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Treponema denticola synthesizes c-di-AMP and encodes the CdaA-type diadenylate cyclase CdaA

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Treponema denticola synthesizes c-di-AMP and encodes the CdaA-type diadenylate cyclase CdaA

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

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List of Abbreviations

°C	Degrees Celsius
ANOVA	One-way analysis of variance
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
B-PER	Bacterial protein extraction reagent
BCA	Bicinchoninic acid
BLAST	Basic local alignment search tool
BCG	Bacille Calmette-Guerin
c-di-AMP	3',5'-cyclic di-adenosine monophosphate
c-di-GMP	3',5'-cyclic di-guanosine monophosphate
CdaA	Cyclic-di-AMP synthase A
CdaM	Cyclic-di-AMP mycoplasma
CdaR	Cyclic-di-AMP synthase regulator
CdaS	Sporulation-specific cyclic-di-AMP synthase
CdaZ	Cyclic-di-AMP synthase in archaea
cGAS	Cyclic GMP-AMP synthase
CC	Coiled-coil
CM	Cellular membrane
CO_2	Carbon dioxide
DAC	Diadenylate cyclase
DisA	DNA integrity scanning protein A
DNA	Deoxyribonucleic acid

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPS	Extracellular polysaccharide
FPLC	Fast performance liquid chromatography
g	Relative centrifugal force
g	Gram(s)
h	Hour(s)
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IFN-β	Interferon-β
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilodalton
Mbp	Million base pairs
MEGA	Molecular Evolutionary Genetics Analysis
mg	Milligram(s)
min	Minutes(s)
mL	Milliliter(s)
mM	Millimolar
MSP	Major outer sheath protein
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
ng	Nanogram(s)
OH	Hydroxide

ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulfate
Sec	Second(s)
SEM	Standard error of the mean
STING	Stimulator of interferon genes
TAE	Tris acetate EDTA buffer
TBS	Tris buffered saline
Td	*Treponema denticola*
TLR	Toll-like receptor
TM	Transmembrane
V	Volts
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar

Abstract

Treponema denticola synthesizes c-di-AMP and encodes the CdaA-type diadenylate cyclase CdaA

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Periodontitis is a form of oral disease characterized by dysbiosis of the oral microbiome, leading to inflammation, bone resorption, and in severe cases, entire tooth loss, affecting 42% of adults in the US. One of the bacteria most associated with periodontal disease progression is *Treponema denticola* (*Td*), an oral spirochete which inhabits the mouth in small quantities during health but which can dominate the biofilms that form during periodontal disease. The ability of *Td* to survive in a disease environment and contribute to the progression of disease requires the use of robust signaling networks. Analysis of *Td* cultures revealed the existence of a potential regulatory signaling network in *Td*, one using the nucleotide-based second messenger molecule cyclic-di-AMP (c-di-AMP), and further allowed for characterizing a diadenylate cyclase enzyme responsible for its synthesis in *Td*, along with a potential regulator of diadenylate cyclase activity. This enzyme was shown to be encoded in the genomes of a variety of

Td strains and other species of treponemes. This opens for study a wide range of signaling pathways and responses which may be based on c-di-AMP, which is capable of regulating growth, physiology, and virulence. These data help provide, for the first time, evidence for the existence of c-di-AMP in *Td*, and presents a new set of targets for treatment of *Td* infection.

Chapter 1: Introduction

1.1 *Treponema denticola* background

1.1.1 Classification

Treponema denticola is an exclusively anaerobic Gram-negative oral bacterium. It is a member of the Spirochaetes, a bacterial phylum characterized by a helical shape and the presence of endoflagella which are located within the periplasm[1] which grant the bacteria a unique form of motility which is enhanced compared to other bacteria in highly viscous environments[2] and which are thought to aid in tissue penetration during oral infection[3]. The genera of Spirochaetes include *Leptospira*, the causative agents of leptospirosis[4], *Borrelia,* which includes the etiologic agents of different forms of relapsing fever, e.g., *B. recurrentis* in the case of epidemic relapsing fever and *B. hermsii* as one bacterium implicated in tick-borne relapsing fever[5], *Borreliella,* which includes the etiologic agent of Lyme disease, *B. burgdorferi*[5] and *Treponema*, which includes the etiologic agent of syphilis, *T. pallidum*[6], as well as the oral treponeme *T. denticola*[7] As an oral treponeme, *T. denticola* is associated with different forms of oral disease, along with other species of oral treponeme like *T. socranskii*[8], *T. lecithinolyticum, T. medium*, and *T. maltophilum*[9] but especially with periodontitis, the inflammation of the space where the epithelial cells of the gum meet the tooth, the periodontium[10].

1.1.2 Morphology and genomics

T. denticola is motile, using its periplasmic endoflagella for motility[3], spiral-shaped[11] (Figure 1), and relatively small compared to other spirochetes, ranging

between 6-16 μm in length and 0.1 μm to 0.25 μm in width[12]. The genome of the *T. denticola* type strain (ATCC 35405) is 2.8 Mbp in size, with a G+C content of 37.9% and 2786 genes identified as protein-coding, including multiple proteases which are important to its ability to degrade host tissue and fulfill its nutritional requirements[13].

Also important to note is the intractability of *T. denticola* to genetic manipulation. Already quite fastidious, attempts to construct mutants of *Td* often meet with failure, either through electroporating the cells or making them chemically competent for heat shock transformation, though both have generated notable mutants in the past[14].

1.1.3 Niche

T. denticola inhabits the oral biofilm which develops where the surface of the tooth meets the gums[8]. The bacterium is present at low levels during oral health, but increased in abundance during oral disease such as periodontitis 8` as it associates with and binds to other periopathogens like *F. nucleatum* and *P. gingivalis* through the expression of its primary surface protein, major outer sheath protein (MSP)[15]. It has also been shown to reside within the epithelial cells of individuals with the disease most commonly associated with *T. denticola*, inflammation of the periodontium, or periodontitis[16].

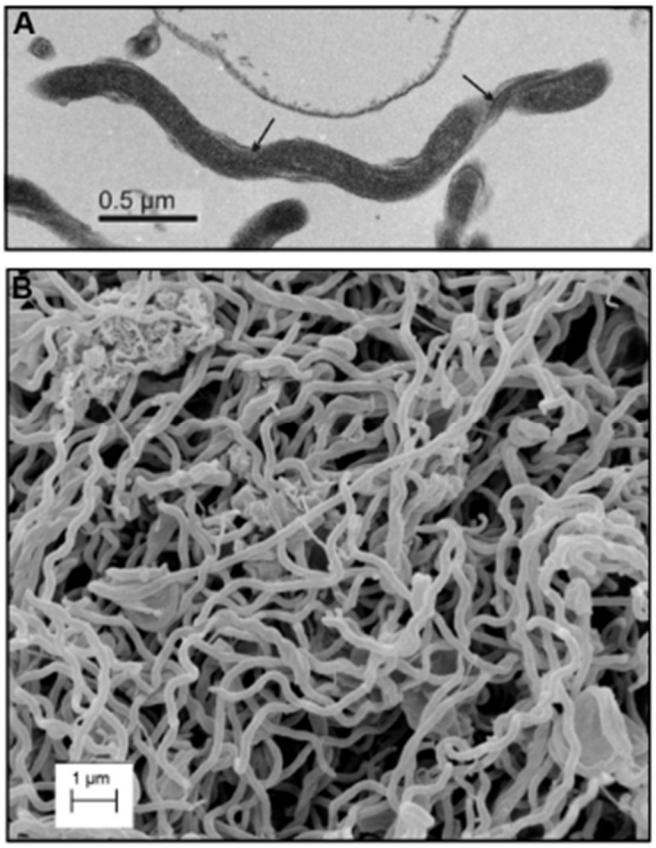


Figure 1. Electron microscope images of *T. denticola*. A) Transmission electron microscopy, with arrows highlighting bundles of endoflagella. B) Scanning electron microscopy of *T. denticola*. Adapted from Frederick *et al.*, 2011[11]. Molecular signaling mechanisms of the periopathogen, *Treponema denticola*. *J Dent Res*. 2011;90(10):1155-1163. Republished with permission from SAGE Journals.

1.1.4 Virulence and infection

Td plays a role in furthering the development of periodontal disease by adhering to fibroblasts and epithelial cells of the periodontium and extracellular matrix components, expression of surface proteins including peptidases, proteinases, and pore-forming proteins[10], and interacting with other oral pathogens to enhance overall virulence[17]. The unique protease complex dentilisin has been studied for its role as a virulence factor, and is suggested to allow enhanced tissue penetration, modulation of host cell responses by degrading certain immune factors, and interacting with *P. gingivalis* fimbriae to support infection[18]. Invasion of host tissues and cells[16] supports disease, and is likely aided by the unique motility of spirochetes[3]. The ability of *Td* to stimulate host inflammation by binding Toll-like receptors TLR2 and TLR4 has been identified[19]. *Td* additionally has a unique profile of antimicrobial susceptibility, showing consistent resistance to fluoroquinolones and susceptibility to the macrolides azithromycin and erythromycin as well as the tetracyclines doxycycline and minocycline[20]. This variety of factors and behaviors are part of the reason *Td* is heavily implicated in the progression of periodontitis.

1.2 Periodontitis

1.2.1 Incidence

Periodontitis is a global noncommunicable disease which represents a massive burden on the world population, with severe periodontitis being found in 11.2% of individuals worldwide and mild to moderate periodontitis affecting a majority of adults

worldwide[21]. It shows a variable incidence by age, peaking at around 38 years old before declining with increasing age[21]. Counting all types of periodontitis, one recent study found that 42% of US adults had periodontitis ranging from mild to severe[22], showing the significant burden which this disease represents for health in the United States.

1.2.2 Clinical manifestations

Periodontitis has been categorized into separate forms, aggressive periodontitis and chronic periodontitis, which primarily differ based on the speed at which the disease occurs, but other differences such as the relationship to systemic disease and association with microbial burden are apparent between the two forms[23]. Both forms are associated with the same major symptoms – inflammation of the gums, formation of hard plaques and calculus at the tooth surface, formation of deep gum pockets in which biofilms form, and alveolar bone resorption which is capable of causing complete tooth loss[23]. This is caused by a combination of factors from the microbes present in the plaques as well as their interactions with the host and the resulting responses by the host epithelium, including the expression and secretion of inflammatory cytokines and recruitment of inflammatory immune cells to the periodontium[24,25,26], as reviewed by Darveau in 2010[27]. These symptoms are driven by a shift in the makeup of the oral microbiome from saccharolytic, aerobic, Gram-positive bacteria to an increased proportion of proteolytic, anaerobic, Gram-negative bacteria[28]. Previously, researchers identified certain bacteria like *T. denticola*, *T. forsythia,* and *P. gingivalis* as being the main drivers of periodontitis and termed them the “red-complex” of bacteria[29], but more

recent work has identified the ability of a vastly increased profile of species to act as pathobionts or opportunistic pathogens in periodontitis, whose virulence and adverse health effects can increase or be activated during the progression of disease by so-called “keystone pathogens” which can even at low abundance have a community-wide impact on the microbiome and begin modulating host responses towards inflammation[30,31], presenting a new model of understanding how periodontitis arises from host interactions with the microbiome.

1.2.3 Association with other diseases

Periodontitis is associated with numerous other systemic noncommunicable diseases. It has been found to be significantly associated with cardiovascular disease[32], to increase the mortality of kidney disease sufferers[33], and to be a risk factor for diabetes and diabetes complications[34]. It has even been found to be a risk factor for oral cancer[35], as invasion of host tissues by bacteria cause irregular cell cycle behavior in host cells, and driver mutations for oral cancer cause the microbiome shift characteristic of periodontitis to occur. Associations with other systemic diseases occur for a variety of potential reasons, such as the increased risk of bacteremia in periodontitis patients[36], and the induction of inflammation in the heart through the production of inflammatory cytokines[37] in response to periodontal pathogens.

1.2.4 Diagnosis, treatment, and prevention

The primary methods for diagnosis involve the calculation of probing depths, the distance a clinician can insert a tool into the space between the tooth and gum, the

presence of bleeding on probing, the degree of bone loss as determined by radiography, and the degree of attachment loss of the gum to the tooth[38]. The results are then graded by severity into mild, moderate, or severe/advanced periodontitis.

Treatment of periodontitis involves a variety of factors, although the most beneficial for most patients are the purely mechanical techniques of scaling, root planing, and cleaning that a clinician can perform to physically remove the biofilms from the surface of the tooth and allow for reattachment of the gums to the teeth and resolution of inflammation[27]. Other therapies for treatment of periodontitis have been studied, from the administration of antibiotics[39], attempts to modify the host inflammatory responses that provide the other set of factors leading to periodontitis[40], to therapies hoping to promote the mechanical therapy traditionally used to treat the disease[41], which have mixed results in terms of efficacy and which come with downsides in the form of side effects or requiring specialized equipment or training to perform. Another promising route is the use of vaccination against certain pathogens that are the most highly associated with periodontitis, such as *P. gingivalis*[42]; work is ongoing to develop a similar vaccine against *T. denticola*[43]. There is a great need for novel methods of treating periodontitis given that the global burden is high and current therapies are limited in their ability to treat disease and prevent recurrence.

Prevention of periodontal diseases like periodontitis is, in the absence of effective vaccines for pathogens involved in its progression, primarily focused on the maintenance of good oral hygiene, especially in individuals with risk factors for periodontitis, such as diabetes[38].

1.3 c-di-AMP

1.3.1 Discovery

The discovery of c-di-AMP was made when a certain DNA repair protein, DisA, was investigated by Witte *et al.* in 2008[44]. Two homologs of DisA, one from *Thermotoga maritima* and another from *Bacillus subtilis*, were noted to control the ability of these bacteria to begin sporulation, and crystal structure analysis yielded results indicating that these enzymes could catalyze the synthesis from two molecules of ATP of a novel nucleotide second messenger, c-di-AMP. This activity was suppressed in the presence of a branched Holliday junction in the DNA to which DisA was bound, implying that DisA was able to signal DNA damage by shutting off c-di-AMP synthesis when it encountered dangerous DNA structures; these enzymes were termed diadenylate cyclases (DACs) for their ability to synthesize c-di-AMP[44]. While c-di-GMP was at this point known as a bacterial nucleotide second messenger[45], this was the simultaneous discovery of the presence of c-di-AMP in bacteria as well as the first diadenylate cyclase enzyme capable of its synthesis. Since then, c-di-AMP has come to be found in a wide variety of bacteria, such as *S. aureus*[46], *L. monocytogenes*[48], and Gram-negative species like *C. trachomatis*[49]. A diadenylate cyclase has even been validated from the archaeal species *Methanocaldococcus jannaschii*[50].

1.3.2 Structure

c-di-AMP is a second messenger dinucleotide synthesized from two molecules of ATP by an enzyme with diadenylate cyclase activity[44], resulting in the formation of two

phosphodiester bonds linking the two AMP moieties at their 3' and 5' carbon atoms. c-di-AMP displays several conformations, ranging from the highly extended conformation shown in Figure 2b. to a highly stacked, U-type conformation where the nucleobases are parallel to each other[51]. This flexibility is suggested to allow it to bind to a variety of molecules, including proteins and c-di-AMP sensing riboswitches[51]. The 3' OH groups on the ribose sugars are responsible for the nucleophilic attack on the corresponding phosphate group which results in the loss of one molecule of pyrophosphate per ATP molecule and the formation of the 12-member ring at the center of c-di-AMP[44].

1.3.3 Biological role

As a second messenger molecule, c-di-AMP acts as an overall sensor in bacteria which synthesize it, with global levels of c-di-AMP functioning to regulate the various molecules it is capable of binding, which are diverse and responsible for a variety of functions, including controlling DNA repair, biofilm formation, and virulence[52].

It is essential for many bacteria which synthesize it, but overly high concentrations of c-di-AMP within bacterial cells can also be lethal, leading it to be termed an "essential poison" for these bacteria[53]. Notably, c-di-AMP is absent from *E. coli*, making the bacterium a powerful potential platform to study the effects of individual genes involved in the metabolism of c-di-AMP without interference from native enzymes[46]. Synthesis of c-di-AMP is performed by diadenylate cyclase enzymes, and degradation is performed by c-di-AMP-specific phosphodiesterases[54].

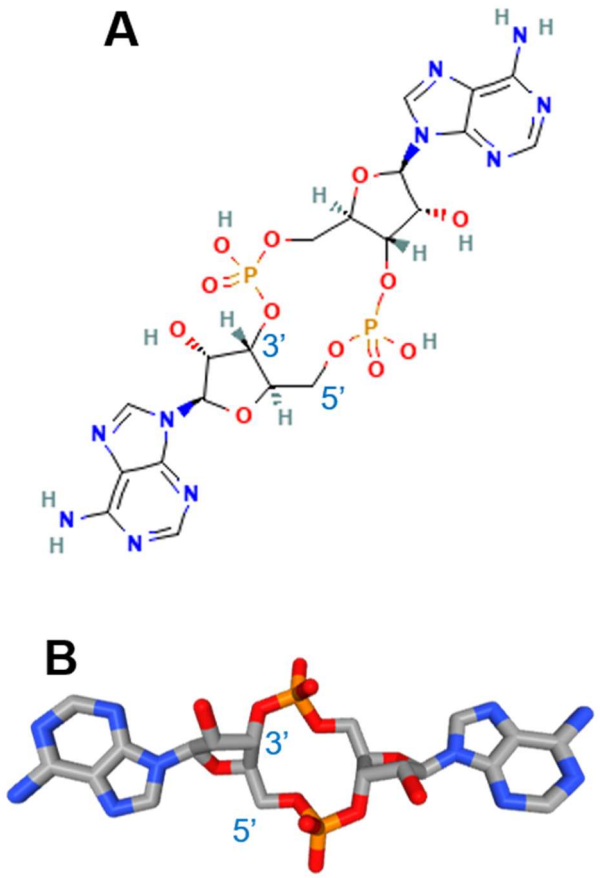


Figure 2. Chemical structure of c-di-AMP. A) The 2D structure of c-di-AMP. The nucleobases, ribose sugars, and phosphate groups make this molecule identifiable as two molecules of AMP joined by bonds between the ribose sugar and the phosphate group at the 3' and 5' carbons of the ribose sugars of the two AMP molecules. B) The 3D structure of c-di-AMP. 3' and 5' carbons of one molecule of AMP are labeled. Structures accessed from PubChem databases.

1.4 c-di-AMP binding partners

1.4.1 DisA and DNA repair

DNA damage sensing was the very first biological role identified for c-di-AMP when it was discovered as a product of the diadenylate cyclase activity of the DNA Holliday junction sensing protein DisA[44]. It was noted to function in response to the initiation of sporulation within *B. subtilis*, scanning for damage, and upon discovery of DNA damage forming a complex with other DisA molecules and managing to inhibit sporulation. Besides a DNA-binding motif, however, it had no previously recognizable enzyme motifs, leaving its role in regulating sporulation unknown.

After structural and functional analysis in which the diadenylate cyclase activity of DisA was identified, the researchers described the diadenylate cyclase motif, identified c-di-AMP as a second messenger in bacteria, and importantly identified many bacterial genes containing a diadenylate cyclase motif, implying the existence of c-di-AMP within a variety of other bacteria[44]. They discovered that the diadenylate cyclase activity of DisA was inhibited upon discovering DNA damage, and that the resulting low levels of c-di-AMP could prevent the bacterium from completing sporulation[44] until the damage was resolved.

Further experiments with a c-di-AMP and diadenylate cyclase-deficient mutant of *B. subtilis* showed that c-di-AMP supplementation was able to restore resistance to DNA damage induction and promote the health of spores in response to DNA damage[55].

1.4.2 Potassium transport

c-di-AMP has been identified as a negative regulator of potassium transport in multiple bacterial species, including *S. aureus*[47], *S. pneumoniae*[56], and *L. monocytogenes*[57]. It binds to different proteins in these species, but a similar phenotype is established in response to c-di-AMP; deletion of c-di-AMP synthesis machinery impairs growth on potassium-containing media[57], while overaccumulation of c-di-AMP by deletion of genes encoding the enzymes responsible for breaking down c-di-AMP renders mutants unable to grow on media with low concentrations of potassium[47], demonstrating that c-di-AMP metabolism is essential for effective osmoregulation within these bacteria. In *S. aureus*, the protein KtrA, a member of the RCK family of potassium transport regulators, was identified as a c-di-AMP binding protein which was required for growth in low-potassium conditions; its ability to open the membrane-bound KtrB potassium transport protein is inhibited by c-di-AMP binding[47]. In *S. pneumoniae*, the CabP protein interacts with the potassium uptake protein SPD_0076 to allow for efficient potassium uptake, unless bound by c-di-AMP which inactivates CabP and in turn reduces potassium uptake efficiency[56]. In showing what role c-di-AMP might have in promoting growth in high-salt environments, *L. monocytogenes* mutants without the capacity to synthesize c-di-AMP effectively were shown to have worsened growth at high concentrations of potassium compared to wild-type *L. monocytogenes*, which was linked to its ability to inhibit the activity of the KimA potassium transport protein[57]. In *B. thuringiensis*, control of expression of the KdpFABC potassium transport system is regulated by a c-di-AMP-binding riboswitch, where c-di-AMP prevents translation of

transcripts from the *kdpFABC* operon unless levels of c-di-AMP decrease in response to low potassium concentrations[58].

1.4.3 Biofilm formation

c-di-AMP has also been implicated in the secretion of extracellular polysaccharide (EPS) and the formation of a biofilm in multiple bacterial species. Both Cheng *et al.* and Peng *et al.* showed in 2016 a role for c-di-AMP in the regulation of biofilm formation in *S. mutans*, although with conflicting results; deletion of the *cdaA* gene which encodes the CdaA diadenylate cyclase responsible for c-di-AMP synthesis appeared to result in increased production of EPS in research by Cheng *et al.*[59], but deletion of the *pdeA* gene which encodes the PdeA c-di-AMP phosphodiesterase responsible for its degradation resulted in increased production of EPS in the data from Peng *et al*. that same year[60]. In a comment on the discrepancy, Peng defended their results as being more reflective of the actual role of c-di-AMP, citing the fact that they observed complementation of the *pdeA* gene as partially restoring the wild-type phenotype and that their data showed expected effects on glucosyltransferases responsible for EPS synthesis[61], as well as identifying the CabPA c-di-AMP binding protein within *S. mutans*. In *B. subtilis*, c-di-AMP appears to have a negative effect on biofilm formation, and the repressor of biofilm synthesis genes SinR was implicated downstream of c-di-AMP as inactivation of SinR prevented high c-di-AMP concentrations from inhibiting biofilm synthesis[53]. Another oral pathogen, *P. gingivalis*, was shown to require c-di-AMP for biofilm formation, along with noting an unusual metabolic system for c-di-AMP where expression of a c-di-AMP phosphodiesterase

increased c-di-AMP concentrations within the bacterium[62]. This is especially relevant in oral disease because *P. gingivalis* interacts with *Td* for synergistic biofilm development, so the mechanisms by which either organism induces biofilm production may have important consequences for periodontal disease driven by these two organisms[17].

1.4.4 Polysaccharide and cell wall biosynthesis

c-di-AMP has been described as positively regulating the synthesis of peptidoglycan in bacterial cells and as regulating the formation of the bacterial cell wall more generally, as noted in the construction of bacterial mutants incapable of synthesizing or degrading c-di-AMP[46,48,63]. In *S. aureus*, c-di-AMP was discovered during analysis of lipoteichoic acid-deficient strains which were still able to grow normally; this was found to be in multiple cases due to a suppressor mutation in the gene for the c-di-AMP-degrading phosphodiesterase, *gdpP*[46]. This mutation resulted in the accumulation of c-di-AMP, an increase in the amount of cross-linked peptidoglycan within the cell wall, and decreased cell size, suggesting that c-di-AMP plays a positive role in peptidoglycan synthesis within *S. aureus*[46]. Showing a similar role for c-di-AMP, a *L. monocytogenes* mutant with a deletion in the *dacA* gene for a diadenylate cyclase enzyme showed greatly increased sensitivity to cefuroxime, a cell-wall targeting β-lactam antibiotic, and higher rates of *in vitro* bacteriolysis in normal BHI media[48]. The *B. subtilis* σ(M) factor, known to provide resistance to several cell-envelope targeting antibiotics, appears to play a role in resistance to cefuroxime that is related to the function of c-di-AMP, as *B. subtilis* mutants with an inactive *gdpP* gene restore cefuroxime resistance to mutants without σ(M)[63].

1.4.5 Control of carbon cycle metabolism

A role for c-di-AMP has been determined in regulation of the central metabolic enzyme pyruvate carboxylase in *L. monocytogenes* and *E. faecalis*, binding to a regulatory site near the enzyme's active site and reducing its ability to convert pyruvate to oxaloacetate[64]. This was found to affect growth rates of *L. monocytogenes* and the production of glutamate and glutamine from oxaloacetate, and to have important effects on bacterial intracellular growth, with mutants of the *dacA* diadenylate cyclase gene growing more slowly and showing higher bacteriolysis within primary bone marrow derived macrophage cell lines[64], likely a consequence of metabolic imbalance as carbon cycle metabolism becomes dysfunctional without c-di-AMP regulation.

1.4.6 Riboswitches

The *ydaO* class of riboswitches has been shown to control a wide range of genes, especially those related to the cell wall metabolism, osmotic stress, and spore germination[65]. It was identified as being a c-di-AMP sensing riboswitch, preventing translation of genes whose mRNA transcripts contain the motif in the presence of c-di-AMP[66], which accords with findings that c-di-AMP regulates proteins involved in the same pathways, suggesting the use of the c-di-AMP-binding *ydaO* riboswitch is another mechanism by which c-di-AMP regulates these pathways. The *ydaO* riboswitch is notable for containing a high number of recurrent motifs such as three-way junctions, and for effectively discriminating against c-di-GMP and 3'3'-cGAMP[67]. This same class of riboswitch was shown to directly regulate transcription of genes in the potassium-

uptake complex *kdp* within *B. thuringiensis*, directly linking the ability of the c-di-AMP riboswitch to bind c-di-AMP to the efficiency of potassium transport[58].

1.5 Diadenylate cyclases

1.5.1 Categories

Diadenylate cyclase proteins serve diverse roles within bacteria and have been identified in Archaea as well[50]. These enzymes catalyze the formation of c-di-AMP from two molecules of ATP, resulting in an increase of intracellular concentrations of c-di-AMP and allowing c-di-AMP to bind and regulate its downstream targets within the cell[68]. There are five currently identified classes of diadenylate cyclases: the DisA class[44], which are involved in DNA repair, the CdaA class[69], important membrane-bound overall synthesizers of c-di-AMP within bacterial cells, the CdaS class[69], which provide c-di-AMP synthesis particularly in the process of sporulation, the CdaM class[70] responsible for c-di-AMP synthesis in *Mycoplasma* and lacking the distinctive N-terminal domains of diadenylate cyclases belonging to other classes, and the CdaZ class[50] responsible for c-di-AMP synthesis in the archaeal species *Methanocaldococcus jannaschii*.

These enzymes can all play roles in c-di-AMP metabolism within a cell, and some species have multiple diadenylate cyclases belonging to different classes, such as *B. subtilis*[69].

1.5.2 Structure and function of CdaA-type diadenylate cyclases and their regulatory partner CdaR

The CdaA-type diadenylate cyclases are the most abundantly found in bacteria[71], and the one with a predicted member in *T. denticola*, encoded by the gene TDE_1909, appears to belong to this class and is labeled CdaA here based on its predicted function as a diadenylate cyclase. These DACs are characterized by the presence of transmembrane domains at their N-terminus, a diadenylate cyclase domain with a conserved HDGA…RHR protein sequence, and two coiled-coil (CC) motifs around the N- and C-terminal ends of the diadenylate cyclase domain[72]. Furthermore, these DACs are often encoded in an operon with a regulatory protein, CdaR, which interacts at the signal peptide and modulates the diadenylate cyclase activity of the corresponding CdaA protein. In *B. subtilis*, CdaR is a positive regulator and increases DAC activity 20-fold[69], while in *L. monocytogenes*, CdaR is a negative regulator of CdaA function and deletion of CdaR improves bacterial resistance to lysozyme[73]. CdaR has an N-terminal signal peptide and 4 YbbR domains of unknown function. It is believed from experiments on truncated CdaA and CdaR proteins that the signal peptide of CdaR interacts with the transmembrane domain of CdaA to regulate its function[73] as shown in Figure 3. The function of the DacA diadenylate cyclase in *L. monocytogenes* was additionally found to require the presence of Mn^{2+} or Co^{2+}, with Mg^{2+} being unable to promote DAC activity, and dimerization of DacA needing to occur for diadenylate cyclase activity[72].

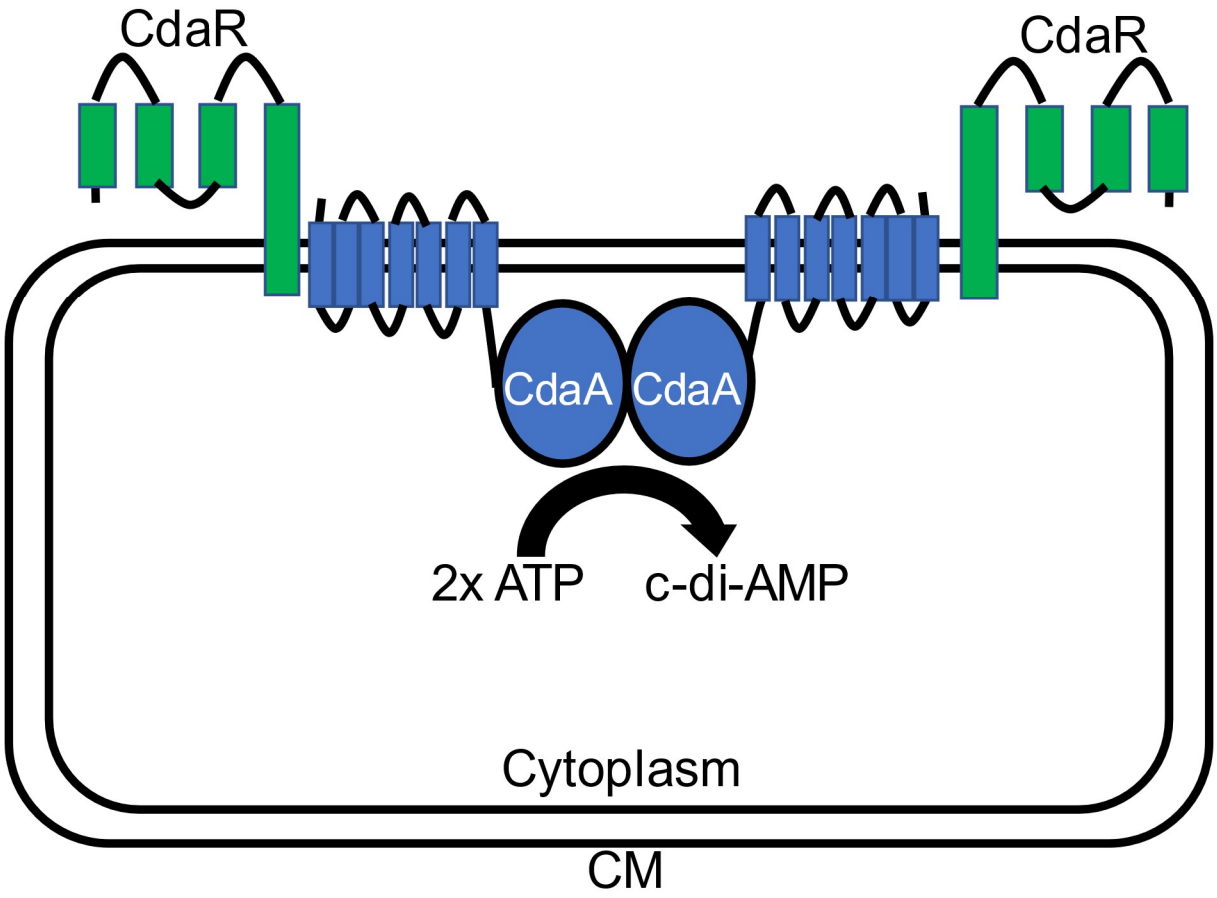


Figure 3. The structure of a CdaA-type diadenylate cyclase and CdaR. A diagram showing the localization of CdaA and CdaR to the cellular membrane of a bacterial cell and the resulting conversion of ATP to c-di-AMP by CdaA as regulated by CdaR. CM: cellular membrane.

1.5.3 Regulation, activation, and inhibition

CdaA genes are often found as part of an operon encoding three separate proteins: CdaA, CdaR, and GlmM. GlmM is an enzyme which, with its partner GlmS, generates glucosamine-1-phosphate, a crucial precursor for peptidoglycan synthesis[53]. In *B. subtilis,* this operon is constitutively expressed at low levels from a promoter upstream of *cdaA*[69]. CdaR is capable of activating or repressing CdaA by interacting at its signal peptide with the TM domains of CdaA, as described[69,73]. Other diadenylate cyclases show autoinhibition, where high concentrations of c-di-AMP bind to the DAC enzyme itself and decrease its activity, as is the case with the CdaS enzyme of *B. subtilis*[74], but such activity has not been reported for CdaA. In *S. aureus*, an additional inhibition mechanism where GlmM binding to the CdaA-type enzyme DacA is capable of inhibiting DAC activity, likely by masking the active site of DacA[75].

1.6 c-di-AMP in infection

1.6.1 c-di-AMP regulation of virulence

An important factor to consider in discerning the pathways of c-di-AMP regulation in bacteria is the resulting impact on virulence that c-di-AMP metabolism has. As c-di-AMP has been shown to be capable of regulating important factors like cell wall biosynthesis (and according sensitivity to cell wall-targeting antibiotics)[48,63], biofilm formation[60], and osmolyte transport[57], understanding the effects that this regulation has on the ability of bacteria to establish and maintain infection is key to understanding some of the clinical applications of this information, and even the potential of c-di-AMP as a drug target given its absence in eukaryotic cells. To that end, multiple pathogens

known to contain c-di-AMP have been studied for the effects of c-di-AMP regulation on virulence.

In the oral pathogen *P. gingivalis*, c-di-AMP was shown to be essential for growth and survival, improving the integrity of the bacterial cell envelope and the structure of the outer lipopolysaccharide layer and increasing biofilm formation, as well as improving the virulence potential of *P. gingivalis* in infection of *G. mellonella* larvae[62]. *L. monocytogenes* infection of macrophage cell lines was similarly impaired by the deletion of the diadenylate cyclase enzyme gene *dacA*, likely because of the decreased cell wall stability that arose[48]. The additional effect of c-di-AMP regulation of central carbon metabolism on the virulence of *L. monocytogenes* was studied by deletion of the pyruvate carboxylase gene *pycA* along with *dacA*, and resulted in improved growth in macrophage cell lines[64]. In the spirochete *B. burgdorferi*, by contrast, deletion of the c-di-AMP phosphodiesterase gene *dhhP* required complementation to survive, and complemented mutants were defective in infecting mice as well as showing slightly reduced resistance to β-lactam antibiotics, implying that c-di-AMP negatively affects virulence in this bacterium[76].

1.6.2 c-di-AMP interactions with host cells

c-di-AMP has been shown to be a pathogen-associated molecular pattern (PAMP) which is capable of activating the cytosolic surveillance pathway, a crucial pathway of the innate cellular immune system which activates NF-κB expression and results in expression of immune molecules like interferon-β (IFN-β); its interaction with c-di-AMP was first described in *L. monocytogenes* infection[77] as the host responded to secreted

c-di-AMP from the bacterium during intracellular infection. A similar mechanism was described for the Group B Streptococcus, with the identification of the cGAS-STING signaling pathway as the one responsible for induction of IFN-β in mouse cells and the identification of an ectonucleotidase CndP, encoded by the bacterium, which degrades secreted c-di-AMP; mutants without CndP resulted in higher c-di-AMP secretion and induction of higher levels of IFN-β, and were less effective at causing bacteremia in mice[78], showing that Group B Streptococcus may attempt to evade immune recognition by degrading secreted c-di-AMP, which is important for causing worsened disease in mammalian hosts. The Gram-negative pathogen *Chlamydia trachomatis* has been shown to activate IFN-β responses through STING, and it has been suggested that secretion of c-di-AMP is the secreted metabolite capable of activating this pathway during *C. trachomatis* infection[49].

Fascinatingly, this has led to the study of c-di-AMP as an immune adjuvant for cancer treatment, with the *M. tuberculosis* vaccine strain BCG used as a platform for overexpression of c-di-AMP, where it was shown to improve antitumor efficacy in a model of bladder cancer[79]. It additionally was shown to significantly reduce *P. gingivalis*-induced atherosclerosis in a mouse model when topically applied to mice, which was theorized by the researchers to be a result of heightened immune training[80].

1.7 Research objective

To determine whether *Treponema denticola* possesses the second messenger molecule c-di-AMP and, if so, what cellular machinery may be responsible for its synthesis in Td.

Chapter 2: Materials and Methods

2.1 Cultivation of *T. denticola* strains

Td strains ATCC 35405 (type strain), SP50, and SP55 were cultured in New Oral Spirochete media (NOS) in anaerobic conditions at 37°C with a gas mixture of 5% H_2, 5% CO_2, and 90% N_2[81].

2.2 Transformation and cultivation of *E. coli* BL21 cells

To generate quantities of recombinant CdaA and CdaR protein for study in HPLC, *E. coli* BL21(DE3) cells were transformed via heat shock protocol where cells were combined with pET-45b plasmid DNA containing the ORF encoding the protein of interest (generated and sequenced by Genewiz in the case of CdaA and in-lab using PCR-based cloning in the case of CdaR), cooled for 30 minutes on ice, placed in a 42°C water bath for 1 minute, and suspended in SOC media before being incubated at 37°C for one hour. After this, 200 µL of heat-shocked cells were plated on LB agar plates containing 50 mg/L of ampicillin and incubated at 37°C overnight. Growing colonies were picked, and in the case of CdaR were subjected to PCR to pick transformants which were positive for the gene of interest. Positive transformants were grown in LB containing 100 mg/L ampicillin at 37°C, and confirmation that recombinant protein expression could be selectively induced upon addition of 1 mM 1-D-thiogalactoside (IPTG) was determined by SDS-PAGE and Coomassie brilliant blue solution staining.

2.3 Reagents

Reagents used include Complete™ EDTA-free Protease Inhibitor (Sigma-Aldrich), Mini-PROTEAN® TGX™ precast 8-16% gels (Bio-Rad), Bacterial Protein Extraction

Reagent (ThermoFisher), Pierce™ Universal Nuclease for Cell lysis (ThermoFisher), ELISA kits for c-di-AMP (Cayman Chemical), and Pierce™ BCA Protein Assay Kit (ThermoFisher).

2.4 PCR, plasmid constructs, and sequencing

The ORFs of the genes for CdaA and CdaR in *Td* were cloned into the plasmid expression vector pET-45b and the dual expression vector pET-Duet-1 using Phusion® DNA polymerase (New England Biolabs) to amplify the gene from purified genomic DNA of *Td* type strain ATCC35405 as the template. After digestion of the plasmids and the amplified ORF sequences with the appropriate restriction enzymes ligation with T4 DNA ligase (Promega) was performed. Plasmids were purified using the Monarch PCR & DNA Cleanup Kit (NEB) and the resulting DNA constructs analyzed via agarose gel electrophoresis. Notably, this version of the ORF for CdaA omitted several transmembrane domains encoded on the ORF for the native gene close to the N-terminus, due to the successful expression and purification of recombinant protein containing transmembrane domains being difficult, as found during the expression of a diadenylate cyclase from *L. monocytogenes* in Rosenberg *et al.*, 2015[72]. Both plasmids also include an N-terminal hexahistidine tag within the open reading frame downstream of the T7 promoter of all multiple cloning sites, which was used for purification.

The CdaR-cloned pET-45b plasmid destined for use in recombinant protein purification was sequenced by Genewiz using their free universal primers for Sanger sequencing to determine which plasmid contained a perfectly faithful ORF for CdaR,

Name	Sequence (5'-3')
TDE1908_F_BamHI	ATCGGATCCAATGAAAATTAGAAAAATTTTTGACCG
TDE1908_R_XhoI	GATCTCGAGTTATTCGGATGTCCTTTGCTTC
TDE1909_Duet_F_NdeI	ATATCATATGATGCTTAGAACCGGAAAGCACTCAAAC
TDE1909_Duet_R_KpnI	ATAGGTACCATTTTCATCTTGTAAGGCCTCCCGC
TDE1908_Duet_F_EcoRI	ATATGAATTCTTTTTATGCCTCCCTCTTGTTATAGAAAATTCGGG
TDE1908_Duet_R_SalI	TATAGTCGACTTATTCGGATGTCTTTGCTTCCTTTTATCTTG

Table 1. Primers utilized in experiments. Primers were used to clone the TDE_1908 gene encoding CdaR in *Td* into pET-45b for recombinant protein expression. They were also used to clone the genes encoded CdaR and CdaA into pET-Duet-1 for dual expression experiments. Underlined bases represent the restriction sites present on the primers.

which was then used to transform *E. coli* BL21 cells. Results of PCR amplification, restriction enzyme digestion, and DNA ligation were visualized through agarose gel electrophoresis and UVA fluorescence on a ChemiDoc MP Imaging system (Bio-Rad).

2.5 Protein sample preparation

Total bacterial lysates were collected through use of B-PER (ThermoFisher). Bacterial cell density in culture was approximated using spectrophotometry to measure the optical density of cultures at 600 nm, and up to 5 mL of *Td* culture or 10 mL of *E. coli* culture were centrifuged at 5000 *g* for 10 minutes before being resuspended in four mL of B-PER. Lysis was performed for 15 minutes at room temperature, and the resulting lysate was centrifuged at 15000 *g* for 10 minutes in order to separate out cellular debris (e.g., cellular membranes). The resulting supernatant was used as a sample to measure c-di-AMP content within bacteria in culture through competitive ELISA.

2.6 Coomassie brilliant blue staining

After preparation by SDS-PAGE using Mini-PROTEAN® TGX™ precast 8-16% gels from Bio-Rad, protein gels were stained with Coomassie brilliant blue staining solution (1.25 g Coomassie Blue R-250 in 200 mL methanol, 50 mL glacial acetic acid, 250 mL H_2O) for 1 hour. Destaining by washing with destaining solution (400 mL methanol, 100 mL glacial acetic acid, 500 mL H_2O) was performed with rocking until the non-protein-containing regions of the gel were clear and bands correlating to proteins after electrophoresis were visible.

2.7 Bicinchoninic acid assay

Protein concentrations in samples collected from bacterial cultures were measured using the Pierce™ BCA Protein Assay Kit (ThermoFisher). Included bovine serum albumin was used to prepare standards and generate a standard curve against which the concentrations of protein in samples were determined using absorbance values generated spectrophotometrically by the SpectraMax iD3 Multi-mode Microplate Reader (Molecular Devices). This information was used to standardize the samples for use in the ELISA as a proxy for the total amount of bacterial cells used to generate the samples, allowing for accurate comparison of the samples against each other as each well was given the same amount of protein during each replicate.

2.8 c-di-AMP ELISA

Both the ORFs for CdaA and the putative CdaR homolog were cloned into the dual expression vector pET-Duet-1 as described above. Transformed *E. coli* BL21(DE3) cells were grown with shaking at 37°C in LB with ampicillin at a concentration of 100 mg/L until they reached an OD600 of roughly 0.3. Expression of both proteins was induced by IPTG added at a concentration of 1 mM and incubated at 37°C for 4 hours. Protein expression by IPTG was confirmed by SDS-PAGE and Coomassie brilliant blue staining of proteins in the resulting gel. To investigate c-di-AMP formation by these bacteria after induction of protein, 10 mL of cultures were collected after induction of protein expression, centrifuged, and lysed in B-PER as above. Samples were then subjected to a BCA assay using the Pierce™ BCA Protein Assay Kit (ThermoFisher) to determine protein content, and standardized with B-PER before being analyzed through

a kit for performing an ELISA for c-di-AMP (Cayman Chemical) to determine the overall c-di-AMP concentrations in the bacteria during incubation.

This assay is based on the principle of a competitive ELISA, through which competition for binding immunoglobulin specific for an analyte present in the sample with a horseradish peroxidase/HRP-linked c-di-AMP tracer molecule allows for detection of c-di-AMP in the sample through spectrophotometric comparison of HRP activity in sample wells to a generated standard curve. Samples were standardized for protein concentration before being added to a 96-well microplate pre-coated with IgG molecules specific for a monoclonal antibody specific for c-di-AMP. Standards were prepared and subsequently added to the plate for generation of a standard curve. After incubation at 37°C of the samples with the monoclonal antibody and the HRP-linked c-di-AMP tracer for two hours, the plate was treated with TMB which was cleaved by the HRP-linked tracer to form 3,3',5,5'-tetramethylbenzidine diimine. The reaction between HRP and the dye was allowed to proceed protected from light at room temperature for 30 minutes. A sulfuric acid-based stop solution was added to inhibit further reaction, and the absorbance values of all wells measured at 450 nm by the microplate reader. Analysis of the resulting data was performed using the spreadsheet provided by Cayman Chemical specifically for their cyclic di-AMP ELISA.

2.9 Purification of recombinant protein

With assistance and equipment from the Marconi lab, *E. coli* cells with cloned expression vectors were grown in 400 mL of autoinduction media (25 mM dibasic sodium phosphate, 25 mM monobasic potassium phosphate, 50 mM ammonium

chloride, 5 mM sodium sulfate, 10 g tryptone, 5 g yeast extract, 10 g glycerol, 1 g glucose, 4 g lactose, 1 mM $MgCl_2$, 100 mg/L ampicillin in 2L) where the lactose in the media is capable of activating T7 RNA polymerase expression under the control of the lac promoter. The resulting culture was then collected after shaking at 24°C for 24 hours, and the bacteria lysed after centrifugation at 5000 x g for 15 minutes performed at 4°C by resuspending the resulting cell pellet in binding/washing buffer with 20 mM NaH_2PO_4, 500 mM NaCl, and 20 mM imidazole calibrated to a pH of 7.4, with a Sigma Aldrich protease inhibitor cocktail at 1% v/v and Pierce™ Universal Nuclease for Cell lysis (ThermoFisher) at a final concentration of 0.5 µg/mL and vortexing the resuspension. Lysis was then completed by passing resuspended samples through an EmulsiFlex®-C3 high-pressure homogenizer three times at 1000-15000 psi at 4°C, and the resulting slurry was centrifuged at 15,500 *g* for 30 minutes at 4°C, after which recombinant protein was purified by FPLC. FPLC was performed by nickel affinity chromatography, using the ÄKTA Pure 25 M platform from Cytiva with a 1 mL HisTrap FF column at a flow rate of 4 mL/min at 4°C[82]. The HisTrap FF column was equilibrated with 5 mL of B/W buffer before samples were loaded into a 10 mL Superloop and washed with 20 mL of B/W buffer. Elution of bound protein was performed using 5 mL of elution buffer at 20 mM NaH_2PO_4, 500 mM NaCl, and 250 mM imidazole and collected in 1 mL fractions from under the observed peak at 280 nm. Fractions were then combined and subjected to dialysis into PBS overnight at 4°C using Spectra/Por 1 dialysis membranes with a 6-8 kDa MW cut-off (Spectrum Laboratories). Purity of protein stocks was determined using Coomassie brilliant blue staining and concentrations by a BCA assay (ThermoFisher).

2.10 High performance liquid chromatography

Investigating the ability of CdaA to synthesize c-di-AMP from ATP *in vitro* was done using HPLC, with assistance and equipment from the Marconi lab. Purified recombinant CdaA enzyme was added at a concentration of 5 µM to a reaction buffer consisting of 50 mM Tris HCl, 0.5 mM EDTA, and 50 mM NaCl, to which was added different metal salts to a final concentration of 10 mM to determine the activity of the recombinant enzyme in the presence of different metal cofactors. As references for the elution time of both the reagent ATP and the intended product c-di-AMP, each was incubated at 37°C for 60 minutes, boiled, centrifuged at 15000 *g* for 2 min, and passed through 0.22 µm syringe filters from Millex. HPLC was performed using a Supelco Supelcosil LC-18-T column on the Agilent 1260 Infinity II system. The two buffers used in the filtration process were a 4 mM tetrabutylammonium H_2S, 100 mM KH_2PO_4 buffer at pH 5.9, and 100% methanol. Nucleotides were separated using a gradient of 0-50% methanol progressing linearly over 20 minutes. The reaction between CdaA and ATP was allowed to proceed at 37°C for 60 min, after which they were boiled, centrifuged, and filtered the same way before HPLC was performed. In an extension of this protocol, the reaction was allowed to continue for a total of 240 min.

2.11 Sequence alignment and distance matrix generation

To compare the similarities of the CdaA protein sequence between different *Treponema* species, a sequence alignment was performed using the protein BLAST algorithm from NCBI to search protein sequence databases and acquire sequences (based on the entry for a given species which had the highest Expect value) for relevant

species and the MEGA 11 software to perform the alignment. Similarity, rather than identity, was then used to generate a distance matrix comparing the overall similarities between the sequences for all the studied species.

2.12 Colony picking and *Td* strain analysis

To determine whether a given colony or strain gDNA sample of Td possessed the ORF for a given protein, PCR was performed using Taq polymerase as part of the GoTaq® Green Master Mix (Promega) and the primers for the relevant ORF (Table 1). Bacterial DNA was supplied either by colony picking using a pipette tip or by addition of 0.1 µL of gDNA. Results were analyzed by agarose gel electrophoresis to determine if a colony or strain was PCR-positive for the gene using the chosen primers.

2.13 Statistical analysis

Statistical analysis was performed via use of one-way ANOVA to compare the c-di-AMP concentrations of *Td* samples per ng of protein, with a Dunnett post-hoc test to determine specifically which groups had significant differences in c-di-AMP concentration, and with a two-way ANOVA test for determining the different concentrations of c-di-AMP per milliliter of *E. coli* samples.

Chapter 3: Results

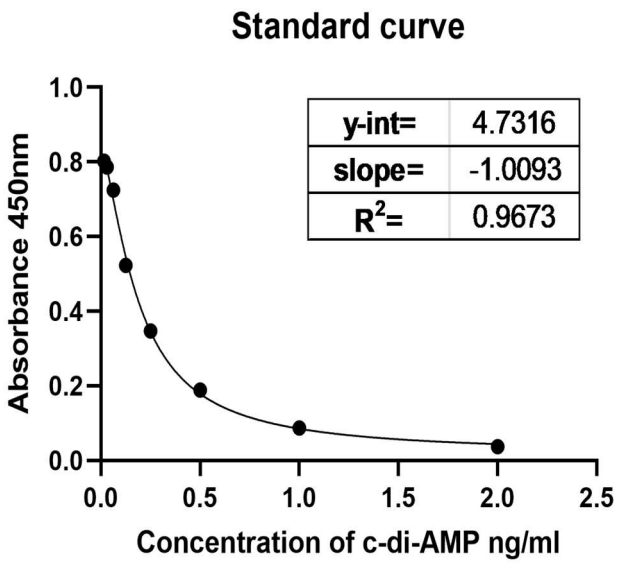
3.1 T. denticola makes c-di-AMP in broth culture

The concentrations of c-di-AMP in samples of *Td* cell lysates grown in NOS were measured using a Cayman Chemical cyclic di-AMP ELISA kit after standardization of samples using a BCA to ensure similar concentrations of bacterial lysate were investigated. Using a standard of c-di-AMP which came from the kit to generate a standard curve, concentrations of c-di-AMP were measured in triplicate at two dilutions for each sample to determine an overall concentration of c-di-AMP for each strain. These results were compared to *E. coli*, a non-c-di-AMP-producing bacterium[46]. While the amount of c-di-AMP fluctuated between strains and between replicates, high concentrations of c-di-AMP were observed consistently in samples containing *Td*, an effect maintained across technical replicates and dilutions (Figure 4).

3.2 The *T. denticola* protein CdaA is a diadenylate cyclase capable of synthesizing c-di-AMP *in vitro*

HPLC was used to investigate the results of incubating recombinant CdaA purified from *E. coli* with ATP, the precursor for c-di-AMP synthesis, in the presence of 10 mM concentrations of Co^{2+}, Mn^{2+}, or Mg^{2+} for 1 hour. Apparent diadenylate cyclase activity was altered significantly by the metal ion being supplied, with the strongest activity being found when CdaA was supplied with Mn^{2+}, modest activity upon addition of Co^{2+}, and no apparent activity upon supplementation with Mg^{2+} (Figure 5). These results show the clear ability of CdaA to synthesize c-di-AMP from ATP *in vitro* when conjugated with the correct metal ions as cofactors. After identifying two metal cofactors which seemed to be satisfactory for CdaA diadenylate cyclase activity, incubation with

A)



B)

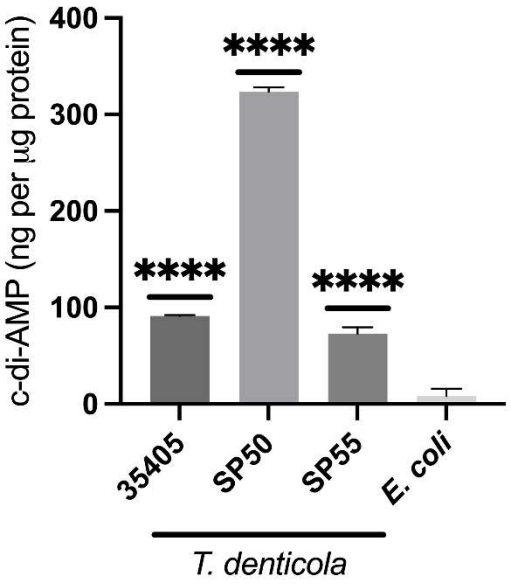


Figure 4. A) Standard curve generated using Cayman Chemical cyclic di-AMP kit to estimate concentrations of c-di-AMP in samples, and corresponding information on the parameters of the line of best fit drawn (sigmoidal, 4PL curve rendered in GraphPad Prism). B) c-di-AMP concentrations of samples taken from three different strains of *Td* grown in broth culture 4 days after passage as measured by competitive ELISA. Figure represents the results of two biological replicates. Results analyzed by One-way ANOVA with Dunnett post-hoc test. Error bars represent standard error of the mean. **** indicates P<0.0001.

ATP was performed with buffers containing these metal ions for 4 hours instead of one. In no condition was complete conversion of ATP to c-di-AMP by CdaA observed.

3.3 CdaR does not increase the diadenylate cyclase activity of CdaA in buffer

Recombinantly expressed and purified CdaA and CdaR were co-incubated prior to the addition of ATP, and the samples analyzed by HPLC to detect the formation of c-di-AMP in the buffer solution. Low but noticeable conversion of ATP into c-di-AMP by CdaA was noticed and compared to the resulting peaks in the condition where CdaR was added, where no difference was observed as a result of CdaR addition (Figure 6).

3.4 CdaA$^+$ *E. coli* cultures show c-di-AMP formation, especially when simultaneously expressing CdaR

Lysates in B-PER from *E. coli* transformed with pET-Duet-1 dual expression vectors cloned with CdaA, CdaR, both, or neither were collected after protein expression for 4 hours. These samples were tested using the ELISA kit used previously to test native c-di-AMP concentrations in *Td*, detecting higher c-di-AMP concentrations in CdaA$^+$ bacteria and even higher c-di-AMP concentrations in CdaA$^+$/CdaR$^+$ bacteria.

3.5 The CdaA sequence is highly conserved between treponemes, especially at the diadenylate cyclase domain

After determining the protein sequence for the diadenylate cyclase CdaA in the type strain of *Treponema denticola*, ATCC35405, from the UniProt consortium, this sequence was used to perform a local alignment using NCBI's BLAST algorithm. The

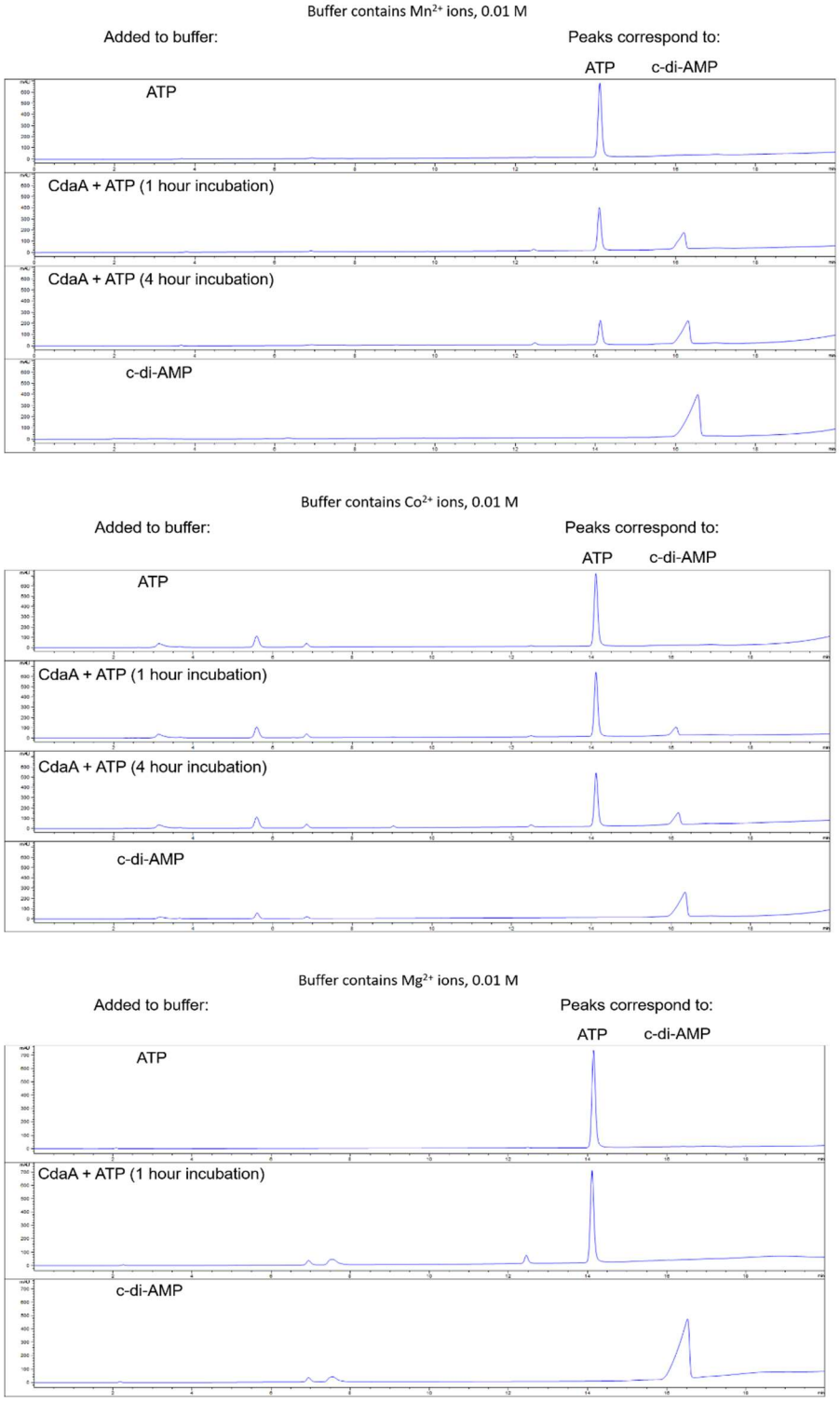


Figure 5. c-di-AMP formation by CdaA when supplied with ATP and metal cofactors. Mn appears to increase activity significantly (A), Co modestly (B), and Mg appears not to be suitable as a cofactor for CdaA activity (C). The 4-hour incubation was not performed with Mg.

A) **B)**

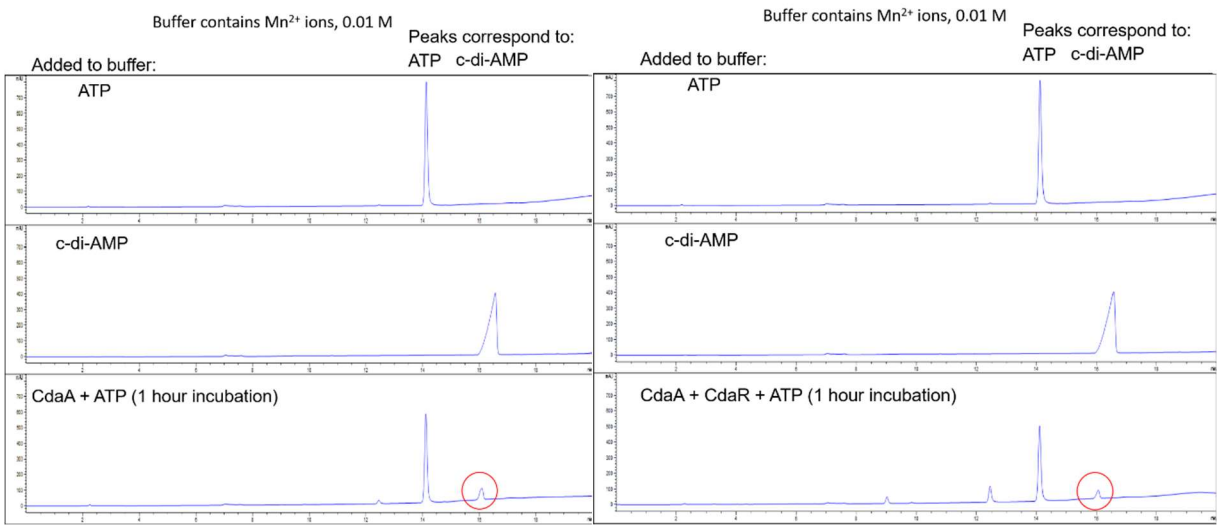


Figure 6. CdaR addition to CdaA in buffer does not increase diadenylate cyclase activity. CdaA and CdaR were co-incubated in Mn^{2+} buffer for 30 minutes prior to 1-hour incubation with ATP. A) Incubation of CdaA with ATP alone B) Co-incubation of CdaA and CdaR with ATP.

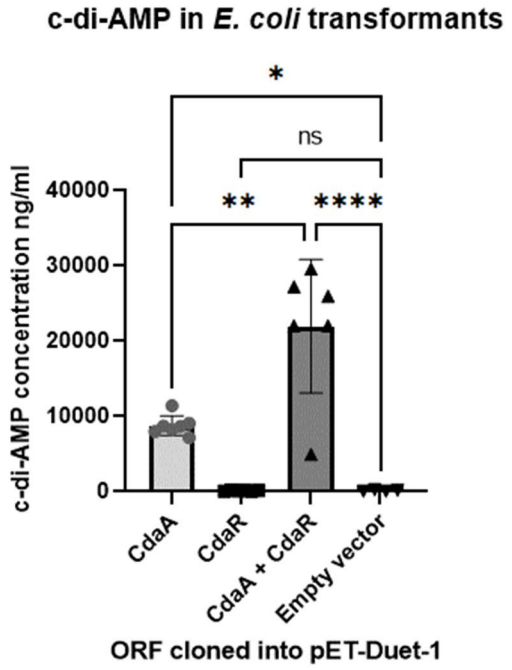


Figure 7. Transformation of *E. coli* with *Td* genes for c-di-AMP metabolism results in c-di-AMP synthesis. The ORF of CdaA, accessory protein CdaR, or both were cloned into the dual expression vector pET-Duet-1. *E. coli* BL21(DE3) cells transformed with these plasmids had protein expression induced and lysates taken to see if c-di-AMP formation could be detected. Lysates from cells with CdaA expression had significantly increased levels of c-di-AMP detected compared to those with CdaR alone or empty pET-Duet-1 vector. Dual expression of both CdaA and CdaR significantly increased measured levels of c-di-AMP compared to CdaA expression alone. Results represent three biological replicates.

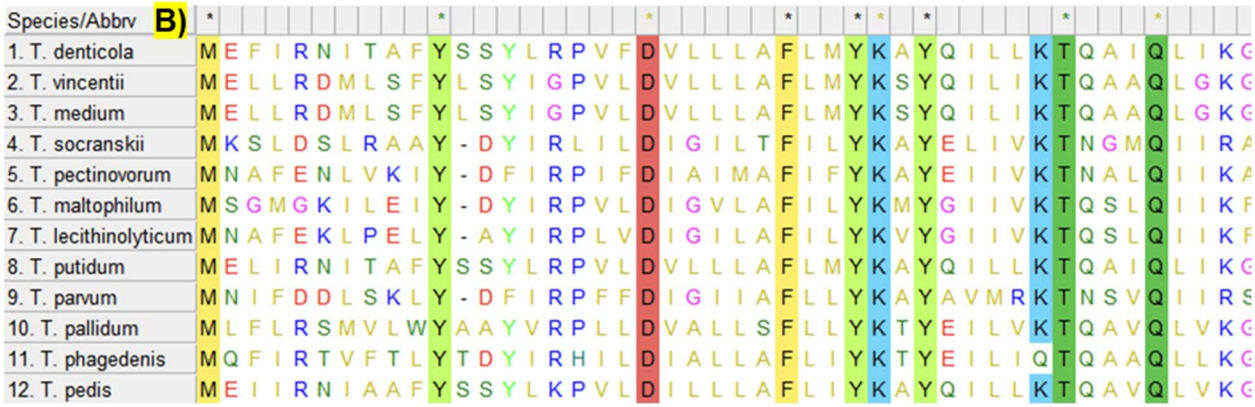
results were then used to identify other *Treponema* species with homologous proteins whose sequences showed the greatest identity to that of the protein in the type strain. One sequence from each additional species was collected based on which result for a given species had the highest E-value according to NCBI's algorithm. Those sequences were then used to generate a distance matrix in MEGA for all species with homologous proteins based on similarity between the protein sequences. Conservation at the crucial diadenylate cyclase domain was noted especially and compared between the recorded sequences in all species (Figure 8).

3.6 Other *T. denticola* strains have CdaA encoded in their genomes

The same primer set used for cloning the CdaA ORF out of genomic DNA from *Td* type strain ATCC35405 was used to perform PCR amplification with Taq polymerase of the CdaA ORF in gDNA samples and whole cell lysates from twelve strains of *Td*. The resulting strains were all noted as CdaA positive, suggesting that they contain an ORF for a diadenylate cyclase with high identity for that in the type strain studied in this research (Figure 9).

A)

	T. den.	*T. vin.*	*T. med.*	*T. soc.*	*T. pec.*	*T. mal.*	*T. lec.*	*T. put.*	*T. par.*	*T. pal.*	*T. pha.*
T. denticola											
T. vincentii	0.3806										
T. medium	0.3843	0.0148									
T. socranskii	0.4428	0.5019	0.5019								
T. pectinovorum	0.4154	0.4644	0.4682	0.3100							
T. maltophilum	0.3954	0.4504	0.4542	0.3346	0.3308						
T. lecithinolyticum	0.4167	0.4639	0.4639	0.3333	0.3068	0.1597					
T. putidum	0.0584	0.3657	0.3619	0.4391	0.4191	0.3840	0.4167				
T. parvum	0.4485	0.4794	0.4757	0.3321	0.3015	0.3612	0.3371	0.4301			
T. pallidum	0.3993	0.4627	0.4627	0.4613	0.4669	0.4373	0.4394	0.3993	0.4779		
T. phagedenis	0.3162	0.4173	0.4173	0.4796	0.4333	0.4221	0.4373	0.3199	0.4741	0.4133	
T. pedis	0.1861	0.3731	0.3769	0.4576	0.4228	0.3840	0.4205	0.1898	0.4265	0.3919	0.3077



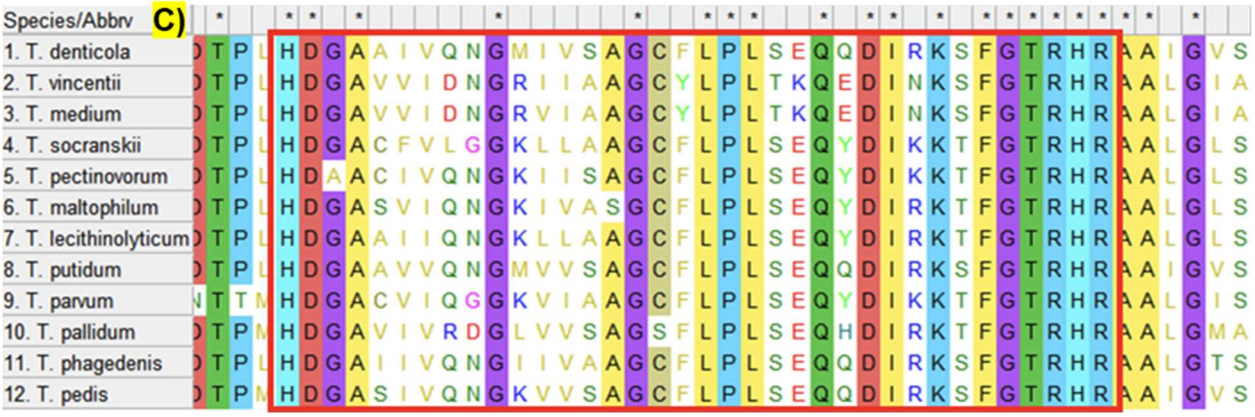


Figure 8. Distance matrix and sequence alignment of CdaA protein sequences from twelve *Treponema* species. A) Distance matrix comparing the similarity of protein sequences to each other, scaled from the least distance highlighted in green to the greatest distance highlighted in red. B) Sequence alignment at the start of the protein sequences, with highlighted residues representing 90% conservation across sequences. C) Sequence alignment around the diadenylate cyclase domain, with a red box depicting the start and end of the domain.

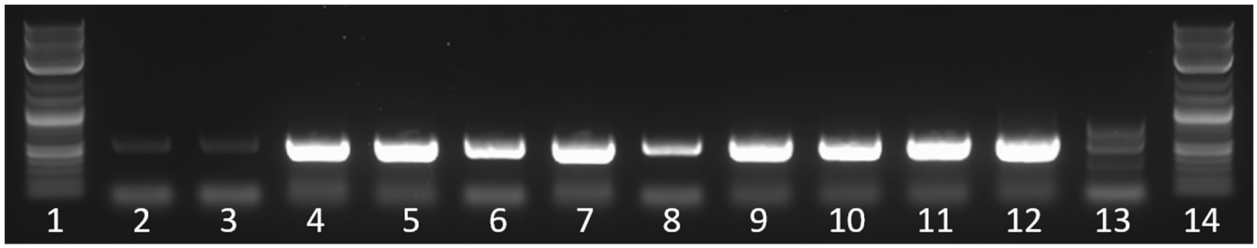


Figure 9. Twelve *Td* strains analyzed by PCR for the presence of the ORF for CdaA in their genome. The lanes represent: (1) 1 kb DNA ladder (NEB) (2) strain SP46 (3) strain SP54 (4) strain SP60 (5) strain SP61 (6) strain SP64 (7) strain SP72 (8) strain 35404 (9) strain SP50 (10) strain SP55 (11) strain 35405 (Type strain) (12) pET-Duet-1 plasmid cloned with CdaA ORF as positive control (13) empty pET-Duet-1 plasmid as negative control (14) 1 kb DNA ladder (NEB). 2-8 represent PCR of gDNA samples, and 9-11 represent PCR of whole cell lysates from broth cultures.

Chapter 4: Conclusions and Future Directions

4.1 Discussion

4.1.1 *Treponema denticola* synthesizes c-di-AMP, raising questions about its role

Many oral bacteria are particularly fastidious, including the oral spirochetes like *Treponema denticola*[8], and difficult to cultivate, genetically manipulate, or test in a variety of models. This does not discount the importance of such organisms; *Td* can represent up to 40% of the bacterial population in diseased pockets of the gums[8], and not by being a commensal member of the microbiome, either. The relationships between *Td* and the host as well as other microbes in the microbiome are worth investigating so that the role it plays in the pathogenesis of periodontitis can be elucidated, giving rise to more effective therapies and treatments that result in real, long-lasting benefits to those with oral disease. This requires understanding, at a fundamental level, the processes by which *Td* responds to its local environment and how it lives and grows during establishment of disease.

This research has demonstrated the existence in *Td* of a molecule, c-di-AMP, which is the basis of essential and far-reaching regulation of gene expression in a wide range of other bacteria, including Gram-positive species like *B. subtilis*[83], and which has evidence suggesting its essentiality in Gram-negative species like *B. burgdorferi*[76]. It has gone further and identified an enzyme encoded by *Td* which is capable of synthesizing c-di-AMP *in vitro*, as well as noting the existence of a homologous gene in several strains of *Td* and other species of *Treponema* entirely. The existence of c-di-AMP in *Td* is of interest as it suggests the existence of an entire regulatory system

based around the use and monitoring of c-di-AMP in the cell to exist within the bacterium, one which in other bacteria has been shown to have far-ranging effects on the ability of the cells to survive and grow and whose receptor molecules serve crucial functions for the cell, including DNA repair, potassium transport, biofilm formation, cell wall biosynthesis, and carbon cycle metabolism[52]. Especially important in the context of *Td* as an oral bacterium is the fact that c-di-AMP is described as regulating biofilm formation, as in many ways the story of oral disease pathogenesis is the story of biofilm formation[28]. As a positive regulator of biofilm synthesis by way of affecting a major glucan-producing enzyme within *S. mutans*, an important oral pathobiont, c-di-AMP may contribute to disease progression at dental caries and the creation of a new equilibrium for the oral microbiome which characterizes disease[60]. The ability of *Td* to participate in and contribute to the formation of biofilms in the context of disease is well-documented as it interacts with other pathobionts of periodontal disease[17], and any regulatory network in *Td* which affects this process is a notable potential target of study to understand the pathogenesis of periodontal disease as it relates to the interaction between the microbiome and the host.

Additionally, it is important to note the context of c-di-AMP as it relates to the ability of other pathogenic bacteria to cause disease. Work with *L. monocytogenes, C. trachomatis,* and Group B Staphylococcus has revealed several important effects on both bacteria and host cells during infection[49,77,78]. The regulation by c-di-AMP of factors within *L. monocytogenes* has a number of important effects; for instance, deletion of the diadenylate cyclase responsible for c-di-AMP synthesis or overexpression of the c-di-AMP phosphodiesterase responsible for its degradation resulting in impaired growth of

L. monocytogenes both *in vitro* and within macrophages, showing its importance for successful replication in the context of infection[48]. Additionally, it appears to regulate cell wall homeostasis, a fundamental process for all bacteria, and deletion of c-di-AMP synthesis was found to increase sensitivity of *L. monocytogenes* to cell wall-interfering antibiotics, important for consideration due to the unique profile of susceptibility to antibiotics that *Td* shows and the high variation between strains of its sensitivity to a given antibiotic[20], where specific treatment of *Td* by adjunctive antibiotic therapy might be enhanced in the context of inhibition of c-di-AMP regulation, improving its sensitivity to β-lactam antibiotics and other disruptors of cell wall homeostasis. The oral pathogen *P. gingivalis* utilizes c-di-AMP to improve its potential for infection[62], making it crucial to understand whether *Td* can do the same. The ability for c-di-AMP to be detected by host cells as a PAMP is also highly interesting in the context of *Td*, as periodontitis is characterized by dysregulated inflammation and c-di-AMP has been shown to activate immune genes in eukaryotic host cells[77].

4.1.2 The *Td* protein CdaA is capable of synthesizing c-di-AMP in multiple models

The evidence presented that CdaA is a diadenylate cyclase protein capable of synthesizing c-di-AMP both in buffer after being supplied with ATP and in bacterial cells expressing the ORF encoded on a plasmid allows for comparisons to other machinery for c-di-AMP metabolism in bacteria and for investigating the circumstances to which *Td* is able to respond using a c-di-AMP-based regulatory network. Diadenylate cyclases belong to multiple classes, each with their own characteristics and structure, and

multiple classes can be present within the same organism, such as in *B. subtilis*, which has enzymes belonging to the DisA, CdaA, and CdaS classes of diadenylate cyclase[68]. While here we have identified a CdaA-class diadenylate cyclase, this opens rather than closes the door to research into the other mechanisms of c-di-AMP synthesis within *Td*. The metal ions which CdaA in *Td* requires as cofactors for DAC activity match the profile of DacA in *L. monocytogenes*, with manganese and cobalt ions being capable of supporting DAC activity and magnesium ions being unable to[72]. This differentiates CdaA in *Td* from other diadenylate cyclases, like those belonging to the DisA class of DACs in *B. subtilis*[44], which rely on Mg^{2+} as a cofactor for activity.

4.1.3 The interaction between CdaA and CdaR produces varying results

The effects of the *Td* homologs of CdaR on the diadenylate cyclase activity of CdaA are mixed. Adding CdaR to CdaA did not appear to improve the ability of the protein to synthesize c-di-AMP from ATP in buffer as measured by HPLC, but dual expression of CdaR with CdaA in *E. coli* did result in a significant increase in c-di-AMP concentrations overall compared to *E. coli* expressing CdaA alone. In other experiments, the combination of CdaA and CdaR from *B. subtilis* resulted in an apparent 20-fold increase in c-di-AMP concentrations in *E. coli* compared to the expression of CdaA alone[69], but the homologous CdaR gene in *L. monocytogenes* has an inhibitory effect on DacA activity[73]. More research is needed to determine what exactly the relationship between CdaA and CdaR in *Td* might be, especially given that the ORF used to synthesize CdaA in this experiment lacks the transmembrane domains through which CdaR regulates DacA function in *L. monocytogenes*[73], meaning any

interaction that would occur between the native forms of these proteins *in vivo* may be obscured in this experiment with the loss of these transmembrane domains, although this truncated form of CdaA was similarly expressed in the *E. coli* transformants which still appeared to show positive regulation of CdaA activity by CdaR.

4.1.4 Prevalence of CdaA protein homologs in other species of *Treponema* and other *Td* strains

The distance matrix of *Td* species and PCR verification of CdaA presence in different *Td* strains both show that this regulatory network may have broader implications for *Treponema* and for its associated diseases than the data from a single type strain and *in vitro* testing might afford. Oral treponemes like *T. maltophilum*, *T. lecithinolyticum*, and *T. vincentii* were found to have CdaA ORF sequences with conservation of the diadenylate cyclase domain, as were treponemes that localize to other niches, including the etiologic agent of syphilis, *T. pallidum*, about which little is known with regards to signaling mechanisms, partially due to difficulty in cultivating the bacterium[84]. Its presence in different *Td* strains speaks to its importance for the bacterium's successful growth and regulation. One shortcoming of the approach used here is that it only confirms the existence of the ORFs within the genome sequences of these bacterial species; if the promoter used to initiate transcription of the gene is mutated in these species so as not to be functional, the resulting protein might not be expressed in the bacterium at all, which would complicate the idea that c-di-AMP metabolism can be suggested in these organisms by the presence of a gene for CdaA within their genome. However, the high degree of conservation at the diadenylate

cyclase motif speaks against this possibility and suggests that diadenylate cyclase activity is beneficial to these organisms.

4.2 Conclusion

c-di-AMP is capable of regulating a variety of functions within bacterial cells and can have profound implications on the ability of bacteria to express virulence factors and establish infection. Understanding the roles of c-di-AMP signaling in bacteria thus allows for understanding the methods by which bacteria cause disease. This research has shown the existence of c-di-AMP in *Td* and demonstrated the ability of the product of a natively encoded gene, *TDE1909 (cdaA)*, to synthesize c-di-AMP both in buffer and in living cells. This opens the door to understanding the regulatory networks within *Td* that give rise to its virulence and ability to cause periodontal disease, as potential c-di-AMP binding partners can be identified and characterized, shedding light on the ability of a small oral spirochete to cause debilitating disease. The importance of c-di-AMP to *Td* and the relevance for future study is further demonstrated by the existence of potential diadenylate cyclase in a wide variety of *Td* strains and other *Treponema* species, including pathogens like *T. pallidum*, the etiologic agent of syphilis. With c-di-AMP's role as an immune adjuvant already being studied for its potential to treat cancer and bacteria-associated cardiovascular disease, this small nucleotide messenger may not just be helpful for understanding *Td*, but one day may be involved in treating *Td* infections.

4.3 Future directions

There is more work to be done in both the short and long term on the role c-di-AMP plays in *Td*, ranging from the confirmatory to the exploratory. Using mass spectrometry to determine c-di-AMP content in *Td* would be a more direct way of confirming its presence and its levels, especially given the fluctuation between strains and between biological replicates observed using the competitive ELISA. Similar techniques could be used to gain insight into the CdaA enzyme itself, even going on to identify the exact active site or conformations it takes with metal ions for activity, as Rosenberg *et al.* performed for the DacA of *L. monocytogenes*[72]. Another question raised by the results reported above is the inability of CdaA to completely convert ATP to c-di-AMP in a buffer solution. This may be due to autoinhibition of CdaA by c-di-AMP, where the concentration of c-di-AMP reaches a level where CdaA is no longer able to synthesize more, a potent and common method of regulation to prevent excess c-di-AMP synthesis which is seen in the *Td* diguanylate cyclase *TDE0125*[85]. Investigating this question further may take the form of an ELISA where wells seeded with CdaA are checked for their ability to bind biotinylated c-di-AMP by addition of HRP-conjugated streptavidin. Another valuable experiment to perform may be to analyze the supernatant from *Td* cultures for c-di-AMP to determine if *Td* secretes c-di-AMP by MS or ELISA, with potential relevance for infection in that c-di-AMP is able to induce host immune responses and act as a microbial-associated molecular pattern when secreted by *L. monocytogenes*[77] Identifying c-di-AMP binding proteins in *Td* would give a diverse set of pathways to study in the future, as in Blötz *et al.*, 2017[70].

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