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ASSESSMENT OF A PREDICTED DIGUANYLATE CYCLASE IN *TREPONEMA DENTICOLA*

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By

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Abbreviations

Δ	Deletion
Asp	Aspartate
Arg	Arginine
BN	Blue Native
cAMP	Cyclic adenosine monophosphate
c-di-GMP	Bis-(3'-5')-cyclic-dimeric-guanosine monophosphate
CGMP	Cyclic guanosine monophosphate
cm	centimeter
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
DGC	Diguanylate cyclase
EAL	Glutamate-Alanine-Leucine
E.	*Escherichia*
gDNA	Genomic Deoxyribonucleic acid
GGD/EEF	Glycine-Glycine-Aspartate/Glutamate-Glutamate-Phenylalanine.
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
Glu	Glutamate
Gly	Glycine
HCl	Hydrochloric acid
HD-GYP	Histidine-Aspartate-Glycine-Tyrosine-Proline
His	Histidine
Hpk1	Histidine sensor kinase 1
Hpk2	Histidine sensor kinase 2
HPLC	High-performance-liquid-chromatography.
IPTG	Isopropylthio-beta-D-galactoside
Kb	Kilobase
kDA	Kilodalton
$MgCl_2$	Magnesium Chloride
$MnCl_2$	Manganese Chloride
mRNA	Messenger ribonucleic acid
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar

mg	Milligram
mL	Milliliter
mM	Millimolar
min	Minute
M	Molar
KH_2Po_4	Monopotassium phosphate
NaCl	Sodium Chloride
ng	Nanogram
Ni-NTA	Nickle-nitrilotriacetic acid
nm	Nanometers
nM	Nanomolar
P.	*Pseudomonas*
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
PdeA	Phosphodiesterase A
PdeB	Phosphodiesterase B
PD	Periodontal disease
pGpG	Linear Dimeric GMP
PlzA	PilZ domain-containing protein A
PlzB	PilZ domain-containing protein B
PlzC	Pilz domain-containing protein C
r-	Recombinant
RNA	Ribonucleic acid
RPM	Revolutions per minute
Rrp1	Response regulator protein 1
Rrp2	Response regulator protein 2
RP-HPLC	Reverse phase-high-performance-liquid-chromatography
SDS	Sodium dodecyl sulfate
T.	*Treponema*
Tris	Tris (Hydroxymethyl) aminomethane
x g	Gravitational force

Abstract

Assessment of a Predicted Diguanylate Cyclase in *Treponema denticola*
By Dhara T. Patel

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2018

Major Director: Richard T. Marconi, Ph.D.
Professor, Department of Microbiology and Immunology

Periodontal disease is a progressive inflammatory condition that is characterized by the reabsorption of alveolar bone, the destruction of connective tissue, and edentulism. It is caused by a dysbiosis in the oral microbiome as a result of a shift from a Gram-positive aerobic bacterial population, to one that becomes more Gram-negative and anaerobic. *Treponema denticola* is thought to drive this diseased state based on its role as a keystone periopathogen. A major component of *T.denticola*'s invasiveness is its motility, which allows the spirochete to penetrate and disseminate through tissues. This motility, which has been seen to be crucial to the invasiveness in other spirochetes through deletion studies, is often regulated by the second messenger, c-di-GMP. In this study, biochemical and biophysical assays were utilized to determine that the predicted

diguanylate cyclase TDE0125 converts GTP to c-di-GMP. This elucidates further function of the c-di-GMP regulatory network in *T. denticola*.

Chapter 1: Introduction

Periodontal Disease

Periodontal disease (PD) is a chronic and progressive inflammatory disease of microbial etiology that culminates in destruction of the periodontium, alveolar bone reabsorption and edentulism (3). The development of PD can be traced to a change in the composition of the oral microflora (17, 35). A transition occurs in the composition of the oral microbiota from predominantly Gram-positive bacteria in the healthy subgingival crevice to Gram-negative bacteria and proteolytic spirochetes of the genus *Treponema* (Figure 1-1). The severity of PD is assessed through measurement of pocket depth of the gums surrounding the tooth. In healthy individuals, the pocket depth ranges from 1 to 3 mm. Individuals with moderate PD have two or more pocket depth measurements greater than 4 mm while those with severe disease have pocket depths greater than 6 mm (3). It is estimated that >70% of the adult human population suffers from moderate to severe PD (79). Risk factors for PD include smoking, poor oral hygiene, socioeconomic status, malnutrition, diabetes, and alcoholism (33, 71). PD also correlates with increased risk of systemic disorders including cardiovascular disease, diabetes, stroke, and certain esophageal cancers (56). The costs of this disease on a larger scale can be summarized by the amount of time (160 million hours) and money lost (greater than 100 billion dollars) from dental visits and treatment (10).

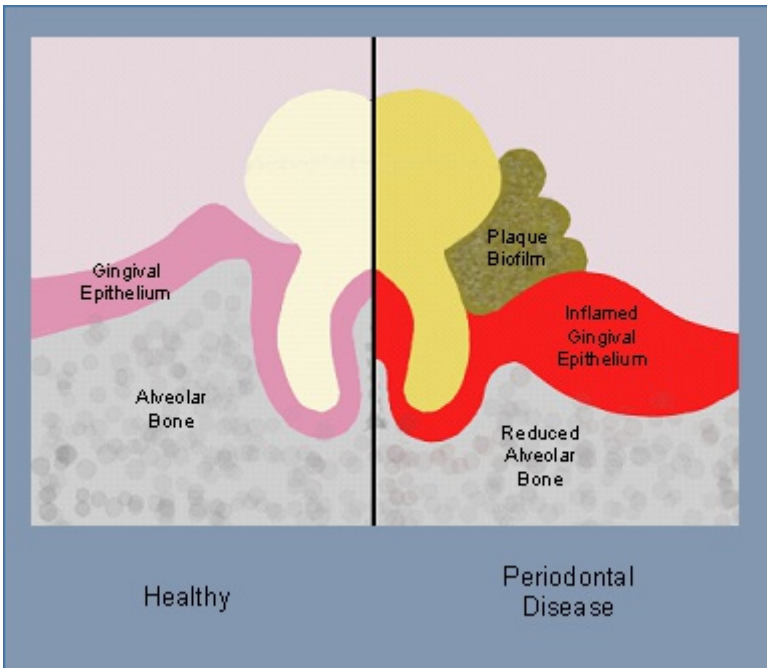

Figure 1-1: A comparison between healthy gums and periodontal disease. The progression of gum disease leads to chronic inflammation which causes damage to the gingival epithelium resulting in deepening of the periodontal pocket and alveolar bone reabsorption.

Treatment of PD typically initially consists of scaling and planing. The purpose of these physical processes is to remove bacteria from the tooth surface and to smooth the tooth surface thereby minimizing crevices that allow for microbial growth. Antimicrobials and chemical treatments are also employed but can be hindered by the resistance of microbial biofilms (53). The establishment of the oral biofilm is extremely important for the progression of the disease as it is a highly ordered process that suggests extensive co-evolution between multiple oral bacterial species in the host (Figure 1-2)(47, 72). The colonization of the tooth begins with the interaction of bacteria, predominantly of the streptococci, on the tooth pellicle. Early colonizers act as binding sites for middle and late colonizers. A study by Soransky et al., described several bacterial consortiums in relation to their location within the biofilm and correlation with disease severity (73). Early colonizers consist of bacteria belonging to "the purple complex,"(*Veillonella parvula* and *Actinomyces odontolyticus*). "the yellow complex" (entirely composed of streptococci) and "the green complex" (*Aggregatibacter acintomycetemcomitans*). "The orange complex" consists of *Fusobacterium nucleatum*, *Prevotella intermedia*, and the *Campylobacter* species, all of which are intermediate colonizers and most closely associated with dental caries. Finally, "the red complex," which consists of *Porphymonus gingivalis, Tannerella forsythia,* and *Treponema denticola*, have the highest correlation with PD progression and severity (73). Species of this complex have been hypothesized to manipulate innate immune responses leading to the disruption of host homeostasis and the progression of PD (19, 17).

As PD develops, that relative proportion of oral spirochetes of the genus

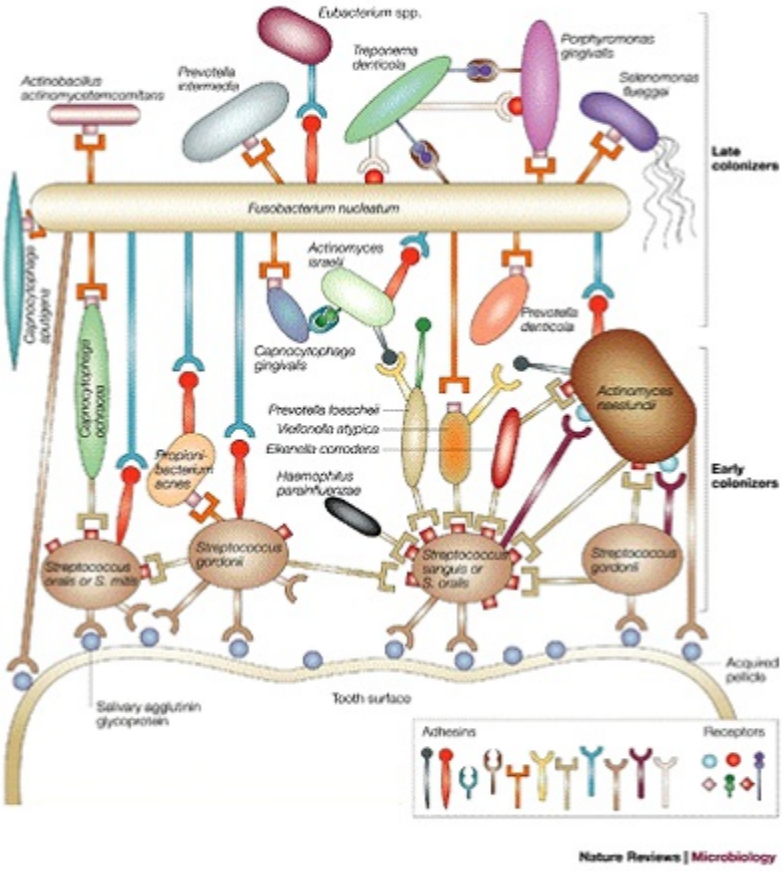

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Figure 1-2: The oral polymicrobial community. Bacterial colonizers in the dental plaque organized by the order in which they colonize the surface of the tooth. *T. denticola* is a late colonizer in the oral microbiome.

Treponema increases in the subgingival crevice and in the periodontal pockets. While oral treponemes constitute less than 1% of the total subgingival crevice in healthy individuals, the bacterial population can increase to as high as 50% of the bacterial population in those with periodontitis (20, 23). Of these treponemes there are upwards of 70 species, yet, only 10 have been cultivated: *T.denticola*, *T. socranskii*, *T. pectinovorum*, *T. medium*, *T. amylovorum*, *T. lecthinolyticum*, *T. parvum*, *T. maltophilum*, and *T. vincentii* (*T. vincentii* is yet to be systematically characterized, though it has been recognized for over 60 years)(23, 40). Multiple studies have examined both the cultivatable and non-cultivatable bacteria of this genus and ultimately determined that they are found more often in those with PD when compared to individuals with a healthy oral microbiome (20, 50, 51, 82). These oral treponemes act as late colonizers and are found deeply in the periodontal pocket (23). At the interface between the polymicrobial biofilm and the gingival epithelium, *T. denticola* directly interacts with host cells, causing extensive cell damage and immune modulation (8, 7, 41). Extensive data has shown that *T. denticola* is resistant to phagocytosis by neutrophils, beta defensins, complement-mediated killing, and can also suppress inflammatory mediators to create a more favorable niche for other periopathogens (8, 7, 9).

Treponema denticola

T. denticola and other oral treponemes belong to a unique group of microbes called spirochetes. Spirochetes have several unique and defining features. While their membrane structure is similar to Gram-negative bacteria (i.e., an inner and outer

membrane), they lack lipopolysacharride (LPS). The structural integrity of the outer membrane is instead maintained by lipoproteins and glycolipids (65). One of the most defining features of spirochetes is a distinctive spiral or flat wave ultrastructure. This shape is imparted by the presence of endoflagella that are anchored to the inner membrane and extend into the periplasmic space (Figure 1-3). The endoflagella exists in two flagella bundles that insert into the inner membrane at opposite ends of the cell where they are connected to the flagellar motor. These bundles extend approximately two thirds the length of the spirochete and wrap around the protoplasmic cylinder in a right handed sense (13). Rotation of the endoflagellar bundles within the periplasm will cause the rotation of the protoplasmic cylinder. This can be referred to as rotational motility (45). Rotational motility causes the cell to move in a wave-like pattern, resulting in translational motility, which can be described as measurable forward or reverse movement (45). For translational motility to occur, the flagellar motors at each end of the cell must rotate in opposite directions. For to the spirochete to change direction, either one motor must stop, or transiently reverse direction. This stops translational motility and causes the cells to flex. The cell will only reverse its direction of movement once both motors change direction (45). Almost 6% of the spirochetal genome is devoted to genes that are involved in motility and chemotaxis (70). Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is a secondary messenger molecule that is often implicated in these processes (1, 5, 42).

C-di-GMP

C-di-GMP was originally found in *Gluconacetobacter xylinus* as an

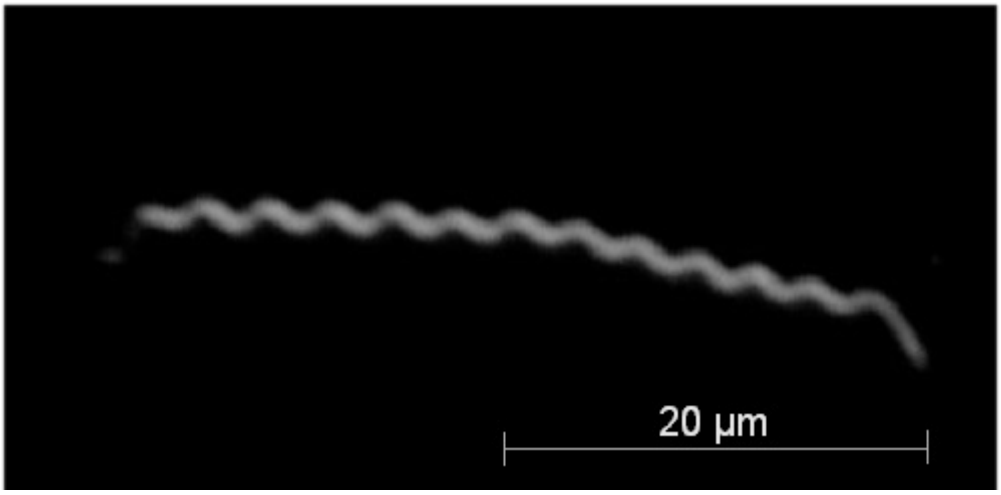

Figure 1-3: The spiral morphology of *T.denticola*. *T. denticola* exhibits the classic spiral morphology of all spirochetes in this image taken via dark field microscopy. Its endoflagella exist as two bundles of flagella that insert into the inner membrane of the cell at both ends and help it move in a corkscrew motility.

important regulator of cellulose synthesis but that is not its only function. (Figure 1-4) (61).This molecule has also been implicated in other bacterial species as a regulator of many cellular signaling pathways relating to motility, virulence factor production, and biofilm formation (1, 5, 42, 64). The synthesis, binding, and degradation of c-di-GMP in the cell are important for c-di-GMP mediated regulation and these functions can be elucidated by the presence of various domains (2, 12, 30, 31, 32). These are the GGDEF, EAL, HD-GYP, and PilZ domains (4, 12, 30, 31, 33). The GGDEF domain, which is named after an amino acid sequence motif (Gly-Gly-Asp/Glu-Glu-Phe), is found in all diguanylate cyclases (DGCs). These domains can have an aspartate or a glutamate residue in the position of the third amino acid. In both cases, DGC function is the same (4). This domain converts two protein-bound molecules of GTP into one molecule of the second messenger molecule c-di-GMP (12, 33, 55, 59, 80). C-di-GMP acts as an allosteric inhibitor to the DGC because of the presence of an inhibitory "I-site" which has an RxxD consensus sequence in the GGDEF domain (12). Proteins known as phosphodiesterases (PDEs) can then break down c-di-GMP into even smaller molecules. There are two types of PDEs, one that contains an EAL domain, and another with a HD-GYP domain (30, 31). These domains are both named after common motifs in their amino acid sequences (30, 31, 32). In the case of EAL domain-containing proteins, one phosphodiester bond of c-di-GMP is hydrolyzed to form linear dimeric GMP (pGpG) (69). Proteins that contain an HD-GYP domain can catabolize c-di-GMP Into its two GMP constituents (14, 29, 30). This is not the extent of the c-di-GMP signaling pathway, however. There are also proteins whose main function is to bind to

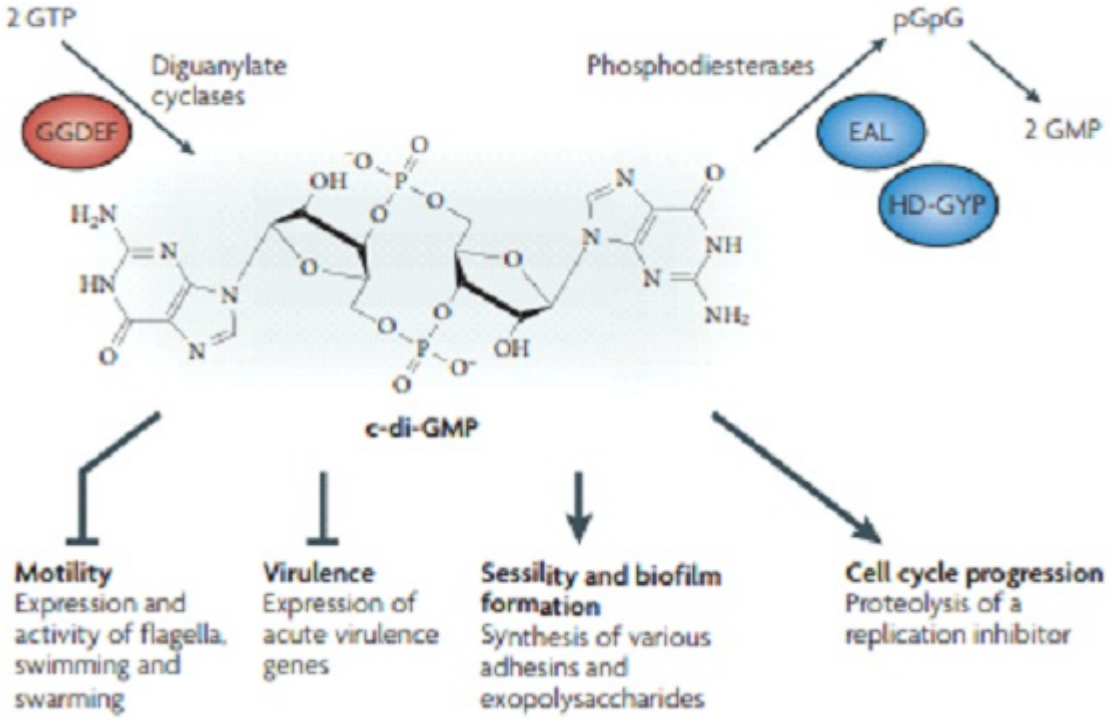

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Figure 1-4: Summary of c-di-GMP and its mechanisms. Two molecules of GTP form c-di-GMP through interactions facilitated by DGCs. The formation of two phosphodiester bonds between the GTP molecules create a twelve member ring out of the ribose sugars and phosphate groups. C-di-GMP can then be used in the cell to cause a variety of cellular responses.

c-di-GMP through the use of a PilZ domain (2, 42). This domain has a RxxxR/DzSxxG sequence motif where x represents any amino acid and z represents any hydrophobic amino acids (2, 42). Proteins containing this domain have been shown to be involved in the regulation of many processes, of which the best characterized have been shown to regulate motility (43, 58, 63).

The enzymatic domains of proteins involved with c-di-GMP signaling typically have sensor domain such as a REC, PAS, or a GAF domain which modulates the function of the protein (16, 66, 68, 81). These sensor domains affect enzymatic activity by sensing oxygen, light, external environmental signals, or binding to regulatory molecules such as phosphate, cAMP and cGMP (16, 31, 78). REC domains often act as a part of two component regulatory systems (31). In these systems, histidine kinases react to an external signal stimulus such as light, or oxygen, which leads to self phosphorylation (31). The phosphate is then transferred to a response regulator for activation, often a REC domain located on the response regulator (31). PAS domains are known to react to the presence of oxygen and light in many bacteria (78). In *T. denticola*, it has been shown in the histidine kinase, Hpk2, to affect the activity of the protein depending on the presence of oxygen (66). GAF domains, which share a similar fold to PAS domains have also been known to react to the presence of oxygen and light, but also to small molecules such as cAMP or cGMP (16, 39, 84). This is seen in PDEs such as human PDE6, in which the cGMP binding to the GAF domain leads to the activation of PDE function and the breakdown of c-di-GMP (84). The importance of these types of domains are highlighted in deletion mutants, where the sensor domains have been knocked out as the mutated domains are unable to modulate the enzymatic

domains on the same protein (37, 66). Sarkar et al. showed this when they created a mutant version of *T. denticola* Hpk2 that lacked the PAS domain and found that under anaerobic conditions, while it could still self-phosphorylate, it could no longer transfer it's phosphate group to its response regulator, Rrp2 (66).

Crystal structure analysis of c-di-GMP has shown that it has two symmetric intermolecular phosphodiester bonds. These bonds result in a 12-member ring formed by the ribose and phosphate groups (68). In addition, the molecule is rigid with the only conformational freedom coming from a rotation around the C-1'-N-9 bond, allowing for the cyclic dinucleotide to exist in an open or a closed conformation (68). C-di-GMP monomers in a closed conformation can associate due to base pair stacking, which allows them to exist as dimers. This means that c-di-GMP mainly exists as a dimer or monomer in physiologically relevant concentrations (68).

C-di-GMP controls cellular functions at multiple stages within the biochemical pathways, including transcription, translation, and post-translational modifications (60). Through its interaction with transcription factors, enhancers, or suppressers, c-di-GMP can positively or negatively regulate the transcription of mRNA (60). Once the targeted genes have been transcribed, translational regulation is controlled by c-di-GMP activated proteins or riboswitches, secondary structures within mRNA which directly bind c-di-GMP to promote or inhibit translation (60). In addition, c-di-GMP can also regulate protein levels and functionality through proteolysis, activation, or deactivation (21).

In terms of c-di-GMP signaling in spirochetes, very little is known compared to other bacteria. However, genomic analyses have indicated that proteins involved with

the synthesis binding and breakdown of c-di-GMP are found in the genomes of various spirochetal genera, such as *Leptospira*, *Borrellia*, *Borreliella,* and *Treponema*.

C-di-GMP Signaling in Spirochetes

GGDEF, EAL, HD-GYP, and PilZ domain-containing proteins have been identified in most spirochetes which indicates that they must have c-di-GMP signaling systems (60, 26, 25, 28, 83). However, our understanding of c-di-GMP functionality in many of these organisms needs much further investigation.

In the Lyme Disease causing bacterium *B. burgdorferi,* c-di-GMP signaling is a major player in infectivity (27, 43, 59, 64). This bacterium has one DGC (Rrp1) and 2 PDEs (PdeA and PdeB). It also has 1 PilZ domain-containing protein, PlzA, which makes *Borreliella* an excellent model to study c-di-GMP signaling (27, 59, 64). However, some isolates of *B. burgdorferi* have a second c-di-GMP binding protein called PlzB. Since there are isolates that do not contain *plzB*, this would suggest that PlzB is not required for the life cycle of the Lyme disease causing spirochete. Changes made to the *B. burgdorferi* c-di-GMP system through the generation of deletion mutants affect the virulence in different ways. Deletions of the only DGC gene, *rrp1,* lead to an inability to colonize ticks because of a decrease in motility (43). Similar phenotypes are seen in *hpk1* deletion mutants because the DGC activity of Rrp1 depends on phosphorylation by Hpk1 (64). Deletion of the PDE genes affected infectivity depending on the PDE. A loss of infectivity was seen in mice, but not in ticks when *pdeA* (an EAL domain-containing PDE) was deleted, but when *pdeB* (an HD-GYP domain-containing PDE) was deleted, there was no loss in infectivity in mice, but there was reduced

infectivity in ticks (74, 75). Lastly, the deletion of *plzA*, led to reduced invasion of ticks and mice (57). In the *plzA* deletion mutant, PlzB did not fill in the role of PlzA, suggesting that it serves a different function in the cell (44). These mutants also exhibited changes in motility and function compared to wild type strains by showing decreased motility and therefore decreased virulence (57, 43, 74, 75). It is clear based on these deletion mutants that these genes and the proteins they encode are important to the overall cellular regulation in spirochetes (43,57, 74, 75).

Due to the fact that the c-di-GMP signaling has been found to affect virulence and infectivity in *B. burgdorferi*, it may do the same in *T. denticola.* TDE0214, a PilZ domain-containing protein that is also a homolog to *B. burgdorferi*'s PlzA, has been elucidated to be important to the infectivity of *T. denticola* by Bian et al. (5). This protein is one of two PilZ domain-containing proteins in *T. denticola* and has been implicated as important for tissue invasion through the use of a deletion mutant (5). Deletion of the gene affects the mutant in several ways. Compared to the wild type, the *tde0214* deletion showed reduced speed and moved at a much lower velocity. In a murine skin abscess model, this lead to reduced virulence as the mutant was unable to move through the muscle tissues and a weaker humoral response in comparison to the wild type (5).

As no other *T. denticola* protein relating to c-di-GMP signaling has been characterized in the literature, it is difficult to assess what stimuli influence which of the many DGCs encoded by *T. denticola* and how this may cause the intracellular pool of c-

di-GMP to fluctuate. Here, we further the understanding this system through the study of TDE0125, a predicted DGC.

Research Objectives

Based on bioinformatic results, TDE0125 has been predicted to be a *T. denticola* DGC that contains an N-terminal GAF domain and a C- terminal GGDEF domain (26). The STRING database, which can be used to predict protein-protein interactions, predicts it to act with the histidine kinase. It is possible that TDE0492, serves as its cognate histidine kinase (77). The primary goal of this study was to characterize the physical properties TDE0125, its distribution among oral treponemes, and its putatitive functional and regulatory activities. Future studies will investigate its interaction with TDE0492 and its *in vivo* biological significance.

Chapter 2: Methods

Cultivation of Bacterial Isolates

T. denticola isolates 35405, GM-1, 33520, SP46, SP49, SP50, SP54, SP55, SP60, SP61, SP64, SP72, CF170, CF171, 33521, and 35404 were used in this study and were grown in new oral spirochete (NOS) medium supplemented with 4 mg of thiamine pyrophosphate^{-1} under anaerobic conditions (5% H_2; 5% CO_2; 90% N_2; 37°C) (49). Dark field microscopy was used to monitor growth. All stains used were previously isolated from human periodontal pockets.

Generation of Recombinant Proteins and Protein Specific Antiserum

Recombinant proteins were generated with N-terminal His tag fusions by polymerase chain reaction (PCR) amplifying the gene from 35405 gDNA with the following primers (Forward primer with a BamHI restriction site: 5'ATAGTCGGATCCCATGAAAACAACTCCAAATGAAAAATTATTAAAAAA 3'. Reverse primer with an EagI restriction site: 5'ATAGTCCGGCCGTTATTAAGCTTTAGGCAAGTTATTATTTTTGGATAATG 3'.) PCR products were then inserted (cloned) into pET45b(+) (Novagen) using the restriction sites BamHI and EagI. Plasmids were sequence verified and transformed into BL21 (DE3) cells for protein expression (Genewiz).

All recombinant proteins were expressed in BL21 (DE3) cells and grown in Lennox Broth (Fisher Scientific) supplemented with 100 µg/mL ampicillin. Cultures of

the transformed BL21 (DE3) cells were grown to mid-log phase at 37 °C. Protein expression was induced with Beta-D-1-thiogalactopyranoside (IPTG; 1 mM; 4-5 hours). Cells were recovered by centrifugation (5,000x g; 15 min; 4°C), suspended in lysis buffer (20 mM NaH_2PO_4, 500 mM NaCl, 40 mM Imidizole, lysozyme, 1 mg/mL), incubated at room temperature for 30 min, sonicated, and centrifuged (15,500x g; 30 min; 4 °C). Protein was purified using an AKTApurifier (GE Healthcare). The final recombinant carried an N-terminal 6xHis-Tag of 2.4 kDa. The purified proteins were dialyzed into Phosphate Buffered Saline (PBS) using Spectra/Por dialysis membranes (Spectrum Laboratories). A Bicinchoninic Acid (BCA) protein assay kit (Thermo Scientific) was used to determine protein concentrations, and purity was assessed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (BioRad), and visualized by staining with Coomassie Brilliant Blue G-250 (CBB-G250) .

The generation of antisera was carried out as follows. During the initial vaccination, 50 µg of recombinant protein was injected into a female Sprague-Dawley rat (n=1) intraperitoneally. Recombinant protein was diluted in PBS and mixed 1:1 with Freund's Complete Adjuvant (CFA) (Sigma-Aldridge). Subsequent vaccinations took place on days 21 and 35 using 25 µg of r-protein with Incomplete Freund's Adjuvant (IFA) mixed 1:1. The rat was exsanguinated by cardiac puncture under anesthesia 9 days post-final injection. Harvesting of serum was completed by separating whole cell blood in Vacuette blood collection tubes (Greiner Bio-One). All animal experiments performed as a part of this study were conducted following the *Guide for the Care and Use of Laboratory Animals* (Eighth edition) and in accordance with protocols peer

reviewed and approved by Virginia Commonwealth University Institutional Animal Care and Use Committee.

Bioinformatics Analysis

TDE0125 sequences were obtained for 17 isolates from the NCBI's Basic Local Alignment Search Tool (BLAST) database (34). The TDE0125 sequence of 12 strains not found in the databases were determined by PCR amplification and sequenced as previously described (49). Translated amino acid sequences were aligned, and phylogenetic analyses were conducted using MEGA7. The phylogenetic tree was generated using the neighbor-joining method and the standard errors of distances were computed by bootstrap analysis using 500 replications.

Triton X-114 Extraction, Phase Partitioning, and Immunoblotting

Triton X-114 extraction and phase partitioning was performed as before (15). Briefly, cells at mid-log phase were centrifuged and the resulting pellet was brought to an optical density at 600 nm of 0.5 and then suspended in PBS with 1% Triton X-114 and incubated at 4°C overnight with gentle rocking. The detergent-insoluble phase was collected by centrifugation (4°C; 15,000x g). The supernatant was incubated (37°C; 15 min) and centrifuged (RT; 15,000x g) to separate the aqueous and detergent soluble phases. Each sample was extracted twice to ensure complete partitioning. The aqueous phase was transferred to a new tube and Triton X-114 (1%) was added. One mL of PBS was added to the soluble phase. Both were mixed well in their respective tubes. Phases were incubated (37°C; 15 min) and then centrifuged (RT;15,000xg). The

desired phases were collected and prepared for separation via SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by solublization in SDS solubilizing solution.

Immunoblot Procedures

T. denticola cells were harvested at mid-log phase and prepared as previously described (48). 100 ng of r-TDE0215 and r-FhbB were also prepared for use as positive and negative controls respectively. Cell lysates and recombinant proteins were screened with TDE0125 rat antiserum at a dilution of 1:10,000 in PBSTM (PBS with 0.2% Tween 20 and 5% nonfat milk). Goat anti-rat horseradish peroxidase (HRP)-conjugated IgG secondary antibody (Pierce) was used as secondary at a dilution of 1:40,000. Detection was achieved using chemiluminescence with Clarity Max Western ECL substrate (Bio-Rad) as instructed by the supplier.

For Triton X-114 cell partitioning, cell fraction and whole cell lysates were screened with TDE0125 rat antiserum (1:1000) in PBSTM. Goat anti-rat HRP-conjugated IgG secondary antibody or Goat anti-rabbit HRP-conjugated IgG secondary antibody (Pierce) were used as secondaries at a dilutions of 1:40,000. Detection was achieved using chemiluminescence with Clarity Western ECL substrate (Bio-Rad) as instructed by the supplier. FlaA rabbit antiserum (1:1000) and FhbB rat antiserum (1:1000) served as controls.

Blue Native-PAGE Analysis of r-TDE0125

The oligomeric state of TDE0125 was assessed by Blue Native-PAGE (BN-PAGE). One µg of r-TDE0125 was diluted in BN-PAGE sample buffer (50 mM Bis-Tris (pH 7.0); 15% glycerol; 0.02% CBB-G250) without ß-mercaptoethanol and

separated on a Bis-Tris acrylamide gel (4 to 16% Native-PAGE; Invitrogen) using 50 mM Bis-Tris (pH 7.0) and 50 mM Tricine-15 mM Bis-Tris (pH 7.0) as anode and cathode buffers, respectively. The gel was run under cooled conditions (100 V for 20 min followed by 200 V for 40 min) with cathode buffer containing 0.002% CBB-G250. The gel was then run at 200 V for an additional 40 min with cathode buffer with no dye. The bands were visualized with CBB G-250.

Assessment of the Potential DGC activity of TDE0125

The ability of r-TDE0125 to produce c-di-GMP was assessed. Protein (5 μM) was incubated with or without 25 mM acetyl phosphate, 50 μm cAMP, or 50 μm cGMP (37°C; 30 minutes) for potential activation in 10 mM $MgCl_2$ or $MnCl_2$, 0.5 mM EDTA,0.5 mM Tris HCl (pH 7.6), and 10 mM NaCl. GTP was added at a final concentration of 150 μm (37°C; 60 min). Reverse Phase HPLC (RP-HPLC) was used to assess the production and purity of c-di-GMP. GTP and c-di-GMP (Sigma-Aldrich) were run as standards. RP-HPLC was performed essentially as described by Ryjenkov et al. (64). In brief, samples were boiled, centrifuged (17,500x g for 2 min), and filtered (22 μm filter) (Millex). Samples (100 μl) were applied to a 150 mm x 4.6 mm Supelcosil LC-18-T column (Supelco) and separated using an AKTApurifier (GE Health Sciences). Nucleotides were separated utilizing buffers A (100 mM KH_2PO_4; 4 mM Tetrabutyl Ammonium Hydrogen Sulfate (pH 5.9)) and B (75% buffer A; 25% Methanol). Nucleotides were detected at a wavelength of 254 nm.

Chapter 3: Results

Analysis of the Distribution of TDE0125 Amongst *T. denticola*

T. denticola 35405 possesses an ORF (TDE0125) that encodes a GGDEF domain. An NCBI BLAST search found that the amino acid sequence for TDE0125 was highly conserved with a sequence identity of at least 92%. Outside of *T. denticola*, there were no other predicted DGCs with similarly high sequence identities. TDE0125 was PCR amplified from isolate 35405 and other isolates listed above. It was found that all isolates tested were PCR positive when using these primers (Figure 3-1).

To assess if residues of TDE0125 thought to be involved in DGC activity are conserved across isolates, TDE0125 amplicons from the PCR analysis of *T. denticola* isolates were sent for sequencing. The amplicons had a size of 1182 base pairs. Nucleotide sequences were converted to their corresponding amino acid residues and aligned for comparison. The relationships between strains are displayed in the phylogenetic tree presented (Figure 3-2). Twenty-five of the 29 isolates were found to group together in one branch, while the other 4 isolates were located on another branch that split off into two further branches. Sequence identity of the 29 total isolates was found to range from 92% to 100% depending on the strain making this a highly conserved gene across *T. denticola* strains. Percent identity and percent similarity of

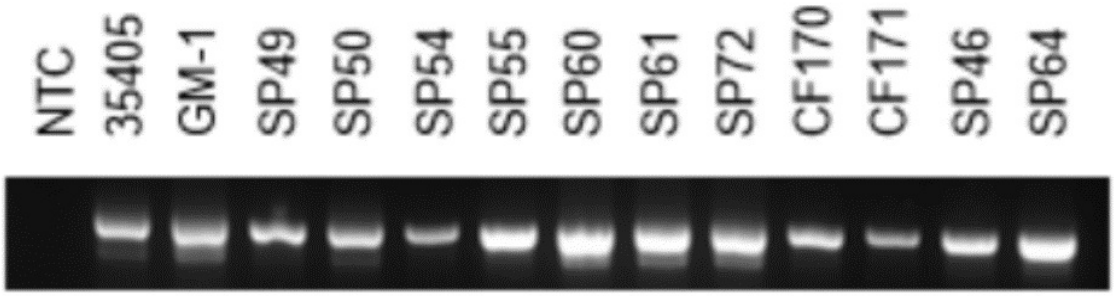


Figure 3-1: Distribution of TDE0125 in *T. denticola.* The distribution of the TDE0125 gene in *T. denticola* was assessed by PCR using full length primers, and products were visualized by SYBER Safe and UV detection. Strain and species designations are indicated on the top.

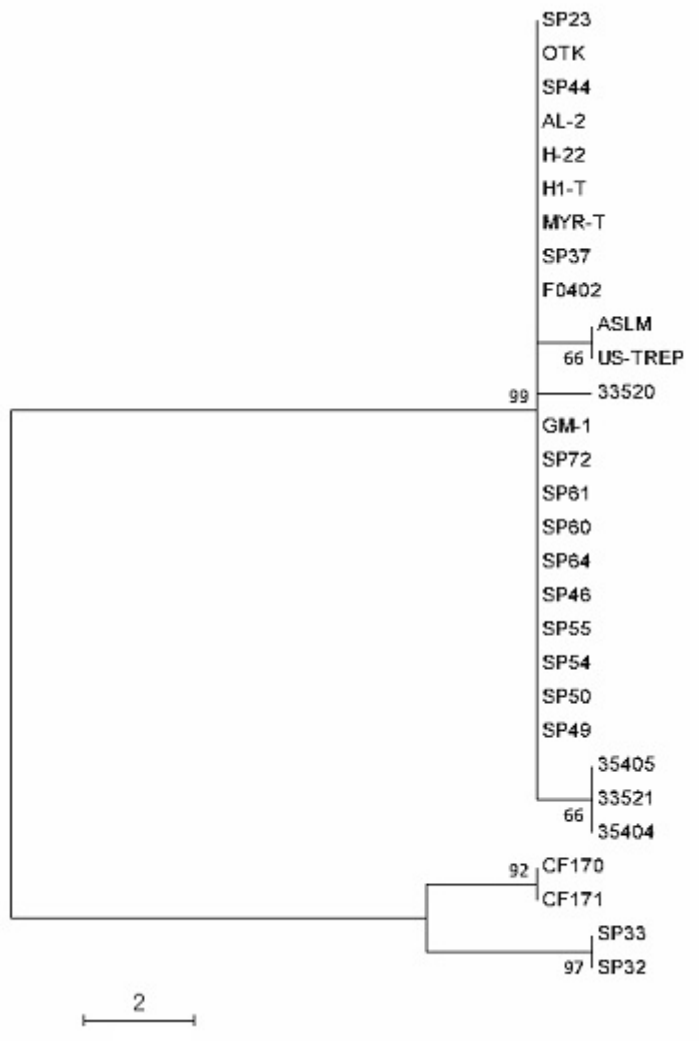


Figure 3-2: Phylogenetic analysis of TDE0125. The TDE0125 gene was sequenced from 12 isolates and phylogenetic analyses were performed with sequences from an additional 17 isolates. The tree was constructed using the neighbor-joining method and the standard error of distances was calculated by bootstrap analysis using 500 replications with MEGA7. Numbers on the branches indicate the percent of replications isolate sequences were found to cluster together. The scale represents distance as the number of amino acid changes per 100 amino acids.

various TDE0125 amino acid sequences of *T. denticola* isolates were used to generate a table. Identical isolates were removed from the table to highlight the high conservation of the most divergent TDE0125 amino acid sequences (Table 3-1).

Analysis of TDE0125 Cellular Location, Native Conformation, and Production During *In Vitro* Cultivation

To determine if TDE0125 is produced during *in vitro* cultivation. Lysates of various isolates were screened with antiserum generated against r-TDE0125 that was amplified, cloned, and purified from *T. denticola* 35405. The protein (42 kDa) was detected in all isolates screened (Figure 3-3). FlaA (38 kDa) was used as a control to ensure lysates were loaded evenly.

Triton X-114 extraction and phase partitioning was also performed to determine the cellular location of TDE0125 (Figure 3-4). Triton X-114 is a detergent that is used to separate cells into their various cellular fractions to detect the location of a protein of interest (15). Here, *T. denticola* was split into its protoplasmic cylinder, periplasm, and outer membrane fractions. These fractions and whole cell lysate were generated and screened by immunoblotting with TDE0125 antiserum. TDE0125 was found to be in the cytoplasm of the cell. FhbB, a known outer membrane protein, and FlaA, a known periplasmic protein, were used as controls. FhbB was found in the cytoplasmic and outer membrane portion of the cell at its known molecular weight of 11.4 kDa. FlaA was found in the periplasmic and cytoplasmic portions of the cell at its known molecular weight of 38 kDa. TDE0125 was located only in the cytoplasmic portion of the cell, at its predicted molecular weight of 42 kDa. This indicates that TDE0125 is a cytoplasmic

Table 3-1: Table Comparing Percent Identity and Percent Similarity of Selected Isolates

Percent Identity

Percent Similarity

	33520	35405	33404	33521	SP64	GM-1	SP32	SP33	CF170	CF171
33520		99.30	99.30	99.30	99.65	99.65	92.66	92.66	93.01	93.01
35405	99.65		100.00	100.00	99.65	99.65	92.66	92.66	93.01	93.01
33404	99.65	100.00		100.00	99.65	99.65	92.66	92.66	93.01	93.01
33521	99.65	100.00	100.00		99.65	99.65	92.66	92.66	93.01	93.01
SP64	99.65	100.00	100.00	100.00		100.00	93.01	93.01	93.36	93.36
GM-1	94.41	100.00	100.00	100.00	100.00		93.01	93.01	93.36	93.36
SP32	94.06	94.41	94.41	94.41	94.41	94.41		100.00	98.25	98.25
SP33	94.06	94.41	94.41	94.41	94.41	94.41	100.00		98.25	98.25
CF170	94.41	94.76	94.76	94.76	94.76	94.76	98.25	98.95		100.00
CF171	94.41	94.76	94.76	94.76	94.76	94.76	98.25	98.95	100.00	

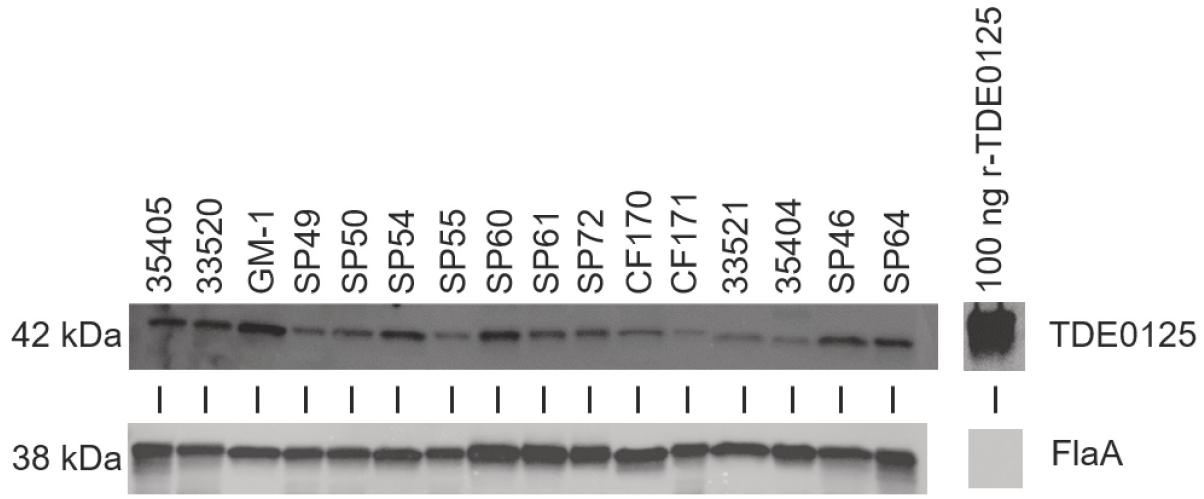


Figure 3-3: TDE0125 is produced *in vitro* by many *T. denticola* isolates. A panel of *T. denticola* isolates was cultivated under standard conditions and then fractionated, transferred and screened with anti-TDE0125 serum at a dilution of 1 to 10000. 100 ng of r-TDE0125 and r-FhbB (not shown here) also served as controls. FlaA served as a loading control.

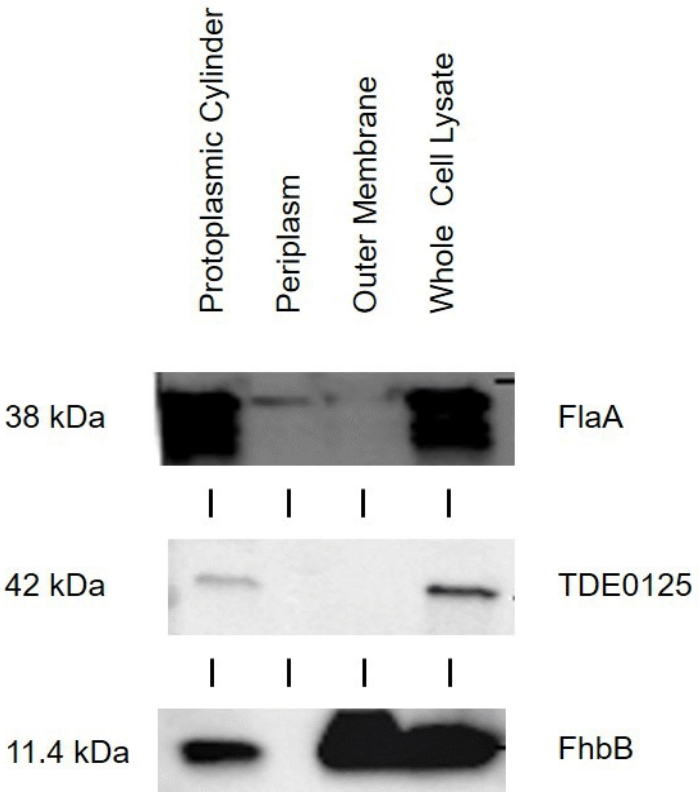


Figure 3-4: Localization of TDE0125 through Triton X-114 partitioning. Triton X-114 was used to extract and partition phases of *T. denticola* resulting in the aqueous, soluble, and insoluble phases which are the periplasm, outer membrane, and cytoplasm of the cell. Identical blots of the fractionated phases and whole cell lysate were screened with antiserum to TDE0125 (1:1000), FlaA (1:1000) (a periplasmic protein), and FhbB (1:1000) (an outer membrane protein).

protein, which is consistent with the cellular location of other DGCs (59).

BN-PAGE was utilized to determine the native conformation of r-TDE0125. In this assay, cysteine bonds are not reduced and proteins keep their native conformation. This allows for visualization of higher order structures (67). It was determined that the recombinant protein was found to exist mainly in a trimeric (~126 kDa) and hexameric (~252 kDa) conformation (Figure 3-5). This is consistent with the native conformation of other r-DGCs which have been found to exist in multimeric conformations (18, 63).

Synthesis of c-di-GMP Using a r-DGC Derived from *T. denticola*

RP-HPLC is a technique that is used to separate molecules based on their hydrophobic properties. The mobile phase in this application is more polar than the stationary phase and the difference in polarity causes hydrophobic molecules to bind to the stationary phase. Switching the mobile phase with an organic solvent then causes the molecule to elute from the stationary phase. Ion pairing RP-HPLC allows for the separation of polar molecules using this type of method. Ion pairing agents, which have a hydrophobic tail and an ionic head act as intermediaries to bind polar molecules to the hydrophobic stationary phase (64)(Figure3-6).

Ion pairing RP-HPLC was used to assess the conversion of GTP to c-di-GMP by r-TDE0125 from the 35405 isolate of *T.denticola.* In this assay, GTP and c-di-GMP were used as standards to determine the relative elution time of each molecule (Figures 3-7A;3-7C; 3-8A;3-C). GTP was found to have an elution time of roughly 27 minutes, while c-di-GMP had an elution time of roughly 31 minutes. After incubation of TDE0125

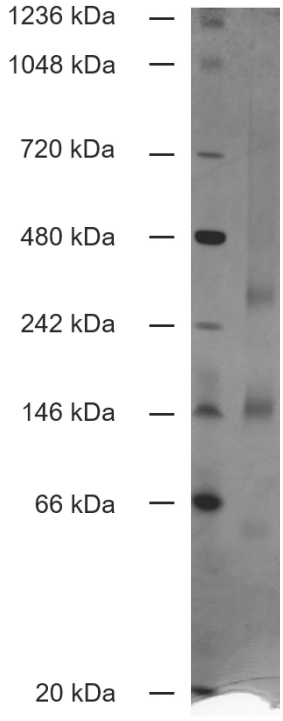


Figure 3-5: Formation of oligomers of TDE0125. R-TDE0125 was separated using Blue Native PAGE under non-reducing conditions. Molecular masses are shown on the left.

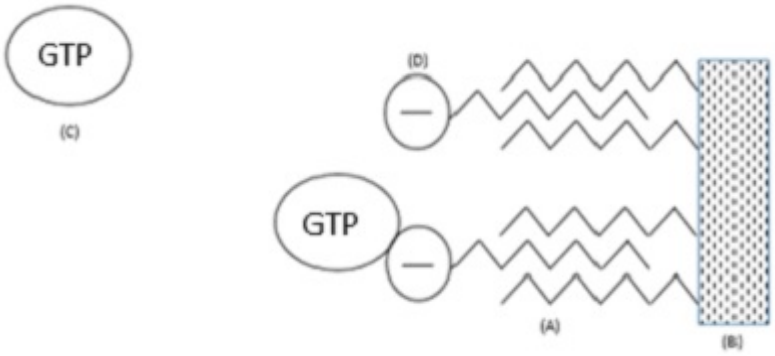


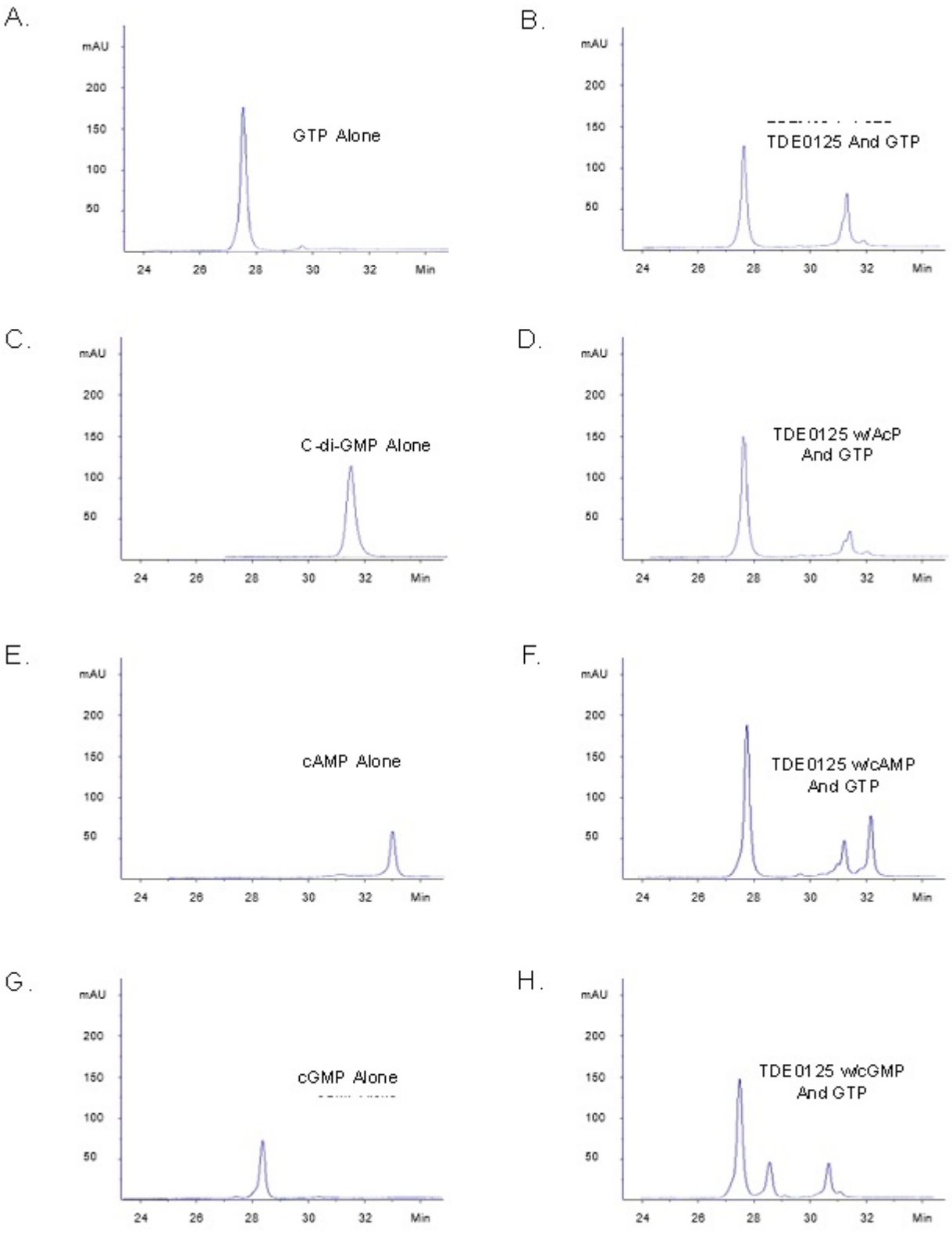
Figure 3-6: Diagram of Ion Pairing RP-HPLC. Ion pairing agents with hydrophobic tails (A) bind to the hydrophobic stationary phase (B). Polar molecules such as GTP or other nucleotides (C) bind to the polar end of the ion pairing molecules (D). Nucleotides elute from the stationary phase upon interaction with organic solvents due to disruption of the polar molecules. Detection of the nucleotides is accomplished by a UV sensor at 254 nm.

with GTP for an hour, there was partial conversion of GTP to c-di-GMP (Figures 3-7B;3-7D;3-8B;3-8D).

To determine the effects of potential activators, samples were also generated with protein incubated with acetyl phosphate, cAMP, and cGMP to potentially activate the protein. A literature review revealed that it could be possible to introduce potential activators to TDE0125 that may have an effect on its DGC activity. *L. interrogans* LIC13137, a DGC with an N-terminal GAF domain and a C-terminal GGDEF domain, was found to only show DGC activity when incubated with cAMP (16). TDE0125 has a similar domain organization, so to test if cNMPs had the potential to activate TDE0125, the recombinant protein was incubated with cAMP as well as cGMP. Acetyl phosphate was used to determine if TDE0125 needed to be phosphorylated based on a STRING search that indicated it may have a cognate histine kinase (TDE0492).

Standards of cAMP and cGMP were run to characterize the elution time of the cNMP's (Figures 3-7E;3-7G;3-8E;3-8G). For cAMP, the elution time was around 32.5 minutes, while the elution time of cGMP was found to be around 28 minutes. The addition of cAMP and cGMP were found to have no increase in the DGC activity of TDE0125 (Figures 3-7F;3-7H;3-8F;3-8H).

Samples were also incubated in solutions containing either magnesium or manganese to determine if DGC activity was affected by the presence of a specific cofactor. DGC activity was determined to be independent of these specific cofactors under conditions in which the protein was not activated, or potentially activated by artificial phosphorylation, cAMP or cGMP (Figures 3-7;3-8).


A.
mAU
200
150
100
50
GTP Alone
24
26
28
30
32
Min
B.
mAU
200
150
100
50
TDE0125 And GTP
24
26
28
30
32
Min
C.
mAU
200
150
100
50
C-di-GMP Alone
24
26
28
30
32
Min
D.
mAU
200
150
100
50
TDE0125 w/AcP
And GTP
24
26
28
30
32
Min
E.
mAU
200
150
100
50
cAMP Alone
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26
28
30
32
Min
F.
mAU
200
150
100
50
TDE0125 w/cAMP
And GTP
24
26
28
30
32
Min
G.
mAU
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cGMP Alone
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Min
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mAU
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150
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50
TDE0125 w/cGMP
And GTP
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26
28
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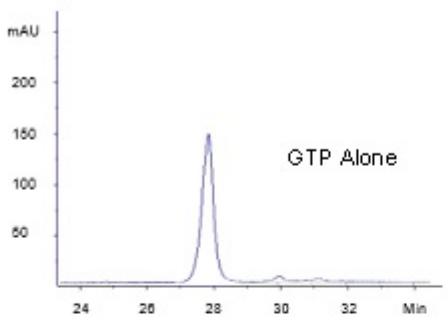
Min

Figure 3-7: HPLC Analysis of c-di-GMP synthesis by TDE0125 (Magnesium). The ability of TDE0125 to produce c-di-GMP in the presence of the cofactor magnesium was assessed via HPLC. GTP (A), c-di-GMP (C), cAMP (E), and cGMP (G) standards are shown to elute out at roughly 27 minutes, 31 minutes, 33 minutes, or 28 minutes respectively. The recombinant proteins were then incubated with GTP and nucleotide products were analyzed by HPLC as detailed in the text. TDE0125 was determined to convert GTP to c-di-GMP (B). This was independent of activation by acetyl phosphate (D) cAMP (F) or cGMP (H).

A.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GTP Alone
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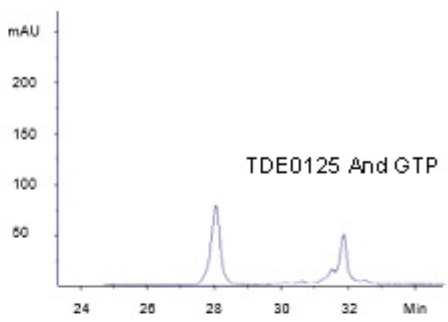
32
Min

B.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TDE0125 And GTP
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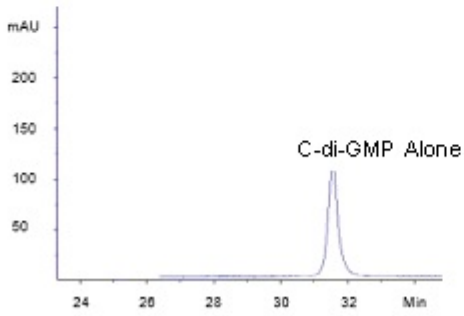
30
32
Min

C.


mAU
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C-di-GMP Alone
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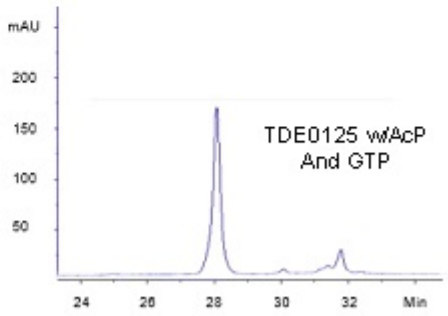
32
Min

D.


mAU
200
150
100
50
TDE0125 w/AcP
And GTP
24
26
28
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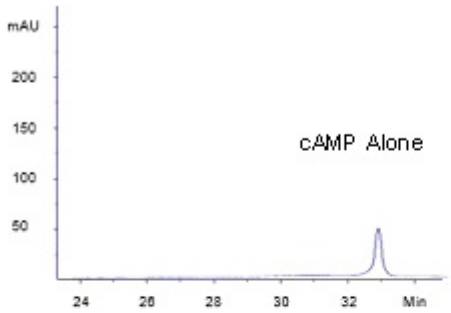
32
Min

E.


mAU
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cAMP Alone
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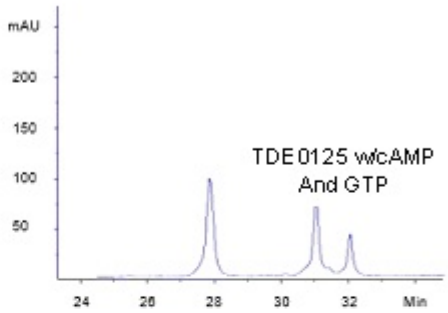
Min

F.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TDE0125 w/cAMP
And GTP
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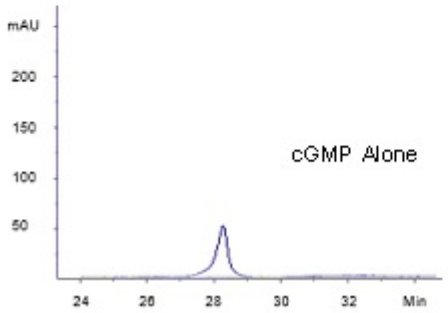
G.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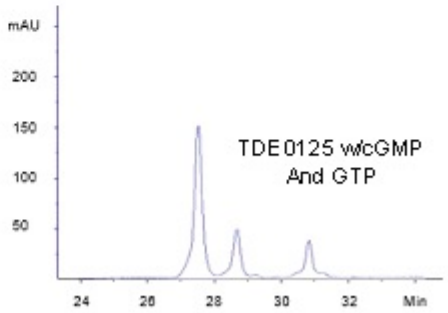
H.


mAU
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TDE0125 w/cGMP
And GTP
24
26
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32
Min

Figure 3-8: HPLC Analysis of c-di-GMP synthesis by TDE0125 (Manganese). The ability of TDE0125 to produce c-di-GMP in the presence of the cofactor Manganese was assessed via HPLC. GTP (A), c-di-GMP (C), cAMP (E), and cGMP (G) standards are shown to elute out at roughly 27 minutes, 31 minutes, 33 minutes, or 28 minutes respectively. The recombinant proteins were then incubated with GTP and nucleotide products were analyzed by HPLC as detailed in the text. TDE0125 was determined to convert GTP to c-di-GMP (B). This was independent of activation by acetyl phosphate (D) cAMP (F) or cGMP (H).

Chapter 4: Discussion

C-di-GMP has been studied as a molecule of interest in second messengers for nearly 30 years (61). In spirochetes, it has been found to be a very important molecule in regards to virulence and infectivity through its impact on motility (46). The turnover of c-di-GMP in the cell is controlled by DGCs, which sythesize c-di-GMP, and the PDEs which break it down. The generation of c-di-GMP is important for the modulation of many factors contributing to host invasion, but it does not carry out these effector functions on its own (60). Instead, it relies on receptors that bind to c-di-GMP (60). This second messenger molecule is extremely important to cell function, yet the literature in regards to c-di-GMP signaling in *T. denticola* is relatively sparse. However, the c-di-GMP signaling mechanisms in *Borrelia* and *Borreliella* spirochetes are much better characterized and can serve as an example for how crucial the second messenger is for motility and ultimately virulence.

Mutation studies have been used to elucidate how c-di-GMP functions in the Lyme disease causing spirochete, *B. bugdorferi*. Rogers et al. was the first to generate an *rrp1* deletion mutant (Δ*rrp1*) thus removing the ability of the spirochete to produce c-di-GMP (59). Transcriptome analysis of Δ*rrp1* revealed that this mutation affected several genes when compared to the wild type. These genes were involved with motility, chemotaxis, and metabolism, of which correlates to 10% of the genome. This suggests that c-di-GMP may be an important regulator of these processes (59).

Hpk1, in addition to Rrp1, has been shown to be upregulated during tick feeding in many other studies as well (27, 52, 59). This implies that the Rrp1-Hpk1 system may be important in the expression of genes significant for spirochete survival in the tick. Additional mutational experiments have shown that Rrp1 and its cognate histidine kinase, Hpk1, are important for *B. burgdorferi* survival in ticks (36, 43). This was confirmed experimentally with tick infection studies in which deletion mutants lacking the *rrp1* or the *hpk1* genes were unable infect the tick host (36, 43) .This proved c-di-GMP's role in *B. burgdorferi* survival within the tick.

It has been shown that intracellular levels of c-di GMP do not only affect the survival of the Lyme spirochete within the tick, but also alter its ability to survive in the mammalian host (43, 74, 75). This was shown experimentally with an Rrp1 overexpressing strain which showed an inability to infect neither the tick nor the murine host (43). In addition, the *pdeA* deletion mutant exhibited an increase in intracellular c-di-GMP accumulation (75). This ultimately led to an inability to infect mice even at very high bacterial loads, however the ability of the mutant to infect the tick remained the same (75). This phenotype within the *Borreliella* is consistent with the rest of the bacterial world where high levels of c-di-GMP correlate with decreased infectivity (60).

Through the use of mutational studies, c-di-GMP levels have been shown to be important for host invasion and ultimately progression through the enzootic cycle in the *Borrelia* as well as in the *Borreliella* (43, 75). C-di-GMP cannot be used in the cell without effector molecules, however, and as such the identification of these factors is important. The presence of a PilZ domain often indicates proteins that bind to c-di-GMP

(2,42). In the *Borreliella* and the *Borrelia*, PlzA and PlzC are the only confirmed PilZ domain-containing proteins which have been confirmed to bind c-di-GMP. Multiple studies have demonstrated that *plzA* deletion mutants exhibit a decrease in infectivity in not only the tick host, but also in the murine host (43,57). Despite invasion of both the vector and mammalian host, albeit mitigated, natural transmission of the spirochete from one host to the other did not occur (57). This suggests, *plzA* mutants are incapable of completing the enzootic cycle, correlating with the phenotypes of other *B. burgdorferi* strains with mutated c-di-GMP associated genes.

It is possible that the reduction in infectivity that is observed in the Δ*plzA* is due to the decrease in motility that is seen when compared to the wild type strain (43). This could be attributed to changes in the transcription levels of *cheY3*, *flil,* and *fligG1* in the Δ*plzA* (43). This phenotype could be accounted for through altered motility gene expression of protein-protein interactions with the flagellar motor. *B. burgdorferi* encodes two motor switch FliG proteins (FliG1 and FliG2) which interact with the flagellar motor to determine the direction of rotational movement. (24). FliG2 localizes to each periplasmic flagellar bundle (46). In comparison, FliG1 will localize to one of the two poles. In Δ*fliG1*, it has been found that only one flagellar bundle is capable of rotational movement even though each bundle is comprised of its normal flagellar filaments(46). This manifests itself as decreased translational motion in the cell (46). In murine infection studies, the *flig1* mutant was determined to be non infectious, reaffirming the significance of motility in spirochetal pathogenicity (6, 46).

Compared to *Borrelia and Borreliella,* there have been very few studies published on c-di-GMP signaling in *T. denticola* even though it has been readily detected in the spirochete (11, 22). As such, we have a weaker understanding of how c-di-GMP functions as a second messenger *in T. denticola* to modulate cellular responses.

The c-di-GMP network is a better studied and understood in the *Borrelia* and *Borreliella* spirochetes although c-di-GMP levels have been detected in *T. denticola* as well. In addition, just as motility plays a role in the virulence of *Borrelia and Borreliella,* it most likely plays a role in *T. denticola* with c-di-GMP implicated as a part of the signaling process. This is seen with TDE0214, one of two PilZ domain-containing proteins in *T. denticola.* This protein has been found to be a homolog of PlzA (26). In *tde0214* deletion mutants, the lack of one of the PilZ domain-containing proteins manifests itself by generating mutants that move with a reduced speed compared to the wild type (5). This leads to a decrease in motility and less invasion of tissues, overall showing a marked reduction in ability to cause infection in the murine skin abscess model (5). This study focused on characterizing the predicted DGC TDE0125 and its ability to generate c-di-GMP as a part *T. denticola*'s regulatory network.

Bioinformatics analysis revealed that TDE0125 has two domains, a GAF domain in the N-terminal region followed by a GGDEF domain at the C-terminus. The GGDEF domain is known to be indicative of a protein that is a DGC; however, proteins with this domain exist that do not have the ability to convert GTP to c-di-GMP (76). To determine if TDE0125 is a functional DGC, r-TDE0125 was constructed, purified and used to generate antiserum. Using this antiserum, we were able to localize the predicted DGC to the cytoplasm through Triton X–114 cell partitioning. The result was in line with other DGCs (59). To confirm TDE0125 is a functional DGC, we demonstrated that r-TDE0125 shows DGC activity that is independent of activation by any external factors. Potential activating factors were identified based on literature reviews and then assessed for their ability as DGC activators.

Based on the STRING database that TDE0125 and the histidine kinase, TDE0492 interact, r-TDE0125 was phosphorylated to mimic any interactions with histidine kinases (77). TDE0492 has 4 domains that could explain how it functions in the cell: A REC domain, a HAMP domain, a HisKA domain,

and a HATPase_c domain. The presence of REC suggests that phosphorylation may play a role in the activation or inactivation of TDE0492 (31). In addition, the HAMP domain may then use the phosphorylation signal to change the conformation of the protein, either causing TDE0492 to act as a histidine kinase, or a phosphotase to regulate proteins involved in the same cellular pathways (31). Interestingly, the recombinant protein incubated with acetyl phosphate showed there was a decrease in DGC activity. During an assessment of the recombinant protein's ability to convert GTP to c-di-GMP when phosphorylated, the peak area associated with c-di-GMP's retention time was observed to be significantly decreased as opposed to that of the non-phosphorylated protein. This suggests that phosphorylation does not play a major role in the conversion of GTP to c-di-GMP.

To further test potential activators of TDE0125, the protein was incubated with cNMPs. The presence of a GAF domain suggests that TDE0125 may become activated upon binding cAMP or cGMP (29) (84). Just like the results in which acetyl phosphate was added to potentially activate TDE0125, the addition of the cyclic nucleotides seemed to decrease DGC activity. This was perplexing as in LIC13137, a GAF and GGDEF domain-containing protein in *L. interrogans*, was only able to convert GTP upon activation by cAMP (16). Furthermore, Rrp1 from *B. burgdorferi* was also only able to convert GTP to c-di-GMP upon phosphorylation (64).

A possible explanation of the reduced DGC activity by all potential activators used may come from the BN-PAGE CBB stain of r-TDE0125. A majority of the recombinant protein was found to exist in a trimeric and hexameric form. This suggests that the recombinant protein exists as an active DGC trimer in solution. Typically, active DGCs exist as dimers so the two GTP molecules can interact and form the characteristic ring of a cyclic nucleotide (54, 55). It is possible that the trimeric form may affect DGC activity because the GTP molecules may fit into the active site differently when compared to the way they would in a dimeric protein (18, 63). In addition, the fact that the level of DGC activity tended to be lower when a stimulus was added to potentially activate the protein may be because the activated state could allow for the increased formation of the hexameric conformation. This conformation may be subject to stronger feedback regulation by c-di-GMP in the inhibitory "I-site" compared to the recombinant protein that was not incubated with any potential activators.

De et al propose such an explanation for r-WspR, which showed similar results when they added beryllium trifluoride, a commonly used compound mimicking phosphorylation and saw a modulation of r-WspR's activity. R-WspR can be active without being phosphorylated as it can exist in the active dimeric state; however, *in vivo* studies show that the protein must be phosphorylated before it can function (18, 38). They believed that during *in vitro* studies, the dimeric recombinant protein was forming a tetrameric structure that caused an overall decrease in activity after an initial burst (18). They argue the initial burst of activity led to an abrupt increase in the cellular concentration of c-di-GMP. Through a mechanism of allosteric inhibition, the c-di-GMP would then bind to the inhibitory "I-site" and stop any further DGC activity. Given that it has been shown recombinant proteins that are purified and have a high concentration can naturally be in a dimeric or trimeric structure, it is possible that r-WspR and r-TDE0125 exist in conformations that can actively convert GTP to c-di-GMP. In addition, as TDE0125 does have an "I-site" located on its GGDEF domain, inhibition of the "I-site" is a possible method of modulation for the protein.

The RPC data that was collected during these series of experiments looked at various methods of activation; however, something else that may affect DGC activity is the presence of oxygen. It is known that GAF domains have a similar fold to PAS domains, which can respond to oxygen or light (78, 84). As TDE0125 is a protein from an anaerobic spirochete, it is possible that the DGC activity of the protein may be enhanced under anaerobic conditions.

This study also examined how conserved the gene was across multiple isolates of *T. denticola*. By initially conducting an amino acid alignment using NCBI's BLAST program, we determined that the isolates found within the database have a near identical sequence to *T. denticola* isolate 35405 (34). 12 more isolates from our collection were also chosen and the TDE0125 gene was PCR amplified from those strains and sent out for sequencing. These results agreed with the BLAST search that the gene was highly conserved. The amino acid sequences of the protein from the 12 isolates, in addition to the 17 amino acid sequences from BLAST, were used to generate a phylogenetic tree. What was interesting was how conserved the gene appeared to be across all isolates. Sequence identity analyses using BLAST showed that the most dissimilar sequences, SP33 and SP32, still had a ~92% sequence identity (34). This suggests that the gene and the protein it encodes may be important to *T. denticola* strains as a whole.

Similar results were seen in an immunoblot of various *T. denticola* isolates was screened with antisera generated against isolate 35405's r-TDE0125. This result confirmed that not only was the protein produced under *in vitro* growth conditions, but that it was readily expressed in all isolates that were screened.

Future directions of this project will attempt to understand the importance and function of TDE0125 in *T. denticola*'s regulatory network through X-ray crystallography and the generation of r-mutants via site-directed mutagenesis. X-ray crystallography will be used to determine the structure and observe how the oligomeric form affects the binding of molecules such as GTP, metal ions, etc. This understanding of the DGC activity of TDE0125 will also be furthered with the generation of mutant proteins as well as mutant strains lacking the TDE0125 gene. The recombinant mutants will give us the ability to learn how the domains and various binding sites affect protein activity and function. Ultimately, these mutants will help us determine how the function of TDE0125 contributes to the overall cellular function of c-di-GMP in the cell as well as under what conditions. As such, these steps will be crucial in advancing our understanding of TDE0125 in regards to *T. denticola's* infectivity.

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Vita

Presentations

January 2018, poster presentation, “Construction and analysis of chimeric *Treponema denticola* FhbB based vaccine antigens for prevention of periodontal disease,” **Dhara T. Patel***,* Nathaniel S. O’ Bier, Lee D. Oliver, Daniel P. Miller, and Richard T. Marconi. Gordon Research Conference. Ventura, CA