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Cyclic-di-GMP Regulates Salmonella Typhimurium Infection of Epithelial Cells and Macrophages

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by

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ABSTRACT

Cyclic-di-GMP Regulates *Salmonella* Typhimurium Infection of Epithelial Cells and Macrophages

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Regulation of the bacterial second messenger cyclic-di-GMP in *Salmonella* Typhimurium allows it to delicately alter phenotypes to optimize invasion and survive intracellularly in epithelial cells and macrophages to become virulent and cause infection. Cyclic-di-GMP concentration is regulated by the presence of external stimuli, sensory diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), and cyclic-di-GMP binding effectors. Previous studies established that maintenance of low cyclic-di-GMP concentrations is required for survival in macrophages, and that deletion of 3 active PDEs reduces this survival. Here I showed that these 3 PDEs also influenced the infection of epithelial cells. Further studies re-established the decreased survival in an immortalized macrophage cell line and determined that cyclic-di-GMP-binding cellulose synthase BcsA was responsible for the decreased survival in macrophages. Finally, I also identified an active DGC whose deletion within the 3xKO restores survival levels, suggesting that this enzyme is responsible for the synthesis of cyclic-di-GMP during macrophage infection.

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CHAPTER 1. INTRODUCTION

Food Poisoning

Food poisoning is among the leading cause of illness, disease outbreaks, and death worldwide. Ingestion of food or drink contaminated with microorganisms including bacteria, fungi, parasites, viruses, and some toxic substances are responsible for this illness. The Centers for Disease Control and Prevention (CDC) reports that 1 in every 6 people in the United States experiences a form of food poisoning, resulting in about 48 million cases, 128,000 hospitalizations, and 3,000 deaths each year.[1] The majority of food poisoning is caused by bacteria, among which includes the family of pathogenic *Salmonella.* Drug-resistant *Salmonella* are currently regarded as a serious threat by the CDC, and CDC's 2019 antibiotic resistance threats report shows a current resistance of 16% and 74% to the essential antibiotic used in treating nontyphoidal and typhoidal *Salmonella* respectively.[2] This study aims at understanding a key pathway that contributes to the survival and pathogenesis of these bacteria so that future studies may target this pathway as an alternate treatment for infection caused by *Salmonella* and other related bacteria.

Salmonella

Salmonella enterica is a rod-shaped, Gram-negative bacterium that causes salmonellosis, a common form of food poisoning. While over 2500 serotypes of *Salmonella* have been identified, fewer than 100 have been demonstrated to cause human infections and 32 amongst these – including the most prevalent, *Salmonella enterica* serovar Typhimurium – are extensively shown to be responsible for causing food poisoning-related symptoms.[1] *Salmonella enterica* is a common pathogen of poultry, farm animals, and humans. As a foodborne pathogen, it seems almost inevitable as it can be detected in many foods, especially raw animal products, fruits, vegetables, and the water we take every day. It causes

infections ranging from gastroenteritis characterized by nausea and diarrhea to acute typhoid fever, chronic, life-threatening immune system disorders and cancer.[3; 4]

Salmonella Typhimurium infections typically arise following ingestion of bacteria, where they then invade the epithelial cells that make up the gastrointestinal tract resulting in gastroenteritis. Most cases are limited to gastrointestinal involvement before the bacteria is passed back into the environment. In particularly dangerous cases, the bacteria can become invasive by spreading throughout the body and taking up residence within phagocytic macrophages.[5; 6] Similar to other microbial pathogens, *Salmonella* Typhimurium regulates different proteins and molecules in response to varying extracellular signals to detect and survive within different environmental constraints, including against the human immune system.[5] The bacteria are then able to evade the killing mechanisms of these phagocytic macrophages and establish a replicative niche causing systemic infection afterwards.[7] We have hypothesized that *Salmonella* Typhimurium has evolved to detect whether it is present within epithelial cells or markedly more dangerous macrophages through the use of sensory regulatory mechanisms. Demystifying these regulatory mechanisms could proffer potential solutions and alternative treatments to the often-occurring infections caused by *Salmonella*.

Cyclic-di-GMP

Cyclic di-guanosine monophosphate, also known as cyclic-di-GMP is a bacterial second messenger, a signaling molecule that regulates the motility and virulence of planktonic free-living cells and their transition to a sessile biofilm.[8; 9] The concentration of cyclic-di-GMP in bacterial cells is regulated in response to external stimuli (first messenger) received by the cells depending on their immediate environment.[10] Environmental conditions including temperature, pH, oxygen level, and nutrients have been studied to be involved in the regulation of cyclic-di-GMP concentration.[11-13] This is accomplished through the

enzymatic activities of cyclic-di-GMP modulating enzymes (CMEs) that sense these external stimuli. Sensory diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) synthesize and degrade cyclic-di-GMP respectively, thereby increasing or decreasing the cyclic-di-GMP concentration as required for the virulence, persistence, and survival of the bacteria (Figure 1).[8] DGCs carry the GGDEF enzymatic domain, while PDEs carry the EAL enzymatic domain. There are also CMEs with both GGDEF and EAL domains i.e., they can carry out the enzymatic activity of both DGCs and PDEs. cyclic-di-GMP molecules subsequently bind to effectors (commonly proteins) to control cell growth and division, activation of enzymes, or induction of cellular structure formation such as flagella or exopolysaccharides. Studies in a range of bacterial species have identified that generally, a high concentration of cyclic-di-GMP is associated with the formation of biofilm and a lower concentration exists in the motile, more virulent state.[14; 15]

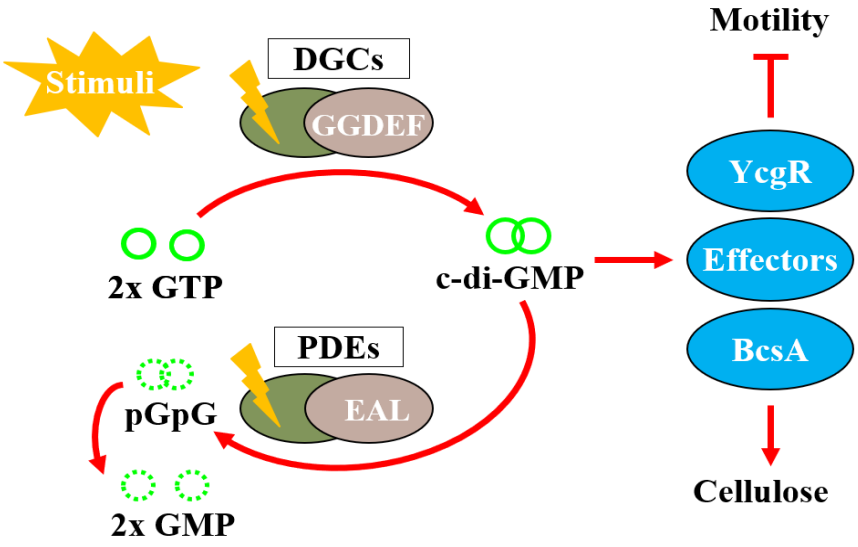

Figure 1: An overview of the cyclic-di-GMP signaling pathway. External stimuli activate sensory domains within DGCs or PDEs to regulate cyclic-di-GMP concentrations. Activation of DGCs induces the formation of cyclic-di-GMP from 2 GTP molecules, while activation of PDEs leads to degradation of cyclic-di-GMP. Synthesized cyclic-di-GMP then binds to effector proteins to stimulate changes in phenotypes including biofilm formation and motility among others.

Cyclic-di-GMP Modulating Enzymes

Salmonella Typhimurium encodes 17 CMEs including 6 DGCs, 9 PDEs, and 2 others with both DGC and PDE enzymatic activity (Figure 2).[11] Some of these CMEs have been studied and identified to be active in regulating cyclic-di-GMP depending on the bacterial cells' environmental constraints. STM1987 is a DGC identified to be very active in rapidly increasing the concentration of cyclic-di-GMP, thereby increasing cyclic-di-GMP-dependent cellulose synthesis in the presence of L-arginine.[11] STM0385 (AdrA) is another DGC shown to activate CsgD-mediated cellulose production and biosynthesis of curli fimbriae by increased synthesis of cyclic-di-GMP in *Salmonella* Typhimurium exhibiting the multicellular behavior red, dry, and rough (rdar) morphotype. STM3388 and STM2123 are

known activators of CsgD expression, while STM1703, STM3611 amongst other PDEs can suppress CsgD expression.[12; 16] STM4551 has been shown to restore survival during desiccation and phenotype requiring fimbriae synthesis – as seen in wild type strain – in the absence of all other DGCs. It is interesting to note that this is independent of its capacity to produce cyclic-di-GMP as cellulose is not synthesized under this condition and is able to confer virulence to the mutant.[17; 18]

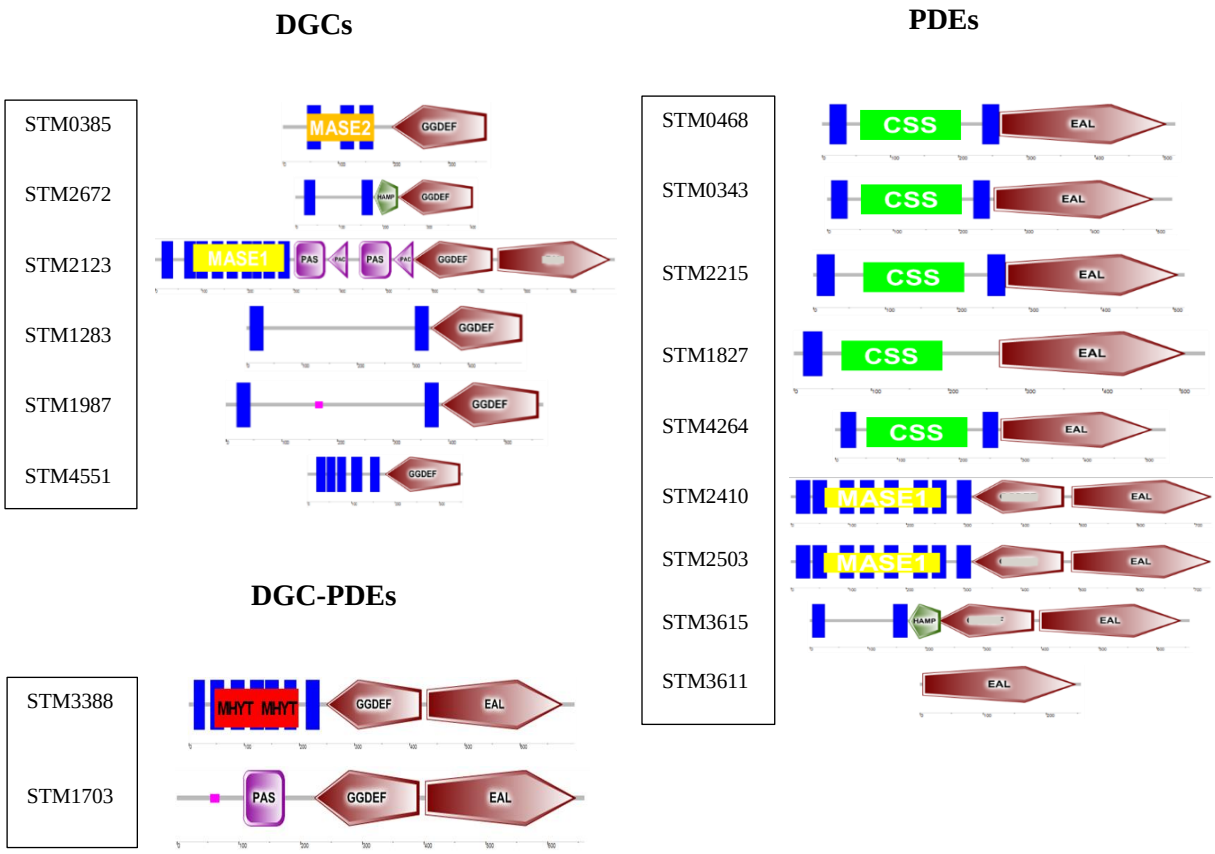

Figure 2: *Salmonella* Typhimurium cyclic-di-GMP metabolizing enzymes. Analysis of the *Salmonella* Typhimurium genome identified 6 DGCs, 9 PDEs, and 2 others with both DGC and PDE enzymatic activity. Many of these encode transmembrane domains (blue bars) to extend within the periplasmic space for sensory purposes. In addition to the enzymatic domains, many also contain predicted sensory and signaling domains to regulate enzymatic activity (HAMP, sensor protein signaling module; MASE1 and MASE2, membrane-associated sensor; MHYT, integral membrane sensory domain with conserved MHYT amino

acid pattern; PAS/PAC, cytoplasmic sensor of oxygen, redox state, or light; CSS motif, PDE-associated sensory domain).[12]

Cyclic-di-GMP Binding Effectors

Salmonella Typhimurium also encodes two well-studied cyclic-di-GMP binding effectors. YcgR is a flagellar brake that binds cyclic-di-GMP when concentrations increase. Cyclic-di-GMP-bound YcgR then binds to the base of flagella, preventing turning and thereby halting motility under high cyclic-di-GMP conditions. BcsA is a cyclic-di-GMP-dependent cellulose synthase that produces cellulose in response to high levels of cyclic-di-GMP. Cellulose is an extracellular polysaccharide necessary for biofilm formation consisting of glucose monomers.[14]

Studies have established that there are multiple CMEs involved in cyclic-di-GMP regulation of *Salmonella* Typhimurium. However, the environments and signals received by these CMEs before regulating cyclic-di-GMP levels are poorly understood. Although cyclic-di-GMP *Salmonella* Typhimurium mutants have been studied to their survival, there is still much to understand about how *Salmonella* uses cyclic-di-GMP to adapt and survive intracellularly, especially in gut epithelial cells and macrophages.[14] Understanding the survival of *Salmonella* as an intracellular pathogen can also serve as a model contributing to better drug development and medical intervention towards combating *Salmonella* and other pathogenic microorganisms.

Cyclic-di-GMP Regulation is Required for Survival in Macrophages

A previous study of cyclic-di-GMP metabolizing enzymes during *Salmonella* infection of macrophages identified three PDEs that were found to be active within host cells. While deletion of any single PDE had no effect on survival within macrophages, deletion of all three reduced survival approximately 10-fold (Figure 3). Mouse infection data suggested

that this reduction in survival was due to the over-production of cellulose related to high cyclic-di-GMP levels, possibly as a consequence of leaving the cell with little glucose to maintain cellular growth and survival.[14] However, further investigation into the signaling mechanisms behind cyclic-di-GMP regulation during intracellular growth is required to understand the complex pathways involved in this phenotype.

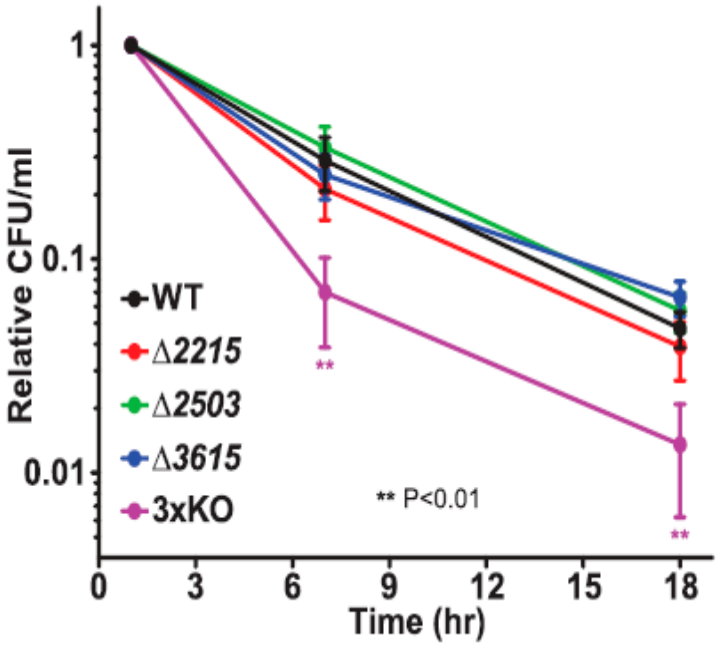

Figure 3: Three PDEs maintain low cyclic-di-GMP levels intracellularly to permit survival within macrophages. Single deletion mutants of each of the three PDEs (STM2215, STM2503, and STM3615), a triple deletion mutant (3xKO), and wild-type (WT) bacteria were tested for their survival within bone marrow-derived macrophages at 1, 7, and 18 hours post infection (n = 3). Only the triple PDE mutant (3xKO) exhibited reduced survival, suggesting that high cyclic-di-GMP levels caused by deletion of these PDEs is detrimental to intracellular macrophage survival. Statistical significance between WT and each strain was determined using paired two-way ANOVA on the log of the inoculum-adjusted bacterial CFU at each time point.[14]

Hypothesis

The main aim of this research is to further investigate the regulation and role of the cyclic-di-GMP pathway in the survival of the pathogen *Salmonella* Typhimurium during intracellular infection of gut epithelial cells and macrophages. To understand the regulation of cyclic-di-GMP in *Salmonella* Typhimurium, it is important to know the CMEs that are actively contributing to its synthesis and degradation during intracellular infection. I hypothesized that *Salmonella* Typhimurium detects its presence within host cells through sensory enzymes that regulate the second messenger cyclic-di-GMP, and that this detection is required for intracellular survival.

Continuing with the established studies that identified 3 active PDEs necessary for intracellular macrophage survival, I first sought to determine whether these same PDEs were required for survival in gastrointestinal epithelial cells and an immortalized laboratory macrophage cell line, and whether the known effectors were responsible for any change in phenotype. I then aimed to identify the DGC(s) responsible for synthesizing cyclic-di-GMP during macrophage infection contributing to the decreased survival in a mutant without the 3 PDEs (3xKO)[14]. Ultimately, this work can further investigations as to how these sensory proteins and pathways can be manipulated to combat *Salmonella* and other related intracellular pathogens.

CHAPTER 2. MATERIALS AND METHODS

Bacterial Strains: Growth and Storage Conditions

All bacterial strains used at different stages in this project were typically grown in 2 to 3 mL of Luria-Bertani (LB) broth with antibiotics (if applicable) overnight (16 – 24 hours) in a shaker at 250 rpm at 37^{o}C. Strains grown on LB agar with antibiotics (if applicable) are usually incubated at 37^{o}C overnight or at 25^{o}C for 36 – 48 hours for counting purposes. The LB constituted 10g/L Fisher BioReagents™ tryptone, 5g/L Fisher BioReagents™ yeast extract, 10g/L Fisher BioReagents™ NaCl, and 15g/L Fisher BioReagents™ bacteriological agar (for agar plates). Media are autoclaved and allowed to cool before adding appropriate antibiotics – ampicillin 100μg/mL; kanamycin 50μg/mL; gentamycin 30μg/mL; and/or chloramphenicol 34μg/mL. Bacterial stocks with appropriate antibiotics were stored by mixing an overnight LB culture with 40% glycerol at 1:1, then freezing at -80^{o}C. Cultures on agar plates were wrapped with parafilm and stored at -4^{o}C.

Generation of Deletion and Site Directed Mutants

The triple PDE deletion strain (3xKO) was previously generated in the *Salmonella* Typhimurium 14028s background. A variant of this strain with two clean deletions and a single remaining kanamycin resistance marker (2215::null, 2503::null, 3615::Kan) was used for construction of the DGC mutations within this 3xKO strain. Gene deletions of each DGC were previously constructed containing chloramphenicol resistance cassettes.[11; 19] These gene deletions were transferred into the 3xKO strain using P22 phage transduction as described elsewhere.[11; 20] In brief, the chloramphenicol-resistant DGC mutants were incubated with P22 mutant phage to acquire the genomic fragment containing the DGC deletion. This phage preparation was used to transduce the DGC mutation into the 3xKO background by selecting

for chloramphenicol resistance. After this strain was cleared of any remaining phage through selective passaging, PCR was used to confirm the presence of the DGC mutation.

STM1703 and STM3388 GGDEF Site Directed Mutagenesis

Two *Salmonella* Typhimurium cyclic-di-GMP metabolizing enzymes (STM1703 and STM3388) contain both predicted DGC and PDE activity. As full gene deletion of this protein would also delete any potential PDE activity, we instead sought to generate a site-directed mutant of the DGC active site within the genomic locus while leaving the PDE activity intact. A suicide plasmid containing the desired mutated DGC sequence (conversion of the active site GGDEF residues to GGAAA) was generated in *E. coli* strain MC1061. This vector contains chloramphenicol for selection of initial integration and the SacB counter selection marker to eventually select for bacteria that have lost the vector sequence. Upon recombination of the vector out of the genome, in 50% of cases the mutated sequence will be left behind, and the presence of the mutated sequence was confirmed by sequencing a PCR product spanning the DGC active site (sequencing conducted at Genewiz Inc.) (Figure 4).

DNA fragments consisting of the coding sequence of STM1703 and STM3388 were amplified using Phusion DNA polymerase from *Salmonella* Typhimurium genomic DNA and the recipe below. This coding sequence was cloned into the pGP704Cam/SacB vector using Gibson cloning,[21] and the fidelity of the sequence was confirmed by sequencing. A second round of PCR using primers containing mutated residues at the GGDEF active site was used to introduce that mutation into the plasmid sequence, which was again confirmed by sequencing. In both cases, plasmid DNA was prepared from 3ml LB+Cam culture using IBI Scientific High-Speed Plasmid Mini Kit according to the manufacturer's protocol and then sent out for sequencing to confirm the appropriate sequence.

Table 1. Components of Phusion DNA polymerase PCR

Component	
5x HF buffer	5ul
dNTPs	1ul
Phusion	0.25
Primer 1 (rev)	1ul
Primer 2 (fwd)	1ul
DNA template	0.5ul
DNAse free water	16.25ul
Total	25ul

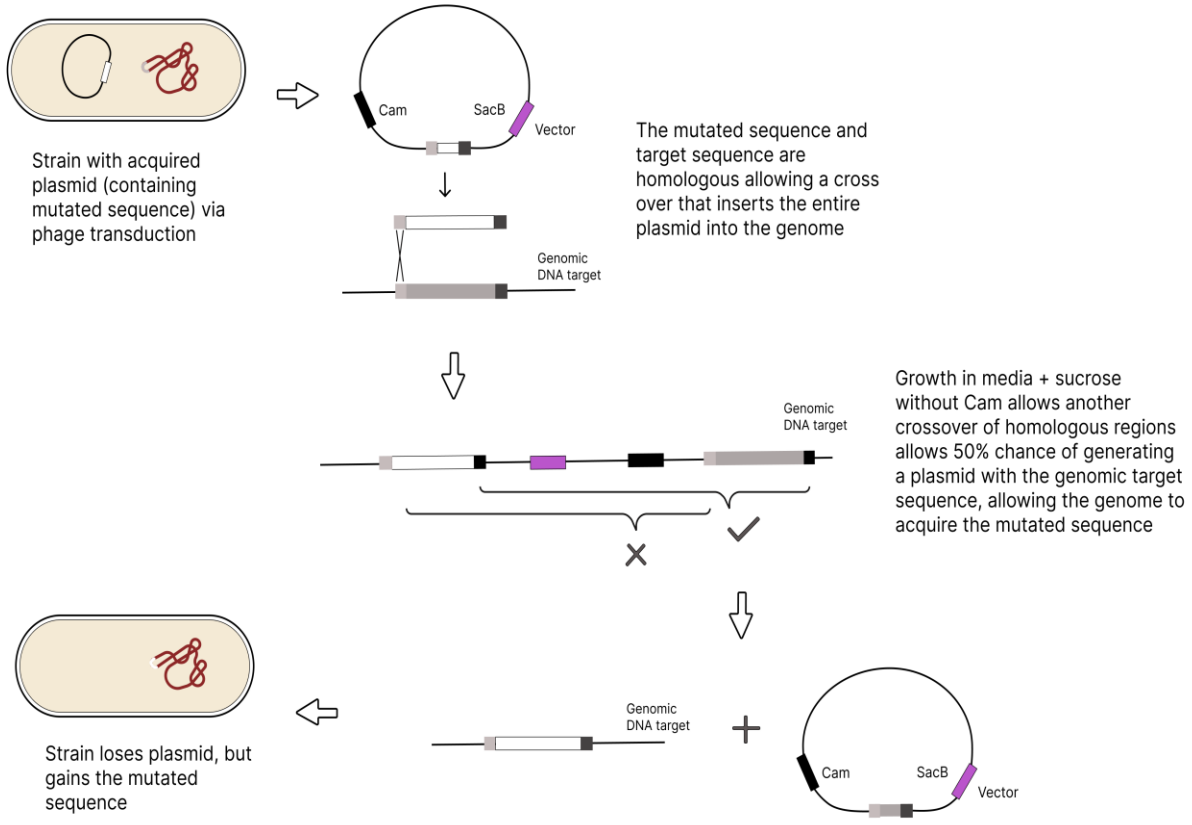

Figure 4: Generation of STM1703 and STM3388 GGDEF site-directed mutants via plasmid recombination. Suicide plasmid containing the site-directed GGDEF mutant of either STM1703 or STM3388 was allowed to recombine into the *Salmonella* Typhimurium genome. Subsequent removal of chloramphenicol and selection with sucrose permits isolation of bacteria that have successfully lost the integrated vector. In 50% of these cases, the mutated active site sequence is left behind, which can be determined by sequencing.

Conjugation

The chemically transformed MC1061 containing the confirmed mutated plasmid serves as a donor strain and was conjugated with a GentR *Salmonella* strain using the KanR helper strain pRK2013. The confirmed plasmid was then allowed to integrate into the genome of the *Salmonella* strain at the DGC site; this forms a CamR/GentR *Salmonella* strain. This strain was then colony PCR'd using the recipe below to confirm the presence of the integrated plasmid (with desired mutation).

Table 2. Components of *Taq* DNA polymerase PCR

Component	
2x Taq MM	12.5ul
Primer 1 (rev)	1ul
Primer 2 (fwd)	1ul
DNA template*	0.5ul
DNAse free water	10ul
Total	25ul
*DNA template was a colony of bacteria boiled in 50ul of DNAse free water	

P22 Phage Transduction

Because loss of the vector sequence would also lose the antibiotic resistance marker, the integrated vector must be moved into our desired strains before removal of the vector within that strain. The plasmid in the CamR/GentR *Salmonella* strain (donor) can be transferred to other *Salmonella* strains (recipients) using phage transduction.[11; 20] P22-HTInt phage was used to transfer copies of the integrated plasmid from the donor strain into new recipients, KanR *Salmonella* strains (3xKO mutants). The resulting CamR/KanR strains from the completion of the phage transduction protocol were then colony PCR'd to confirm the presence of the integrated plasmid.

Plasmid Recombination

The KanR 3xPDE mutants now have the mutated GGDEF sequence (inactivated DGC) as an integrated plasmid with a Cam/SacB backbone.[22; 23] This strain was grown in the absence of chloramphenicol to permit the integrated vector to recombine out of the genomic DNA, thus allowing the mutated sequence from the plasmid to remain in the genome in half of the cases. The cultures were then streaked onto 6% sucrose plates and incubated at 30ºC for 48 hours to allow selection of single colonies. This preferentially permits survival of bacteria that have lost the *sacB* gene within the vector backbone, as SacB will convert sucrose to a toxic metabolic byproduct.[24] These colonies were then patch-plated between LB+Kan and LB+Cam plates with grids, incubated for 24 hours at 37ºC to select from KanR/CamS colonies. The CamS colonies were then colony PCR'd for a fragment containing the DGC active site and sequenced to confirm the generation of the site-directed mutants of STM1703 or STM3388 within the 3xKO background.

Generation of STM0385 Expression Vectors

An expression vector for STM0385 was constructed to permit complementation of the STM0385 deletion. Primers were designed to generate a fragment containing the STM385 coding sequence and 270 base pairs upstream of the start codon to include the native promoter. A second set of primers was designed to introduce a mutated active site (SDM) within the STM0385 coding sequence. The STM0385 fragment was amplified from *Salmonella* genomic DNA template using Phusion PCR. The size of the fragments was confirmed by agarose gel electrophoresis before subsequent use. The STM0385 coding sequence/promoter fragment was then cloned into the pMMB67EH (pMMBA) vector using Gibson assembly. After construction of the pMMBA-STM0385 expression vector and confirmation of the gene integrity by sequencing, a second set of PCRs was generated using

the active site mutation primers to introduce that genetic mutation into the STM0385 sequence (STM0385SDM).

Both STM0385 and STM0385SDM vectors were constructed in a similar manner. In brief, the fragment of STM0385 generated from the genomic DNA template or the mutated fragments generated from the pMMBA-STM0385 vector were proportionately mixed with pre-purified pMMBA fragments and Gibson assembly master mix and incubated in the thermocycler at 50ºC for 60 minutes.[21] Plasmids generated from the Gibson assembly were then transformed into chemically competent cells derived from *E. coli* – DH5α.[25]

After transformation, the culture was spread with beads on LB+Amp plates and grown overnight to select for DH5α colonies containing p385comp and p385SDM. Selected colonies from LB+Amp plates were colony PCR'd for confirmation of the presence of the plasmids in the cells, after which they were grown in 4ml LB+Amp for freezing, plasmid miniprep, and sequencing for confirmation.

Electroporation

After confirmation of the intended sequence, p385comp and p385SDM derived from minipreps were electroporated into the WT, 3xKO, and 3xKO/Δ385 strains.[26] Overnight cultures of strains to be electroporated were subcultured at 1:100 in LB for 2 hours to achieve growth of 0.5 OD600. Cultures were centrifuged in 1.5 mL tubes and washed thrice with 750 µL of chilled DNAse free water to desalt cell pellets. The pellets were resuspended in DNAse free water at a volume of 100 µL/ sample and aliquoted into 1.5 mL tubes. Each electroporation sample contains 95 – 97 µL of cells and 3 – 5 µL of plasmid DNA. Samples were electroporated at 1800v, diluted in 750 µL of SOC, transferred into 5 mL tubes and incubated at 37ºC for 2 hours in a shaker. After incubation, 150 µL of the culture was spread with beads on LB+Amp plates and grown overnight for strains containing p385comp and

p385SDM. Selected colonies from LB+Amp plates are grown in 4mL LB+Amp for freezing and PCR for confirmation of the presence of the plasmid. For strains with little or no electroporation success, triparental mating with KanR helper strain pRK2013 was employed in generating a CamR/AmpR strain as the recipients are already chloramphenicol resistant.

Cell Culture

The general cell culture guidelines used in this study are as described in the ThermoFisher or ATCC product and safety data sheets.[27-29] These protocols were carried out under proper aseptic technique in a class II biosafety cabinet with disinfection of surfaces and materials with 70% ethanol before use. All incubation of cell lines was conducted in a CO_2 incubator at 37^{o}C, 5% CO_2, and 95% humidity.

Media Preparation

BioWhittaker RPMI-1640 was the main ingredient in the cell culture media used throughout this study. The cell culture media constituting 500mL RPMI was supplemented with 50mL 10% Cytiva HyClone Cosmic calf serum and 5mL BioWhittaker L-Glutamine. 5mL BioWhittaker Penicillin-Streptomycin was also added to the media to prevent bacterial contamination only during passaging and freezing; it was not included for media used in cell infections. The complete RPMI was then labelled with all constituents, date of preparation, and stored at 4^{o}C for subsequent use. Freezing media for storage of these cell lines in liquid nitrogen was prepared by adding 5% DMSO in complete RPMI.

Thawing Frozen/ Received cells

HeLa and RAW 264.7 cell lines used in this study were acquired directly from ATCC. Upon receipt, cell vials were thawed in a 37^{o}C water bath and sprayed with 70% ethanol before transferring into the hood. The contents of the cell vials were emptied into 9 mL of warmed complete RPMI (with penicillin-streptomycin) in a 15 mL tube and centrifuged at

125g for 5 mins to remove any cryoprotective agent present. The supernatant was removed, and the pellet was resuspended in 10 mL of complete RPMI (with pen-strep). The entire volume was transferred into a T75 flask and incubated at 37^{o}C in a 5% CO_2 incubator.

Standard Cell Passaging

Immortalized cell lines cultured in T75 flasks were observed under a light microscope to determine confluency and when to passage. Cells were allowed to reach a confluency of 70-80% before passing. To passage cells, the media was removed from the flask, and the growth surface was washed twice with 10mL BioWhittaker 1X Phosphate Buffered Saline (PBS). 2mL of BioWhittaker Trypsin-Versene mixture was added and incubated for 5 mins at 37°C to lift the cells. 10–20mL of PBS was used to dislodge the cells by repeated pipetting, and the flask was then emptied into a 15 or 50mL tube and centrifuged at 200g for 5-10 mins. The supernatant was discarded, and pellet was resuspended in 10mL of culture media. Cells were passaged at 1:5 and 1:10 dilution. For 1:10 dilutions, 1mL of the resuspended cells was transferred into a new T75 flask containing 9mL of the complete RPMI, while 2mL of cells and 8 mL of complete RPMI was used for 1:5 dilutions. The new flask was labelled with the cell type, passage number, and date, then incubated at 37^{o}C in a 5% CO_2 incubator.

Freezing Cells

Following the same procedures for standard cell passaging; after cell lifting and centrifuging, pellets were resuspended in 1mL freezing media per T75 flask used in obtaining the cells. Resuspended cells were transferred into 1.5mL cryovials with appropriate labels. Cryovials were then placed in an isopropanol freezing chamber overnight at -80^{o}C, after which they were transferred into liquid nitrogen storage.

Cell Infections with *Salmonella* Typhimurium

Cells were lifted from their culture dishes, counted on a hemocytometer using Trypan Blue staining, and diluted in RPMI (without pen-strep) to a concentration of 1e5 cells/mL before transferring 1mL into 24-well CELLSTAR tissue culture-treated plates (Greiner Bio-One). The bacterial strains to be used for cell infections were grown in LB (with antibiotics if plasmids were present) and incubated overnight in a shaker at 250 rpm at 37ºC. The 24-well culture plates were labelled with each bacterial strain to be used with corresponding time points. Each bacterial strain gets 6 wells; duplicates for each time point studied (1-, 4- and 24-hours post-infection) (Figure 5).

Bacterial cultures were diluted and added to cell cultures at a multiplicity of infection (MOI) of 10 i.e., 1e6 bacterial cells:1e5 cells. Overnight bacterial cultures were used for RAW macrophage infections. Unlike RAW macrophages that can engulf bacterial cells, HeLa epithelial cells require bacterial cells at log-phase with active Type III secretion system. For this, overnight bacterial cultures were subcultured at 1:100 and incubated for 2 hours before used in infection. Bacterial cultures were measured and diluted in LB to 0.5 OD600. 16 ul of each 0.5 OD600 bacterial culture was added to 4 mL of complete RPMI to make an infection culture (approximately 2e6 CFU/mL) to generate the 10MOI infection culture. This infection culture was quantified for actual CFU/ml concentration to ensure the starting infection dose was known. The complete RPMI in the overnight 24-well plates were replaced with the infection culture – 0.5 mL (1e6 CFU) per well, centrifuged at 500g for 5 minutes to enhance cell contact, and incubated for 25 minutes at 37ºC in a 5% CO_2 incubator.

After incubation, the infection culture was replaced with 1mL complete RPMI+Gent^{50} and returned into the incubator for 30 mins (1-hour p.i.). Complete RPMI+Gent^{50} kills any bacteria not yet internalized by the cells. At 1-hour p.i., the complete RPMI+Gent^{50} in the

1-hour wells was removed, wells were washed twice with 0.75 mL BioWhittaker 1X Phosphate Buffered Saline (PBS), and cells were lysed with 0.5 mL 0.1% Triton X-100 in 1xPBS. This process was repeated at 4- and 24-hour p.i. The Triton X-100 solution was used to lyse the infected cells in the wells by repeated pipetting, and the lysates were used in the enumeration of the bacterial CFU.[14; 30]

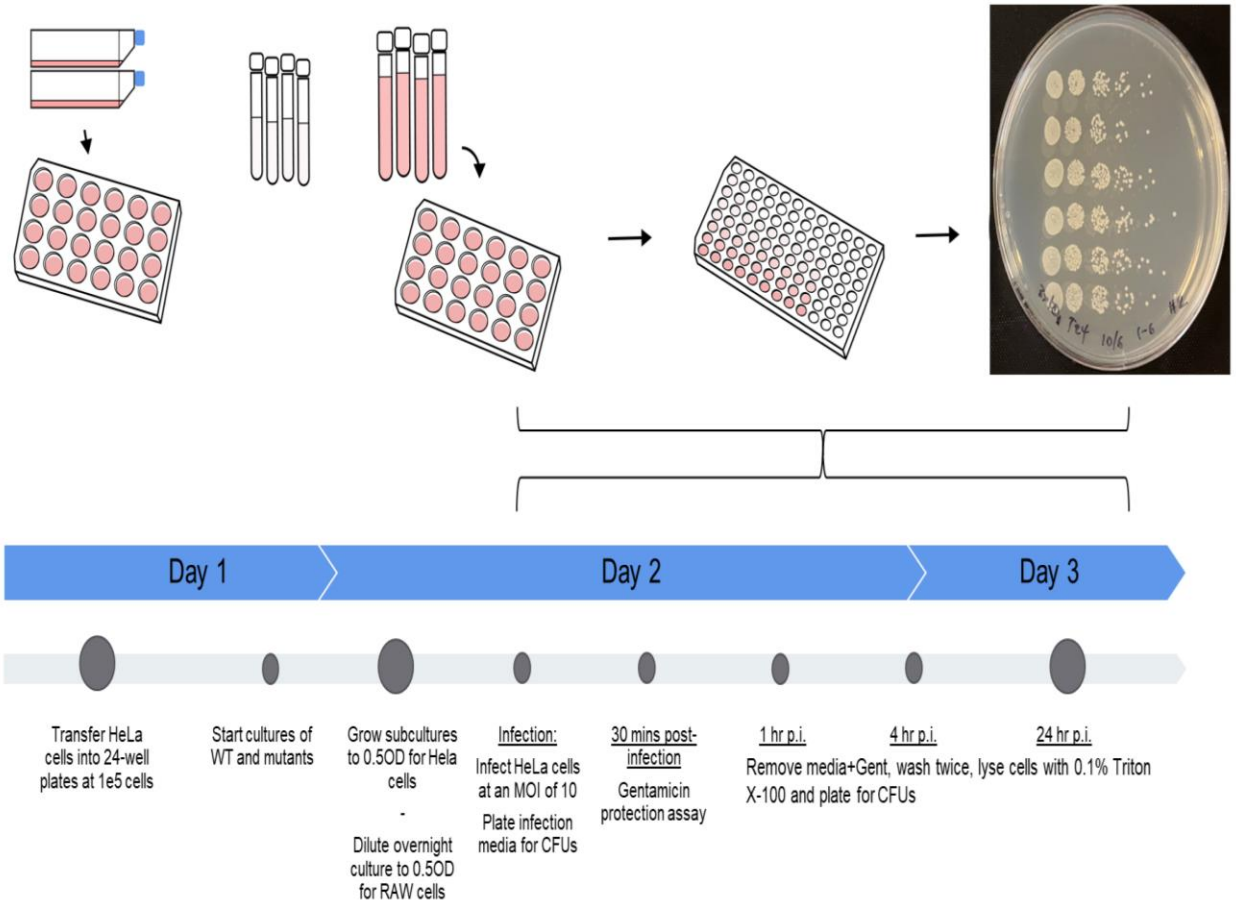

Figure 5. Infection assay for determining *Salmonella* Typhimurium intracellular survival. Cells were seeded at a concentration of 1e5 and infected at an MOI of 10 with either stationary phase (RAW macrophages) or log phase (HeLa epithelial cells) *Salmonella* Typhimurium cultures. *Salmonella* infection cultures were quantified to determine the

CFU/ml. After gentamycin killing of non-internalized bacteria and at the indicated time points, infected cells were lysed to release intracellular bacteria for quantification.

To determine the intracellular invasion and survival of the bacterial strains used, the infection culture and lysates at different time points were diluted at 1:5 in 96-well plates, plated 6x6 on LB plates, and incubated at 37^{o}C until colonies are countable (Figure 6). To dilute samples, 200 μL of each sample (infection culture or lysates from 1, 4, 24 hr p.i.) was transferred into column 1 in 96-well plates. Other columns (2-8) were filled with 160 μL of 1xPBS. Then, 40 μL from column 1 was transferred to column 2 with repeated pipetting to ensure homogeneity, then 2 to 3 in the same procedure till column 8. To plate samples, multi-channel pipette was used in transferring 7 μL of samples in dilution 3-8 unto LB plates in 6 replicates (6x6). Dilution 1-6 was transferred onto LB plates for the time points lysates. Plates were allowed to soak the spotted samples into the agar and incubated at 25^{o}C overnight to slow colony growth till individual spots are visible.

After incubation, dilution spots with up to 100 colonies in total were counted and used in calculating bacterial CFU/ml for both the infection and lysate samples. CFUs depicting intracellular invasion and survival were used to construct graphs and check for statistical significance using GraphPad prism v9. In order to properly examine the full time course of infection, the area under the curve (AUC) of the line generated from the 1, 4, and 24 hour time points was also determined. These values from separate experiments These data were analyzed by one-way ANOVA against the indicated strain to monitor changes within infection time course assays.

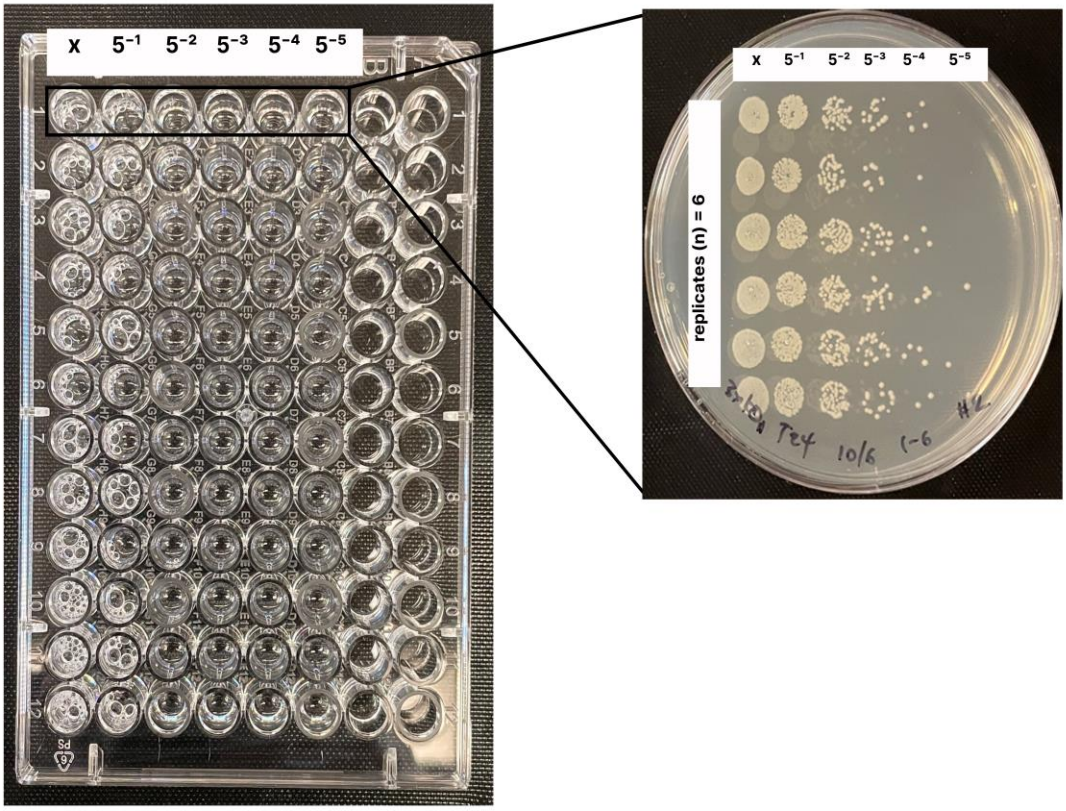

Figure 6. Bacterial enumeration using 96-well plates for 5-fold serial dilution and 6x6 drop plate method. Either the infection culture or time point lysates of infected cells were transferred into column 1 of a 96-well plate and serially diluted 5-fold in 1X PBS. 7 μL from six consecutive dilutions were plated in 6 replicates on an LB plate for quantification after growth at 25°C for 24-36 hours until colonies were appropriately sized.

CHAPTER 3. RESULTS

Cyclic-di-GMP Regulates *Salmonella* Typhimurium Infection of HeLa Epithelial Cells

It was previously shown that three PDEs were required for proper survival of *Salmonella* Typhimurium during intracellular infection of bone marrow-derived macrophages.[14] In contrast to macrophages with cytotoxic capabilities, epithelial cells contain numerous amino acids, carbon compounds, and salts that can serve as nutrient for the proliferation of any internalized organism – such as proliferation of *Salmonella* Typhimurium in the gut to cause gastroenteritis. The first aim of this study was to understand the effect of cyclic-di-GMP and reliance of cyclic-di-GMP effectors during epithelial cell infection. Wild type *Salmonella* Typhimurium (WT), the triple PDE deletion strain (3xKO), and the triple PDE deletion strain additionally deleted for the two cyclic-di-GMP-binding effectors (3xKO/Δ*bcsA* and 3xKO/Δ*ycgR*) were used for HeLa cell infection. The intracellular CFU of each strain from lysates at 1-, 4- and 24-hour time point were analyzed, normalized to the CFU of the infection culture, and compared to WT using two-way ANOVA with Dunnett's post hoc test (Figure 7A). The area under the curve (AUC) from each infection trial was quantified to convert the time course into a single value. These data points were analyzed against WT using one-way ANOVA with Dunnett's post hoc test (Figure 7B).

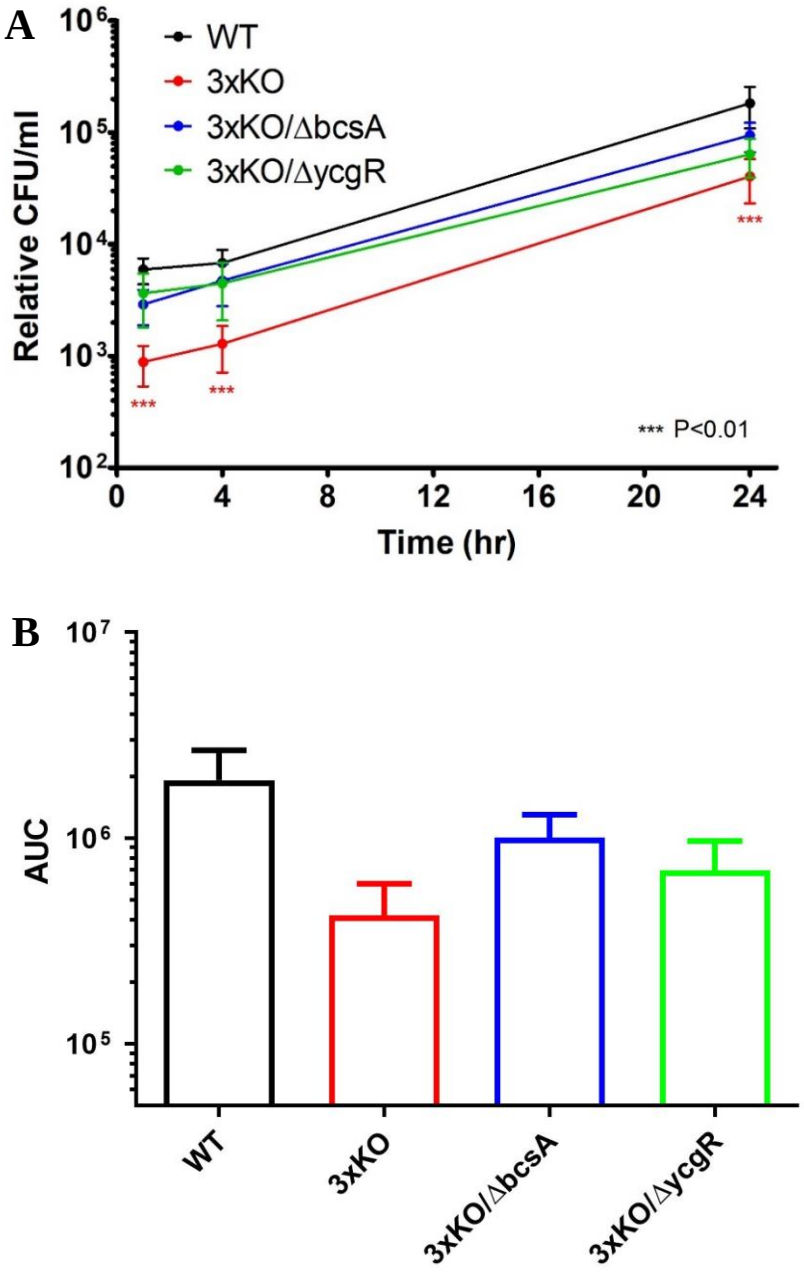

Figure 7. Cyclic-di-GMP regulates infection of *Salmonella* Typhimurium of HeLa epithelial cells. (A) Intracellular survival of WT, triple PDE deletion (3xKO), and deletion of effectors with 3xKO (3xKO/Δ*bcsA* and 3xKO/Δ*ycgR*) at 1, 4, 24 hours post infection was quantified within HeLa epithelial cells (n=5, ± = SEM). Statistical significance between WT and each strain was determined using paired two-way ANOVA on the log of the inoculum adjusted bacterial CFU at each time point. (B) Area under the curve (AUC) was used to depict the overall survival of each strain over the entire period of internalization (n = 5, ± = SEM).

Significance was determined using one-way ANOVA with Dunnett's post hoc test on the mean AUC of each strain.

Similar to the previous results from bone marrow-derived macrophage infections, data shows that the triple PDE deletion (3xKO) has a significant reduction in number of bacteria within epithelial cells at all time points. Deletion of both YcgR and BcsA effectors within the 3xKO strain partially restored these levels, but not as high as that of the WT. High cyclic-di-GMP levels cause YcgR to bind cyclic-di-GMP and halt flagellar motility, so deletion of YcgR is predicted to restore motility to this strain. Similarly, high cyclic-di-GMP levels bind BcsA and result in production of cellulose, so deletion of BcsA results in no cellulose formation. This possibly suggests that both cellulose formation and motility reduction in the 3xKO strain cause a decrease in epithelial cell infection.

Salmonella Typhimurium Requires Low Cyclic-di-GMP To Prevent Cellulose Over-Production and Reduced Survival in RAW 264.7 Macrophages

Previously published data identified 3 active PDEs maintaining the survival of *Salmonella* in bone marrow-derived macrophages.[14] In contrast with bone marrow-derived macrophages used in the previous study, a less cytotoxic but immortalized mouse macrophage cell line - RAW 264.7 - was used in this study. I first sought to re-confirm the phenotype of the WT *Salmonella* Typhimurium and the 3xKO mutant within this alternative cell line. Similar to the results seen for bone marrow-derived macrophages (Figure 3), deletion of the three identified PDEs (3xKO) significantly reduces survival within RAW 264.7 macrophages (Figure 8).

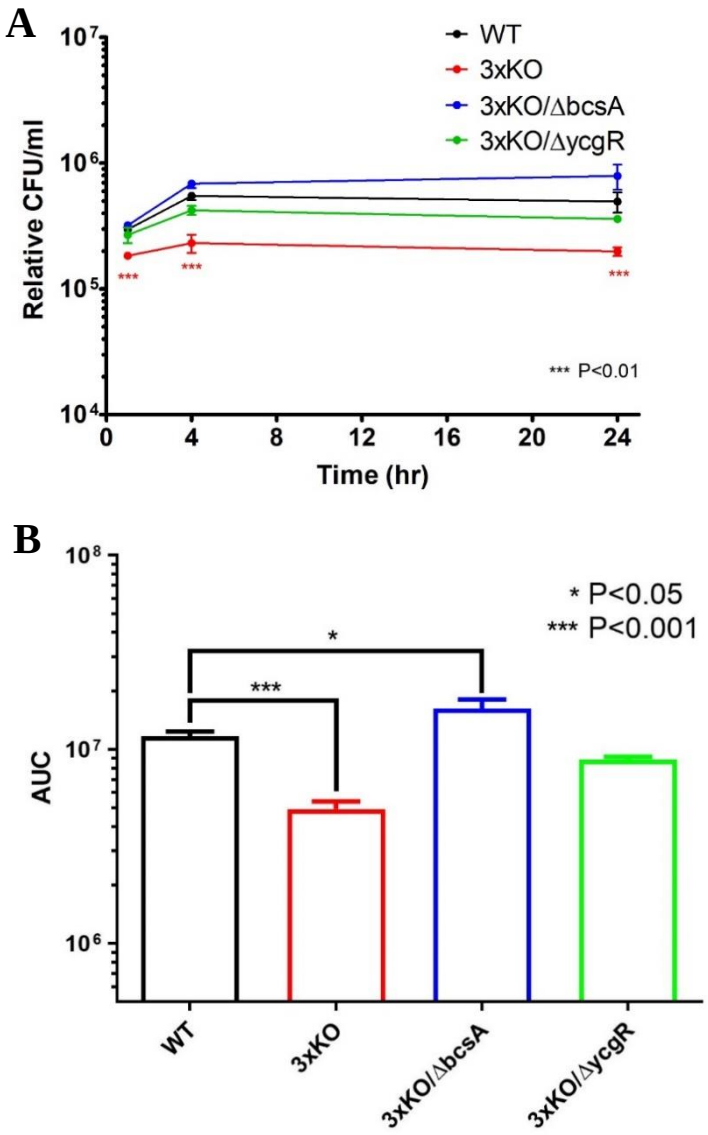

Figure 8. Three PDEs lower cyclic-di-GMP levels to maintain survival within RAW 264.7 macrophages in a BcsA-dependent manner. (A) Intracellular survival of WT, triple PDE deletion (3xKO), and deletion of effectors with 3xKO (3xKO/Δ*bcsA* and 3xKO/Δ*ycgR*) at 1, 4, 24 hours post infection was quantified with RAW 264.7 mouse macrophages (n = 6, ± = SEM). Statistical significance between WT and each strain was determined using paired two-way ANOVA on the log of the inoculum adjusted bacterial CFU at each time point. (B) Area under the curve was used to depict the overall survival of each strain over the entire period of internalization (n = 6, ± = SEM). Significance was determined using one-way ANOVA with Dunnett's post hoc test on the mean AUC of each strain.

Previous findings during mouse infections also found that deletion of these three PDEs resulted in reduced virulence within a mouse model, and that this phenotype could be reversed by deletion of the cyclic-di-GMP responsive cellulose synthase BcsA.[14] This previous data suggested that over-production of cellulose caused by high cyclic-di-GMP levels in the 3xKO mutant reduces virulence in mice, but it was unknown whether this had an effect on macrophage survival directly. To test whether cellulose synthesis or cyclic-di-GMP-induced reduction in motility were responsible for this phenotype, I infected RAW macrophages with mutants in these effectors within the 3xKO mutant – 3xKO/Δ*bcsA* and 3xKO/Δ*ycgR* (Figure 8). While deletion of YcgR in the 3xKO background partially restored survival within RAW macrophages, deletion of BcsA completely returned the 3xKO phenotype to that of wild type levels. This suggests that reduction in motility may play a small role in macrophage survival, but that cellulose over-production in the high cyclic-di-GMP 3xKO strain is the primary cause of reduced survival in RAW macrophages.

The DGC STM0385 is Required for Reduced Macrophage Survival in the Triple PDE Deletion Strain

The next aim was to identify the active DGC(s) responsible for synthesizing cyclic-di-GMP that activates BcsA within the triple PDE mutant in a macrophage infection. This DGC would be assumed to be active during intracellular infection, also suggesting that it was responding to some sort of signal within this condition. To identify these DGC(s), quadruple mutants – DGC/3xKO were generated by making single deletion of all DGCs, including GGDEFSDM of dual domain CMEs (DGC-PDEs), within the 3xKO mutant.[11] These mutants were then used for RAW macrophage infections, and the intracellular survival was analyzed paired to the 3xKO and wild type (WT) strains. Intracellular survival was analyzed using area under the curve (AUC) of the infection time course, and each mutant was compared to the

3xKO using one-way ANOVA with Dunnett's post hoc test to identify those mutants that showed a reversion towards the WT phenotype (Figure 9).

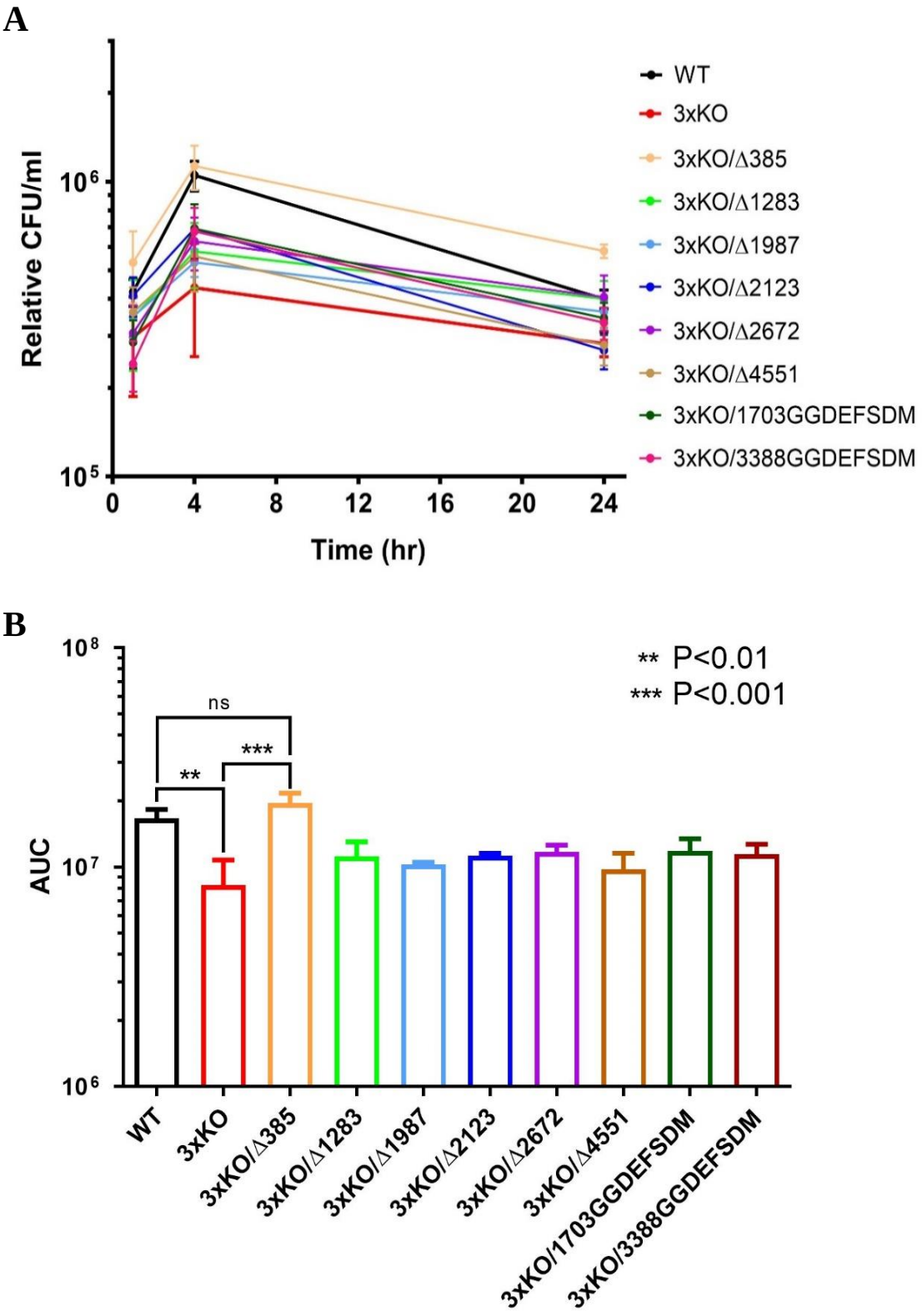

Figure 9. STM0385 is the DGC responsible for reduced survival in the triple PDE deletion strain during macrophage infection. (A) Intracellular survival of WT, triple PDE deletion (3xKO), and quadruple mutants (3xKO/ΔDGC) at 1, 4, 24 hours post infection was quantified within RAW mouse macrophages (n = 3, ± = SEM). Statistical significance

between 3xKO and each strain was determined using paired two-way ANOVA on the log of the inoculum adjusted bacterial CFU at each time point. (B) Area under the curve was used to depict the overall survival of each strain over the entire period of internalization (n = 3, ± = SEM). Significance was determined using one-way ANOVA with Dunnett's post hoc test on the mean AUC of each strain.

The result shows that the deletion of only a single DGC - 3xKO/ΔSTM0385 - had a significant change in survival from that of the 3xKO parent strain. Hence, this suggests that the high levels of cyclic-di-GMP found within the triple PDE mutant is synthesized by the DGC STM0385.[14] This also may indicate that STM0385 plays a key role in the synthesis of cyclic-di-GMP during intracellular survival of *Salmonella* Typhimurium in macrophages, possibly as a result of detecting a particular signal that indicates the bacteria is in an intracellular environment.

To further support the involvement of STM0385 in regulation of cyclic-di-GMP for intracellular survival, a genomic fragment consisting of the STM0385 coding sequence, and its native promoter was amplified from *Salmonella* Typhimurium genomic DNA. These fragments were introduced into a vector - pMMBA - via Gibson cloning. A second vector was then generated in which the active site of STM0385 (GGDEF) was mutated to an enzymatically inactive variant (GGAAA = SDM). Both plasmids were then transferred into WT, triple PDE deletion (3xKO), and the quadruple mutant (3xKO/Δ385) via electroporation. An empty vector pMMBA was also transferred into each strain to serve as a control for observing any changes in the phenotypes previously established in the WT, 3xKO, and 3xKO/Δ385 strains. These complementation vectors were used to infect RAW macrophages to determine their survival (Figure 10).

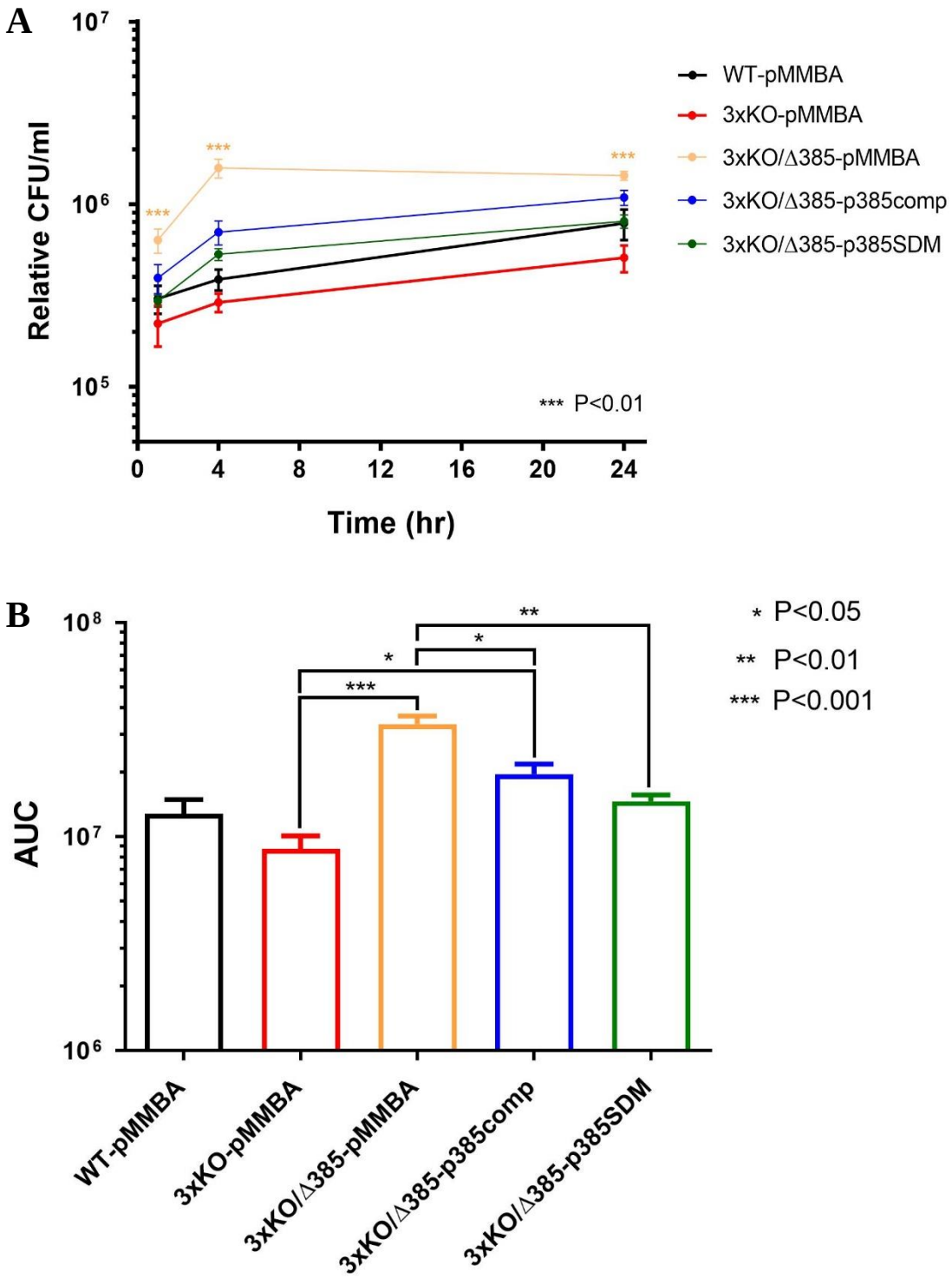

Figure 10: Complementation of STM0385 partially restores the phenotype of the 3xKO mutant. (A) Intracellular survival of WT, triple PDE deletion (3xKO), and quadruple mutants (3xKO/ΔDGC) with either the empty pMMBA vector, 385 complementation vector (p385comp) or the inactive 385 complementation vector (p385SDM) at 1, 4, 24 hours post infection was determined within RAW mouse macrophages (n = 3, ± = SEM). Statistical

significance between 3xKO-pMMBA and each strain was determined using paired two-way ANOVA on the log of the inoculum adjusted bacterial CFU at each time point. (B) Area under the curve was used to depict the overall survival of each strain over the entire period of internalization (n = 3, ± = SEM). Significance was determined using one-way ANOVA with Dunnett's post hoc test on the mean AUC of each strain.

The expression of a functional STM0385 (p385comp) in 3xKO/ΔSTM0385 shows a partial reversion towards the 3xKO phenotype as expected. However, the presence of an inactive STM0385 (p385SDM) also shows a reversion towards the 3xKO phenotype, suggesting that either we have not fully removed enzymatic activity from this mutant, or that enzymatic activity may not be required for this phenotype. There are examples in the literature of DGC proteins possessing phenotypes beyond their enzymatic activity, and further investigation into STM0385 will be required to determine its role in reduced survival in the 3xKO mutant.[17; 18; 31]

CHAPTER 4. DISCUSSION

Cyclic-di-GMP is a widely conserved bacterial second messenger that modulates a wide range of physiological and metabolic characteristics that accounts for the exhibition of varying colonization and survival, acute and chronic virulence, various forms of motility, and of sessility.[32] These characteristics are all dependent on intrinsic and extrinsic factors – host immune system, antimicrobials, temperature, nutrients, pH, active/inactive cyclic-di-GMP modulating enzymes, transcriptional regulators, and cyclic-di-GMP effectors – that regulate the concentration of cyclic-di-GMP within the bacterial cells. Interestingly, all CMEs, despite being classified as either cyclic-di-GMP synthesizing or degrading enzyme, function at different levels dependent on the aforementioned factors. Regulation of a wide range of phenotypes, including motility and biofilm formation, requires different CME(s) to be activated.[12; 14; 33] One crucial impact of cyclic-di-GMP is its contribution towards the pathogenicity and virulence of bacteria. It has been generally established that high concentration of cyclic-di-GMP results in biofilm formation which confers adhesion, surface colonization, tolerance to antimicrobials, and evasion of immune cells and components.[32; 34] On the other hand, low concentration of cyclic-di-GMP is required for motility and occasionally virulence. The capability of pathogenic bacteria to delicately shuffle between being motile and sessile is important to determining its survival and virulence intracellularly. Regardless, both motile and sessile phases at the right time can be harmful to the body.[33; 35]

Our focus in this study was to understand how *Salmonella* Typhimurium uses its cyclic-di-GMP signaling system including DGCs, PDEs, and effectors to regulate cyclic-di-GMP concentrations and optimize its invasion and survival in host cells. 3 PDEs were previously found to be actively required for survival in bone marrow-derived macrophages (Figure 3) and their combined deletion results in a significant decrease in survival compared to the wildtype. Unlike macrophages with phagocytic traits, during infection of the

gastrointestinal tract, *Salmonella* Typhimurium interact and gain access into the epithelial cells via Type III Secretion System (T3SS) interaction.[9] Our result shows that the 3xKO mutant has a significantly lower infection rate in HeLa epithelial cells. The high level of cyclic-di-GMP in the triple PDE deletion could activate both the YcgR flagellar brake and the cellulose synthase BcsA. Deletion of either effector within the 3xKO (3xKO/Δ*bcsA* and 3xKO/Δ*ycgR*) showed a much closer infection level as that of the wild type (Figure 6). From this, it appears that both the absence of motility or presence of extracellular cellulose could affect the interaction with the host cell. As an alternative hypothesis, although T3SS is not regulated by cyclic-di-GMP, a study on gastrointestinal HT-29 cell infection overexpressed AdrA in *Salmonella* Typhimurium to cause over production of cellulose and discovered that this phenotype inhibits invasion by showing decreased cell surface attachment.[9; 36]

As described above, previous work found a requirement for these three identified PDEs during infection of bone marrow-derived macrophages.[14] RAW 264.7 mouse macrophages are an immortalized cell line that is easier to handle within the lab, but exhibits altered killing ability compared to bone marrow-derived macrophages.[37; 38] I was able to confirm that the triple PDE mutant also exhibits reduced survival within RAW macrophages, and I also identified the cyclic-di-GMP-binding effector BcsA as responsible for this reduction in intracellular macrophage survival (Figure 6). One hypothesis to this phenotype is that over-activation of BcsA during macrophage survival produces an excess of cellulose that removes the crucial nutrient glucose from the bacterial cytoplasm, resulting in decreased bacterial survival.[14; 39]

As high cyclic-di-GMP concentrations in the triple mutant appear to activate BcsA and lead to a reduction in macrophage survival, we sought to identify the active DGC accounting for this high cyclic-di-GMP production[14] We identified that the DGC STM0385 was responsible for the reduced survival of the triple PDE deletion (3xKO) in macrophages

and that the deletion of this single DGC within the 3xKO restored intracellular survival to wild type *Salmonella* Typhimurium level. No other single DGC deletion showed this phenotype. We initially considered that this may have suggested STM0385 was particularly adept at producing cyclic-di-GMP during macrophage infections. However, subsequent complementation tests of the 3xKO/STM0385 mutant with both the wild type STM0385 and a variant of STM0385 that was predicted to have lost its enzymatic activity showed that both were able to partially restore the phenotype of the 3xKO mutant. This possibly suggests that STM0385 participates in intracellular signaling in a manner independent of cyclic-di-GMP synthesis, which could explain why no other DGC was found to influence the reduction in survival of the 3xKO strain.

STM0385, also known as AdrA in *Salmonella* Typhimurium and YaiC in *Escherichia coli*, has been found to activate cellulose biosynthesis in vivo, to allow cells to exhibit different morphotype based on the level of its expression, and its expression has also been shown to correlates with increased cyclic-di-GMP levels and decreased motility.[12; 40] Future studies will need to investigate how STM0385 is helping to regulate intracellular cyclic-di-GMP levels, and why STM0385 causes reduced virulence in the 3xKO strain. We may also expand these findings to mouse infections to determine the role of STM0385 during an in vivo study, examine the regulatory interactions and signals that activate STM0385 during intracellular growth, and determine whether STM0385 similarly is responsible for the reduced infection of the triple PDE mutant in HeLa cells indicated here.

In conclusion, our results showing active DGCs, PDEs, and effectors during *Salmonella* Typhimurium's intracellular survival in epithelial cells and macrophages would enable us to take a step further in understanding the importance of the cyclic-di-GMP signaling pathway. This could also lead to the identification of those external stimuli/ first messenger responsible for activating its components. There exist a number of new routes to

continue this work, all of which will hopefully lead to new avenues to combat not just *Salmonella* infections, but also other enteric pathogens with the cyclic-di-GMP signaling pathway.

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APPENDICES

Appendix A: Cell Lines Used in This Study

Name	Code	Description	Source
HeLa	ATCC CCL-2	Standard epithelial cell line from cervical adenocarcinoma	ATCC
RAW 264.7	ATCC TIB-71	Immortalized mouse macrophage cell line	ATCC

Appendix B: Strains Used in This Study

Strain	Genotype[1]	Source
EM012	Wild type *Salmonella enterica* serovar Typhimurium 14028	ATCC
DH5α	Invitrogen DH5α Competent Cells	Invitrogen
EPST299	14028 RcsD::TnGent	This study
EPST388	14028 2503::Cam/2215::Kan/3615::null	11
EPST452	14028 2215::null/2503::null/3615::Kan	11
EPST456	14028 1703::pGPCS-1703-GGDEFSDM	11
EPST444	14028 1703::pGP704CS-1703GGDEFSDM/ RcsD::Gent	11
EPST474	14028 2215::null/2503::null/3615::Kan /1703::pGPCS-1703GGDEFSDM	11
EPST481	14028 2215::null/2503::null/3615EALSDM::Kan /1703::pGPCS-1703GGDEFSDM	11
EPST488	14028 2503SDM::null/3615SDM::null/2215SDM::Kan /1703::pGPCS-1703GGDEFSDM	11
MAST002	14028 2215::null/2503::null/3615::Kan/1703GGDEFSDM::null	This study
MAST003	14028 2215::null/2503::null/3615EALSDM::Kan /1703GGDEFSDM::null	This study
MAST001	14028 2503SDM::null/3615SDM::null/2215SDM::Kan /1703GGDEFSDM::null	This study
EPST465	14028 3388::pGPCS-3388-GGDEFSDM	11
EPST447	14028 3388::pGP704CS-3388GGDEFSDM/ RcsD::Gent	11
EPST475	14028 2215::null/2503::null/3615::Kan /3388::pGPCS-3388GGDEFSDM	11
EPST482	14028 2215::null/2503::null/3615EALSDM::Kan /3388::pGPCS-3388GGDEFSDM	11
EPST489	14028 2503SDM::null/3615SDM::null/2215SDM::Kan /3388::pGPCS-3388GGDEFSDM	11
MAST006	14028 2215::null/2503::null/3615::Kan/3388GGDEFSDM::null	This study
MAST004	14028 2215::null/2503::null/3615EALSDM::Kan /3388GGDEFSDM::null	This study
MAST005	14028 2503SDM::null/3615SDM::null/2215SDM::Kan /3388GGDEFSDM::null	This study
EPST477	14028 2215::null/2503::null/3615::Kan/BcsA::Cam	13
EPST476	14028 2215::null/2503::null/3615::Kan/YcgR::Cam	13
EPST471	14028 2215::null/2503::null/3615::Kan/385::Cam	13
EPST492	14028 2215::null/2503::null/3615::Kan/1283::Cam	13
EPST472	14028 2215::null/2503::null/3615::Kan/1987::Cam	13
EPST494	14028 2215::null/2503::null/3615::Kan/2123::Cam	13
EPST495	14028 2215::null/2503::null/3615::Kan/2672::Cam	13
EPST497	14028 2215::null/2503::null/3615::Kan/4551::Cam	13

EPST042	14028 /pMMBAmp	11
EPST424	14028 2503::Cam/2215::Kan/3615::null /pMMBA	11
MAST013	14028 2215::null/2503::null/3615::Kan/385::Cam/pMMBA	This study
MAST007	14028 /pMMBA-385comp	This study
MAST008	14028 2503::Cam/2215::Kan/3615::null/pMMBA-385comp	This study
MAST009	14028 2215::null/2503::null/3615::Kan/385::Cam /pMMBA-385comp	This study
MAST010	14028 /pMMBA-385SDM	This study
MAST011	14028 2503::Cam/2215::Kan/3615::null/pMMBA-385SDM	This study
MAST012	14028 2215::null/2503::null/3615::Kan/385::Cam /pMMBA-385SDM	This study
[1]Genotype antibiotic resistance marker: Cam = Chloramphenicol, Kan = Kanamycin, Amp = Ampicillin, null = unmarked deletion removed with Flp recombinase		

Appendix C: Plasmids Used in This Study

Plasmid	Extended name	Antibiotic Rx[2]	Parent strain	Source
pEP321	pGP704Cam-SacB	Cam/Sac	MC1061	11
pEP394	pGP704CamSacB-3388	Cam/Sac	MC1061	11
pEP401	pGP704CamSacB-3388GGDEFSDM	Cam/Sac	MC1061	11
pEP397	pGP704CamSacB-1703	Cam/Sac	MC1061	11
pEP398	pGP704CamSacB-1703GGDEFSDM	Cam/Sac	MC1061	11
pRK2013		Kan	DH5α	11
pBTK27	pMMBAmp in Mach1	Amp	Mach1	11
pMA001	pMMBA-385comp	Amp	DH5α	This study
pMA002	pMMBA-385SDM	Amp	DH5α	This study

[2]Antibiotic resistance marker (Ab Rx): Cam = Chloramphenicol, Kan = Kanamycin, Amp = Ampicillin, Sac = SacB marker for sucrose counterselection

Appendix D: Primers Used in this Study

Primer	Sequence	Use
pGP704CS-1703 GFwd	TAGGAACTTCGAAGCAGCTCCAGCCTACACATG GTA CGC ATG ATG AAA CAG ATT CAG GAA	Primers to generate STM1703 fragments for insertion in pGP704CamSacB via Gibson cloning
pGP704CS-1703 GRvs	TGTGGAATTGTGAGCGGATAACAATTTGTGTTA ACG CAT TTT TTT CGT CTG ATA ACG TTT	
pGP704CS-3388 GFwd	TAGGAACTTCGAAGCAGCTCCAGCCTACACATG CCG GTT AGT GAG TAC AAC CAC ATC CTT	Primers to generate STM3388 fragments for insertion in pGP704CamSacB via Gibson cloning
pGP704CS-3388 GRvs	TGTGGAATTGTGAGCGGATAACAATTTGTGGCG GAA GAA GCC TGA GAT TAT TTT GAG TTG	
1703 Int Fwd	CCTGCCCAATCGCAATGC	Sequencing primer for STM1703
3388 Int Fwd	GTTGGCGCTGCATGATACG	Sequencing primer for STM3388
STM1703 Seq R	GGCAATTCGTTGTGCTTCG	Primers to sequence STM1703 deletion
STM3388 Seq F	CCG ATC TGG AGT AGC GAA C	Primers to sequence STM3388 deletion
STM3388 Seq. Rev NEW	CTTTATAAGCAGGCGCCTG	
pGP704 Seq 3’	GAAGCCCTTAGAGCCTCTC	Primer to sequence pGP704 inserts
rrnB Term GFwd	GGCTGTTTTGGCGGATGAGAGAAG	Primers to generate pMMBAmp fragments to be used in Gibson cloning for generating STM0385 expression vectors
pMMB2GFwd1	CATGCCGGAGTTCGTCG	
pMMB2GRvs1	GCGTTCATACAGGTCGGC	
pMMB2GFwd2	CGATACCGGGTGCTCTATCG	
pMMB2GRvs2	CCTGCGCCCATCATGG	
Ptac MCS GRvs	CGAATTCTGTTTCCTGTGTGAAATTG	

pMMMB Seq F	ATG TGT GGA ATT GTG AGC GG	Sequencing primer for pMMBA and pMMB vectors
385-Comp_GFwd	ATAACAATTTCACAC AGGAAACAGAATTCG GCA AGC GTT GAA AGG CG	Primers to generate fragments of STM0385 from *Salmonella* genomic DNA to be used in Gibson cloning for generating pMMBA-385comp
385-Comp_GRvs	GAAAATCTTCTCTCA TCCGCCAAAACAGCC GCG GTA ATA GAA GCG ATG ACG	
385SDM_GFwd	G CGC TTT GGC GGC GCC GCA GCT GCG GTG ATT ATG T	Primers to generate fragments of STM0385SDM (with mutated active site) from *Salmonella* genomic DNA or pMMBA-385comp to be used in Gibson cloning for generating pMMBA-385SDM
385SDM_GRvs	A CAT AAT CAC CGC AGC TGC GGC GCC GCC AAA GCG C	

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