent to evaluate the factors that determine its virulence. The messenger molecule cyclic-dimeric-Guanosine Monophosphate (cyclic-di-GMP) is a signaling molecule that has been found to bind receptors that regulate virulence factors, such as genes involved in biofilm formation and motility.

Previous studies have also shown that cyclic-di-GMP determines the expression of genes that are pertinent to adhesion and invasion potential into intestinal epithelial cells (6,7). One previous study on the microorganism *Vibrio cholerae*, also revealed that cyclic-di-GMP plays a significant role in the tolerance of the bacterium to reactive oxygen species, such as hydrogen peroxide, which is encountered within the bacterium's marine environment. Since the bacterium relies heavily on this aquatic environment in order to find a new host to inhabit, this mechanism of resistance is important (8).

Within the cell, diguanylate cyclases create molecules of cyclic-di-GMP while phosphodiesterases break down this molecule, making these two enzymes responsible for the regulation of intracellular concentrations of cyclic-di-GMP (7). One study on the pathogen *Mycobacterium tuberculosis* showed that expression of the diguanylate cyclases increased and phosphodiesterases decreased in response to anaerobic conditions (9). A study on *Escherichia coli* suggested that the coupling of diguanylate cyclases and phosphodiesterases senses and responds to environmental signals such as the presence of oxygen for the control of cyclic-di-GMP (10). Based on these previous findings, it was hypothesized that anaerobic conditions prompt the upregulation of diguanylate cyclases and the downregulation of phosphodiesterases in *L. monocytogenes*, which leads to an increase in the intracellular concentration of cyclic-di-GMP and virulence. Additionally, studies in *Vibrio cholerae* and *L. monocytogenes'* virulence triggers indicated a link between reactive oxygen species and biofilm formation (8, 11). Due to this, it was also hypothesized that hydrogen peroxide impacts intracellular concentrations of cyclic-di-GMP. Determining the regulation of expression of these two classes of enzymes that impact intracellular cyclic-di-GMP concentrations will potentially aid in the goal of preventing *L. monocytogenes* from expressing virulence factors during host invasion.

Chapter 2: Review of Literature

Most, if not all, cases of *Listeria monocytogenes* infection are caused by the ingestion of contaminated food sources, such as soft cheeses, milk contaminated post-process, and raw meat products. Upon infection, this bacterium has the ability to enter the lumen of the small intestine through Peyer's patches and multiply within nonphagocytic cells, allowing for the invasion of tissues. In addition, the pathogen is able to disseminate from the gastrointestinal tract and infect other organs as well, including the spleen and brain, which may cause meningitis, and the fetus of pregnant women which may cause miscarriage (12).

2.1 Internalization Mechanism and Intracellular Life Cycle of *Listeria monocytogenes*

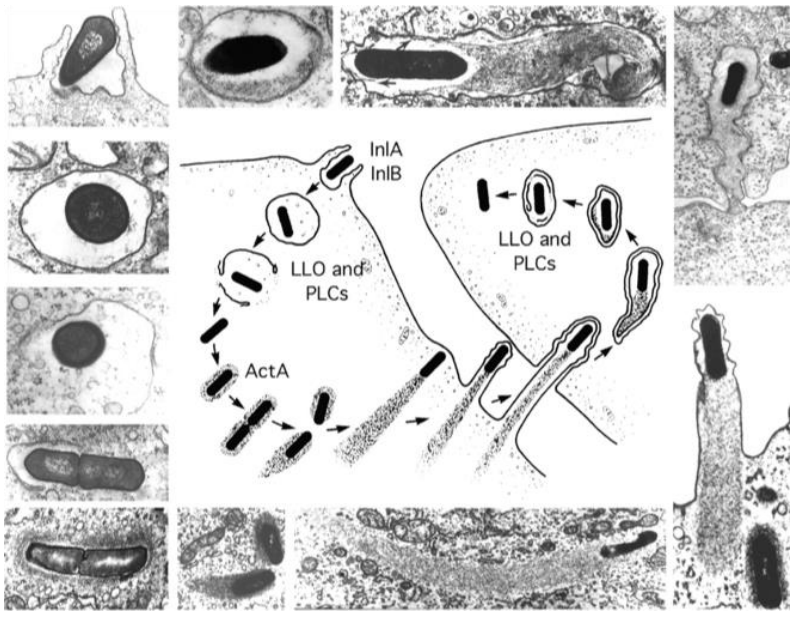
Listeria monocytogenes utilizes the zipper mechanism of internalization, in which a bacterial ligand interacts with a surface molecule on the host cell, allowing the pathogen to be engulfed by the plasma membrane. Intracellular entry allows for the pathogen to escape into a protected area and avoid initial host immune defenses, such as circulating antibodies (13). This admission into mammalian cells involves the utilization of two proteins on the surface of the bacteria, internalins A and B (*inlA* and *inlB*). It has been established that the surface protein InlA is required for the invasion of *L. monocytogenes* into gastrointestinal enterocytes, while InlB is needed for hepatocytes invasion and fetal infection. However, studies have shown that the presence of just one of these proteins can be sufficient to gain entry into a host cell if its appropriate receptor is expressed (14).

The transmembrane glycoprotein E-cadherin has been identified as the host receptor for InlA that works as a cellular adhesion molecule and mediates the internalization process of *L. monocytogenes* into mammalian cells (15). InlB targets the hepatocyte growth factor, Met, a transmembrane tyrosine kinase receptor on the surface of host cells. Met is expressed

ubiquitously throughout the human body, while E-cadherin is mainly expressed within cells of epithelial origin; InlB can mediate entry into a much broader range of cell types than InlA which mainly mediates entry across the intestinal barrier (16). In addition to Met, InlB may also interact with the multifunctional glycoprotein, gC1qR, which is involved in InlB-dependent entry into cells, though that mechanism is so far unknown (17).

Once internalized, the bacterium must escape from the vacuole using the pore forming protein listeriolysin O and phospholipase C to mediate exit into the cytosol of the cell. In the cytosol, *L. monocytogenes* utilizes ActA, a protein that triggers host actin filaments to be polymerized. This polymerization of actin enables actin-based motility within the cell, and eventually mediates cell-to-cell spread to adjacent cells, successfully evading host defenses and reinitiating the intracellular life cycle within the next cell (18). This sequence from internalization to intercellular spread is delineated in Figure 2.1.

Figure 2.1 Intracellular life cycle of *Listeria monocytogenes* (18). Diagram depicts the stages in the intracellular life cycle of *L. monocytogenes*, beginning with internalization using proteins InlA and/or InlB. Escape from the vacuole is facilitated by protein listeriolysin O (LLO). After, actin-based motility is deployed by actin nucleation enabled by ActA, which allows for cell-to-cell spread (Figure reprinted from 18).



2.2 Cyclic-di-GMP regulates virulence factors

The messenger molecule cyclic-di-GMP has been found to be crucial to the success of intracellular invasion and persistence of *L. monocytogene*s, as it regulates the expression of several virulence factors, including motility, adhesion, biofilm formation, and invasion (19). In the 1980's the molecule was first established as an allosteric activator of a cellulose synthase enzyme present in the bacterium *Gluconacetobacter xylinus*. Since then, cyclic-di-GMP has been established as a key player in the regulation of virulence in many pathogens, especially regarding the transition from aggregating within biofilms to sessile motility and vice versa (19). In general, higher concentrations of cyclic-di-GMP have been found to promote biofilm formation and inhibit flagellar motility while lower concentrations tend to cause biofilm dispersion and virulence as evidenced in several model pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella enterica* (20, 21). In several bacterial species, the formation of biofilms has assisted with surviving reactive oxygen species. For example, a previous study found cyclic-di-GMP to be integral in the tolerance of the causative agent of cholera, *Vibrio cholerae*, to the reactive oxygen species hydrogen peroxide. This aids in its ability to persist in both host and marine environments as the bacterium exits a host into an aquatic environment to find a new one, continuing its infectious cycle (8).

Cyclic-di-GMP has been documented to modulate cellular function at several concentrations, including allosteric regulation of enzyme activity or protein function and regulation of gene expression of one or more transcription factors (21). In addition, in many bacteria cyclic-di-GMP has been shown to be able to control the translation and transcription of genes through the existence of specific riboswitches, domains of mRNA that control gene expression (22). An example of this is seen in the bacterium *Vibrio cholerae*, the causative agent

for the disease cholera. Through the use of one of two cyclic-di-GMP riboswitches present in *V. cholerae,* its type IV secretion system, a structure that injects toxic proteins into neighboring cells upon contact, is partially controlled (23). However, in the case of *L. monocytogenes*, there is no known cyclic-di-GMP riboswitch, so the detailed method of control is less clear. Nonetheless, prior studies have indicated that cyclic-di-GMP plays a key role in the regulation of virulence in Firmicutes similar to the more explored Proteobacteria and Mycobacteria. For instance, one prior study on the molecule showed that elevated concentrations of cyclic-di-GMP can induce the synthesis of an exopolysaccharide that increases tolerance of *L. monocytogenes* to disinfectants and desiccation and promotes cell aggregation (24). These factors likely aid the pathogen in persisting in food production plants until it is able to infect a host.

2.3 Changes in cyclic-di-GMP concentrations are triggered by environmental signals

Cyclic-di-GMP is synthesized by diguanylate cyclases bearing catalytic GGDEF domains from two GTP molecules and degraded by phosphodiesterases possessing EAL domains, making these two groups of enzymes responsible for regulating intracellular concentrations of cyclic-di-GMP (25). This regulation of cyclic-di-GMP through rates of synthesis and degradation is detailed in Figure 2.2.

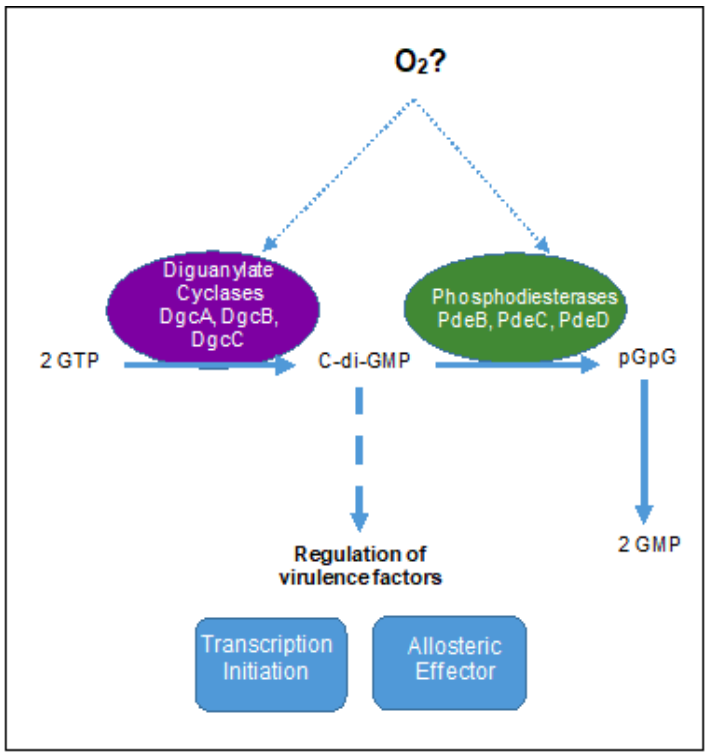


Figure 2.2. Diguanylate cyclases and phosphodiesterases regulate the concentration of cyclic-di-GMP inside the cell. Diguanylate cyclases synthesize cyclic-di-GMP from two molecules of GTP, and phosphodiesterases degrade cyclic-di-GMP into pGpG. *Listeria monocytogenes* has three diguanylate cyclases: *dgcA*, *dgcB*, and *dgcC*, and three phosphodiesterases: *pdeB*, *pdeC*, and *pdeD*.

Most diguanylate cyclases and phosphodiesterases are multi-domain proteins harboring sensing domains, which implies that they are likely triggered through environmental signals. These proteins have been found to sense environmental signals in one of two different ways: 1. the presence of a transmembrane sensor domain that responds to external stimuli or 2. the existence of a histidine kinase capable of phosphorylating a response regulator domain within the multidomain protein (26).

The actual environmental signal that triggers diguanylate cyclases or phosphodiesterases is not clear. Previous studies on other bacteria on the cyclic-di-GMP signaling pathway have shown that the lack or presence of oxygen can affect these enzymes' activity. For example, one study on *Pseudomonas aeruginosa* found increased concentrations of cyclic-di-GMP in anaerobic conditions due to elevated diguanylate cyclase activity (27). Other research has shown that the absence of oxygen elevates cyclic-di-GMP concentrations in the infectious agent *Mycobacterium tuberculosis*, which provokes dormancy and makes the treatment of the disease tuberculosis more difficult (9). Additionally, a prior study on *Escherichia coli* revealed a two-gene operon that produces a complex to manage rates of synthesis and degradation of cyclic-di-GMP based on oxygen concentration (10). Similar to all of these previous findings, initial research in Dr. Janet Donaldson's lab has suggested that the lack of oxygen can trigger increased cyclic-di-GMP production in *Listeria monocytogenes*. Therefore, this thesis proposes that the presence of oxygen is an environmental signal utilized to regulate the quantity of cyclic-di-GMP within *L. monocytogenes.*

Furthermore, other research suggests that another environmental signal may trigger regulation of the molecule cyclic-di-GMP within certain conditions, such as the presence of reactive oxygen species within the environment. This is especially of interest due to the process of oxidative burst. This action involves the tendency of host cells to generate and release reactive oxygen species upon the occurrence of bacterial infection in an attempt to kill bacterial cells. Consequently, the resistance of pathogens to these compounds can prove to be crucial to continued survival and infectious potential (28). Several studies investigating *Listeria monocytogenes* indicated a link between reactive oxygen species and virulence factors such as biofilm formation. In particular, one former study found that the food-borne pathogen's ability to

create biofilms was positively affected by the presence of hydrogen peroxide, suggesting that cyclic-di-GMP concentrations were likely altered (12). Due to these previous findings, it was hypothesized in this study that the addition of hydrogen peroxide to *Listeria* results in increased cyclic-di-GMP concentrations in comparison to controls.

Chapter 3. Materials and Methods

3.1 Bacterial Culture Conditions

Listeria monocytogenes strain F2365 was utilized for the experiments outlined in this study. This strain was frozen and stored within a -80°C freezer when not being used. F2365 was streaked onto Tryptic Soy Agar (TSA) prior to creating overnight cultures for study, and the streaked plate was placed into an incubator at 37°C for 24 hours. The colonies present on the plate were then used to inoculate 2 mL of Tryptic Soy Broth (TSB). TSB cultures were placed into a shaker incubator at 37°C at 200 RPM overnight (about 14-20 hours). After incubation, 1 mL of each culture was utilized to inoculate 20 mL of TSB, and each sample was allowed to grow for approximately two hours to reach the mid log phase or until the optical density (OD_{600}) measured between 0.3 to 0.5. Aerobic midlog cultures were grown within a shaker incubator at 37°C at 200 RPM in normal conditions, while anaerobic mid log cultures were grown at 37°C at 200 RPM within a Coy Laboratories Anaerobic Chamber which fosters an environment free from oxygen through the use of a mixture of 5% hydrogen and 95% nitrogen gases. Under each condition, at least three independent replicates were performed.

3.2 RNA Isolation and qPCR

RNA was isolated from the 20 mL F2365 cultures in mid log phase under aerobic and anaerobic conditions. Once cultures reached OD_{600} of about 0.3 to 0.5, tubes were briefly centrifuged at 7500 *x g* at 10°C for 5 minutes, creating a pellet. The pellets were treated with 2 mL of ice-cold Phosphate Buffered Saline (PBS) and 4 mL of RNAprotect (Qiagen), and each was incubated at room temperature for 5 minutes. The mixture was then pelleted again by centrifuging at 7500 *x g* at 10°C for 5 minutes. RNA was isolated using a Qiagen QIAshredder column and a RNeasy Plus Mini Kit per manufacturer's instructions. Bacterial cells were lysed

by adding the resuspended pellets to 0.5 mm Zirconia beads and adding a 100:1 mixture of lysis buffer and B-mercaptoethanol to the cells. The tubes were then placed within a beadmill bead beater and bead beat two times for 2 minutes at 5 meters per second with a 1-minute rest period between sessions. After washing and eluting into RNAse-free water, the concentration of RNA isolated for each sample was measured using a Nanodrop One Spectrophotometer. qPCR was performed utilizing a TaqMan 1-step RNA kit, the RNA samples, and the gene assays for each gene to be studied. Cycle threshold (Ct) values were obtained and recorded from qPCR results, and fold change calculations were performed.

3.3 Fold Change Calculations

Fold change calculations were used in this study to determine the impact of oxygen on the expression of each diguanylate cyclase or phosphodiesterase. The equation used to calculate fold changes was $2^{-\Delta\Delta CT}$, and this formula was used to determine the differential gene expression between two different samples (Sample A and Sample B). In this case, Sample A was the anaerobic sample mean for the respective gene while Sample B was the aerobic sample mean when performing calculations. The expanded form of this equation is:

$$2^{-\Delta\Delta CT} = [(C_T \text{ gene of gene interest} - C_T \text{ internal control}) \text{ of Sample A}$$
$$- (C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ of Sample B}] \quad (30).$$

A fold change larger than the value of 1 reveals an increase in expression of that gene, while values less than 1 indicate a decrease. If a value is less than 1, the reciprocal expression, $1/(2^{-\Delta\Delta Ct})$, is then used to calculate the fold change for the decrease in expression of the gene.

3.4 Isolation and Enumeration of Cyclic-di-GMP

Listeria strain F2365 was streaked on a TSA plate, and the resulting colonies were used to make 2 mL overnight cultures in aerobic conditions. Fifty microliters of overnight cultures

were utilized to inoculate 5 mL of TSB, and tubes were incubated for 3 hours to allow them to reach mid log growth phase. After incubation, 3% hydrogen peroxide was added to three 5 mL culture tubes (Samples D, E, and F) and tubes were incubated for an additional 30 minutes. OD_{600} was then checked and concentration adjusted until the value read 1.8 (i.e., if OD is 0.9, 2 mL would be centrifuged to reach 1.8). Cultures were centrifuged at the maximum speed for 5 minutes at 4°C, and supernatant was removed. Pellets were resuspended in 100 microliters of PBS, and samples were transferred to microfuge tubes. Cultures were then incubated at 100°C. After five minutes, 186 microliters of 100% ethanol were added, and tubes were vortexed for 15 seconds. Samples were centrifuged at maximum speed for five minutes at 4°C, and supernatant was transferred to a new tube. The extraction was repeated with the pellet and supernatants were combined. Supernatants were then dried down with a vacuum centrifuge. Pellets were stored at -80°C. Samples were then analyzed by mass spectrometry by Pharos Diagnostics. A calibration curve was utilized to determine concentrations of cyclic-di-GMP within each sample. Three independent replicates were analyzed.

Chapter 4. Results

4.1 Oxygen availability impacts expression of diguanylate cyclases and phosphodiesterases in *Listeria* strain F2365.

In order to investigate the effect of oxygen on expression of genes that produce and degrade cyclic-di-GMP in strain F2365, RNA was isolated from both aerobic and anaerobic culture conditions. The isolated RNA was used to perform qPCR to explore differences in expression of diguanylate cyclases *dgcABC* and phosphodiesterases *pdeBCD*. Those average Ct values are shown in Table 4.1 along with average Ct values of *L. monocytogenes* housekeeping gene 16S rRNA under both conditions. 16S rRNA was used as the housekeeping gene for this bacterium because many studies have shown it to remain stable under a large variety of circumstances (29, 30).

Table 4.1 Average Ct Values of F2365 under Aerobic and Anaerobic Conditions

Gene	Aerobic F2365	Anaerobic F2365
16S	7.402	7.766
dgcA	34.418	34.064
dgcB	35.473	34.741
dgcC	35.594	31.758
pdeB	21.49	18.586
pdeC	25.66	25.228
pdeD	22.439	19.203

Ct values were utilized to calculate fold changes of each gene studied to determine if there were any variations in expression when cells are grown in an environment lacking oxygen. These results are displayed in Figure 4.1. Of the diguanylate cyclases analyzed, *dgcC* showed the

greatest increase in expression under anaerobic conditions, with *dgcB* being slightly elevated. Phosphodiesterase B (*pdeB)* also increased in a biologically significant manner in terms of expression when conditions were lacking oxygen, but *pdeC* did not change. *pdeD* had a slight increase in expression. Fold change ratios were also analyzed via a two-tailed T test. The increases in expression of *dgcC* and *pdeB* under anaerobic conditions in comparison to aerobic conditions were found to be statistically significant ($p < 0.05$); the changes in the other tested genes showed no significant difference between the two groups ($p > 0.05$).

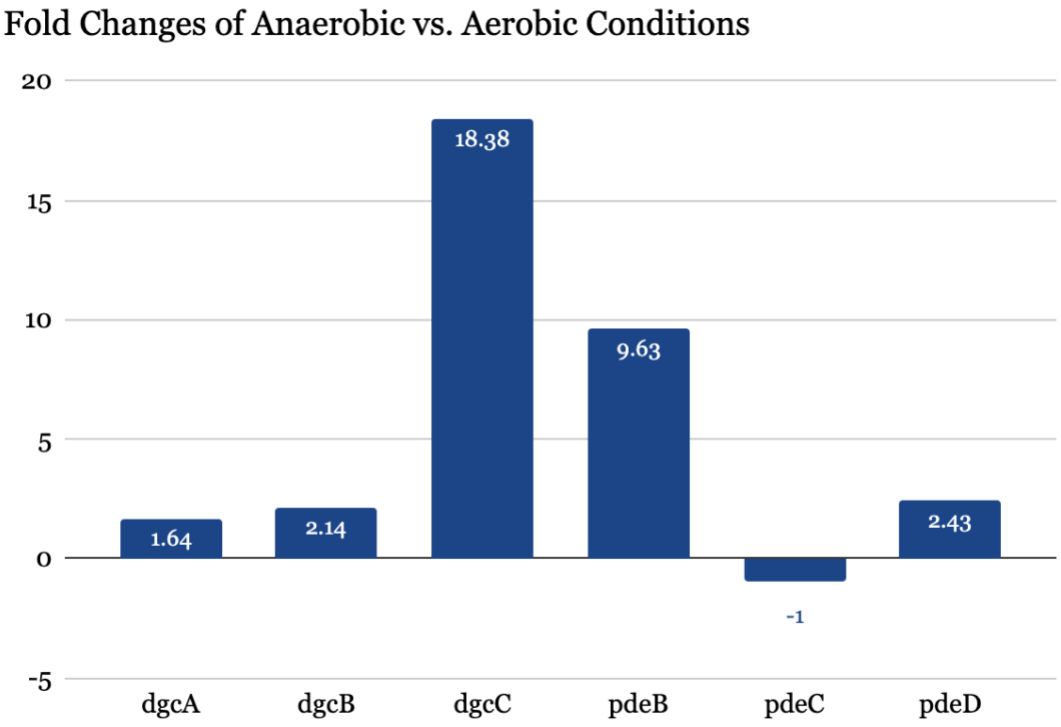


Figure 4.1. Fold changes in expression of diguanylate cyclases and phosphodiesterases in anaerobic cultures compared to aerobic cultures. Diguanylate cyclases *dgcB* and *dgcC* were upregulated in response to anaerobic conditions. Phosphodiesterases showed varying responses, with *pdeB* being upregulated similar to diguanylate cyclases B and C. Increases in expression of *dgcC* and *pdeB* were found to be statistically significant ($p < 0.05$).

4.2 Presence of hydrogen peroxide affects the concentration of cyclic-di-GMP in the cell.

Cyclic-di-GMP was isolated from aerobic cultures of *Listeria* strain F2365 treated with or without the reactive oxygen species hydrogen peroxide. Samples D, E, and F were treated with 3% hydrogen peroxide in order to determine the effect reactive oxygen species had on the expression of cyclic-di-GMP (Table 4.2). Samples A, B, and C were controls. Concentrations of cyclic-di-GMP are displayed in Table 4.2. Samples with hydrogen peroxide showed significantly higher concentrations of cyclic-di-GMP within the cell in comparison to non-treated controls ($p < 0.005$).

Table 4.2 Concentration of Cyclic-di-GMP

Sample	Calculated Amount pmol	(2 μL injection) pmol/μL	Total cyclic-di-GMP (in 200 μL) nmol
A	35.43	17.71	3.54
B	20.16	10.08	2.02
C	27.63	13.81	2.76
D (H_2O_2)	640.32	320.16	64.03
E (H_2O_2)	698.39	349.19	69.84
F (H_2O_2)	584.51	292.25	58.45

Samples D, E, and F were treated with 3% hydrogen peroxide while Samples A, B, and C were untreated aerobic cultures. Treated samples showed much higher concentrations of cyclic-di-GMP.

Chapter 5. Discussion

Listeria monocytogenes is a dangerous infectious agent characterized by a high mortality rate among vulnerable individuals that have ingested contaminated food products (3). As in many pathogens, the survival of the bacterium and severity of the infection relies on the regulation of several virulence factors, such as those that synchronize the transformation from biofilm formation to sessile motility or vice versa. The secondary messenger molecule cyclic-di-GMP has been shown to regulate expression of many of these virulence factors. Cyclic-di-GMP is produced by diguanylate cyclases and subsequently degraded by phosphodiesterases to maintain intracellular concentrations. Production and degradation within the cell are triggered in response to environmental signals, though the exact signal *Listeria* responds to is unclear (6, 7).

Previous research has revealed that oxygen availability could be the signal that other pathogens respond to in order to regulate the expression of virulence (9,10). Prior studies in Dr. Donaldson's lab have suggested a similar response may occur in *Listeria monocytogenes* as well. Based on these results, this study hypothesized that anaerobic conditions upregulate diguanylate cyclases and downregulate phosphodiesterases, therefore increasing concentrations of cyclic-di-GMP within the cell. In order to test this hypothesis, the expression of the three known diguanylate cyclases, *dgcA*, *dgcB*, and *dgcC*, and three phosphodiesterases, *pdeB*, *pdeC*, and *pdeD* were observed under both aerobic and anaerobic conditions, with the housekeeping gene 16S rRNA being utilized as an internal control.

qPCR was used in order to determine if there were variations in expression among the genes tested based on the availability of oxygen within the environment. This raw data is displayed in Table 4.1 as cycle threshold, or Ct, values. However, these values alone do not show us the relative expression of each gene in context with the internal control. Therefore, the

comparative Ct method was utilized in this study to present qPCR data in terms of relative gene expression. This method operates on two vital assumptions: 1. The efficiency of the PCR used is close to the value of one and 2. The efficiency of the target gene is similar to the internal control, which is 16S rRNA in this case. The comparative Ct method consists of calculating fold changes using the expression, $2^{-\Delta\Delta C_T}$. The calculated value represents the variation in expression between the targeted gene based on the internal control in the treated sample in comparison to the untreated sample (30). In this experiment, the treated samples were the cultures grown in anaerobic conditions while aerobic cultures were used as the untreated control samples.

In the *L. monocytogenes* strain F2365, it was observed that two diguanylate cyclases, *dgcB* and *dgcC*, increased in expression under anaerobic conditions in comparison to cultures grown with oxygen available. Phosphodiesterase *pdeB* was also shown to be upregulated in anaerobic conditions. However, expression of *pdeC* was not altered. This differs slightly from what was hypothesized since it was assumed all phosphodiesterases would be downregulated in response to lack of oxygen. One study on *Pseudomonas aeruginosa* may shed some light on why the phosphodiesterases had different responses to anaerobic conditions. This study revealed that while PDE enzymes are all presumed to regulate intracellular concentrations of cyclic-di-GMP, there is not redundancy in phenotypes. This means that the specificity of each enzyme may be related to the site of activity, which could result in differences in regulation of cyclic-di-GMP among the three putative phosphodiesterases. A similar occurrence could be being observed in the case of *L. monocytogenes*, but additional research is needed to determine if this occurs (31). The differential expression observed in these results still supports the idea that oxygen availability is a pertinent environmental signal that governs many aspects of *Listeria*'s virulence and survival capabilities.

This study also aimed to interpret whether the introduction of reactive oxygen species, such as hydrogen peroxide, affected concentrations of cyclic-di-GMP within the cell, thereby also impacting the regulation of virulence factors in *Listeria monocytogenes*. Since oxidative stress is one of the many challenges infectious agents must face in order to persist within the host environment, it is vital to attempt to understand the mechanisms utilized to withstand these stressors. One study on *Pseudomonas aeruginosa* revealed that the addition of hydrogen peroxide to cultures increased amounts of intracellular cyclic-di-GMP, leading to biofilm formation in order to persist in the environment. It also suggested that the presence of reactive oxygen species within the environment, as seen within a host, drives the selection of variants of the pathogen that are able to form biofilms. Since this is one of the factors that allows it to remain in the lungs and cause the morbidity associated with the disease cystic fibrosis, it is important to decipher these methods allowing for duration (32). This experiment hoped to begin to determine whether *Listeria* may have similar adaptations when faced with oxidative stress derived from the presence of reactive oxygen species. The results of this study showed an increase in the concentration of cyclic-di-GMP within the cell in response to the addition of hydrogen peroxide to aerobic cultures. This suggests that there is a differential expression of virulence factors induced by increasing the concentration of cyclic-di-GMP in response to oxidative stress. Since higher concentrations of this second messenger molecule have been linked to biofilm formation in previous studies, it is likely that the addition of hydrogen peroxide had a similar effect. This formation of biofilms would increase bacterial resistance in *L. monocytogenes* to reactive oxygen species such as hydrogen peroxide, though the exact mechanisms involved in the impact of reactive oxygen species require further research to interpret (11, 32). Future research would also benefit from exploring whether similar results are

observed in anaerobic conditions as observed within the gastrointestinal tract that *Listeria* encounters during its infectious cycle.

Ultimately, the outcomes of this research provide evidence to substantiate the idea that oxygen availability is a crucial environmental signal controlling expression of diguanylate cyclases and phosphodiesterases and consequently concentrations of cyclic-di-GMP, resulting in the modification of virulence factors of *L. monocytogenes*. Results also corroborate the concept that reactive oxygen species play a role in the regulation of amounts of cyclic-di-GMP within the cell, with the presence of hydrogen peroxide driving the elevation of concentrations. It will be important to determine if this also results in an increase in biofilm production. These findings will likely prove to be useful as the body of knowledge regarding this food-borne pathogen grows in size, substance, and depth. However, supplemental investigation into the exact mechanisms of the cyclic-di-GMP signaling pathway must be performed in order to formulate a clearer schematic involved in *Listeria*'s virulence. Since many stressors, such as lack of oxygen and presence of reactive oxygen species investigated here, affect the intracellular survival and therefore mortality rate associated with this disease, these discoveries can also be utilized in the future to develop more efficient treatments. The application of these results to further experimentation in the field will likely aid in the production of novel medicinal methods of battling this dangerous infectious agent.

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