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Selenomonas sputigena: Growth Conditions and Projected Virulence Factors of an Emerging Oral Pathogen

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Selenomonas sputigena: Growth Conditions and Projected Virulence Factors of an Emerging Oral Pathogen

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By

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Abstract

Periodontal diseases are ubiquitous diseases. They can affect people through a chronic infection that happens over a lifetime, but also as an aggressive infection that afflicts the younger population. It not only results in tooth loss and a lower quality of life, but it can also lead to fatal secondary infections like cervical cancer, heart disease, and Alzheimer's disease. This study looks at *Selenomonas sputigena*, an emerging oral pathogen, that has been hypothesized to contribute to periodontal disease. It has been linked to fatal septicemia and has been found in increased abundance within the oral biofilm during peak infection. Though *S. sputigena* had been discovered decades ago, there is little to no knowledge about its growth factors, both in vivo and in vitro, let alone virulence factors and disease-causing ability. In addition to establishing basic growth conditions for this understudied organism, understanding potential virulence factors for *Selenomonas sputigena* as an oral pathogen was the focus of this study. These potential factors for this organism include the ability to sense and make swift changes to the oral environment when it comes to pH, temperature, and oxygen concentration, sense and respond to other oral bacteria along with formation of biofilms, cell wall and potassium homeostasis, adhesion, motility, and flagellar assembly. Some of these virulence factors are proposed to be dependent upon the understudied cyclic dinucleotide, cyclic-di-AMP. Cyclic-di-AMP is the proposed secondary messenger that *S. sputigena* utilizes. A diadenylate cyclase, SELSP_1610, a phosphodiesterase, SELSP_2051, and two TetR family transcriptional regulators, SELSP_1770 and SELSP_1836, were all proposed based off of sequence comparison to other pathogenic oral bacteria, with special emphasis placed on SELSP_1610.

Throughout this study, we established previously unknown growth conditions within the laboratory for *S. sputigena*. Growth rates and growth characteristics were studied both in broth

and on agar plates. The determination of oxygen sensitivity was also noted along with sensitivity to different antibiotics. Next, the focus turned to the characterization of virulence factors by studying biofilm development with self, hemagglutination and hemolysis, and binding of *S. sputigena* to gingival epithelial cells. After these virulence factors had been assessed, the cloning process was started and extensive assay development using reverse phase chromatography was performed. This was used to better characterize cyclic-di-AMP metabolism in *S. sputigena* specifically to study the enzyme, SELSP_1610, and its potential activity as a diadenylate cyclase when converting two molecules of ATP into cyclic-di-AMP. While diguanylate cyclase had been successfully studied in the past using HPLC, diadenylate cyclase had not been. This new approach required development of the assay to assess diadenylate cyclase activity by extensively adjusting the variables and conditions used. Here, we optimized enzyme kinetics, buffer conditions and concentrations, as well as the activating cations. Further results are needed to support that SELSP_1610 fits as a diadenylate cyclase. Furthermore, a competitive ELISA using a known control is also being utilized to further support the hypothesis that SELSP_1610 is a diadenylate cyclase. This foundational research will be built upon in the future to further characterize *Selenomonas sputigena* as a periodontal pathogen.

Chapter 1: Introduction and background information

Periodontal disease

Periodontal disease is a chronic inflammatory disease that affects the tissue and bone that support the teeth (5,57,61). It is a progressive disease that affects approximately 47.2% of adults, as of 2015, within America alone (1,2,76). This disease not only affects people in an economic aspect all over the world, but it also contributes to a reduced quality of life due to pain, tooth loss, and trouble with mastication (1,29,68). Periodontal disease has two aspects to it: gingivitis and periodontitis (57,61). Gingivitis, the reversible stage, is defined as inflammation in the gingiva due to the progressive buildup of plaque over time (Figure 1-2)(57). This inflammation can worsen the epithelium and connective tissue around the tooth, eventually leading to its erosion if proper treatment is not sought (4,57,61). The tooth is unharmed at this stage; however, if left untreated it can progress to periodontitis (57,61). Unlike gingivitis, periodontitis is more difficult to treat and results in chronic inflammation and the destruction of periodontal ligaments (Figure 1-1) (57). Once the support from the tooth has been removed, the gap begins to grow between the tooth and gums (57). Destruction of teeth and the underlying alveolar bone begins, and the loss of ligaments results in edentulism, or loss of teeth (57,71). Severe tooth loss, losing one tooth or more, can come from this progression of disease (57). Periodontitis has two types: chronic and aggressive (55,57,61).

Figure 1-1 Progression of periodontal disease

Progression of periodontal disease from healthy, to gingivitis, to periodontitis exhibiting the inflammation of the gingival epithelium and the resection of alveolar support eventually resulting in destruction of gums and teeth.

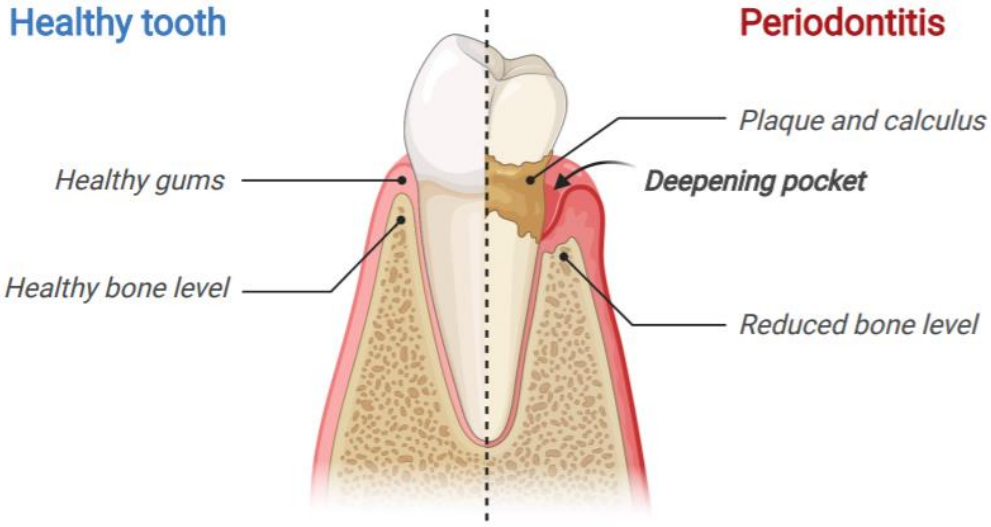


Figure 1-2 Plaque Formation in the Subgingival Sulcus

Progression of periodontal disease from healthy to disease state with respect to the buildup of plaque, first on the tooth, in the gingival sulcus and then subgingival space as inflammation increases and gum and bone loss occurs (60,61,70).

Chronic periodontitis is progressive and spans the lifetime of the afflicted person (57,68). It comes from poor hygiene and is usually found in the adult demographic later in life (57,61). The prevalence increases by 70.1% in adults over the age of 65 and usually presents in adults over the age of 30 (1,2,68,76). Within the United States, half of the nation's adults over 30 have chronic periodontal disease (1,2,53,68,76). It appears to be linked to the gradual shift from synergy of oral microorganisms to dysbiosis (6,7,57). This shift from healthy to disease state is essential to this process and will be expanded upon later in the introduction (7). Its dependence on oral microbial dysbiosis allows for preventative measures to be taken to reduce the chances of development (6,7). There seems to be a reduced genetic link when it comes to this form of periodontitis in comparison to its counterpart.

However, unlike chronic periodontitis, aggressive periodontitis does have a strong genetic component to it (57,61). It mainly affects people under the age of 30 and mitigation of the disease, not prevention, is the only suitable option (57). In addition to the genetic component, *Aggregatibacter actinomycetemcomitans* has been implicated in the progression of aggressive periodontitis due to its ability to allow other oral bacteria to proliferate (7,50,55). This Gram-negative commensal bacterium has a myriad of virulence factors that makes it the leading cause of this type of periodontal disease (7). If left untreated, it can lead to infections like endocarditis and bacteremia (57). Though treatment is a necessity after infection has occurred, unfortunately aggressive periodontitis does not have the ability to be prevented.

Certain factors can increase the risk of developing periodontal disease. As chronic periodontal disease is developed over time, poor oral hygiene, as previously mentioned, can contribute to development of this disease (57,68,71). The consumption of high amounts of sugar and carbohydrates without regular brushing/mechanical abrasion put those with this kind of diet

at high risk (57,68,71). There are other preexisting conditions as well that contribute such as dry mouth, caused by stress or medications, and diabetes (68,71). Smoking can also increase the risk (68,71). Lack of access to proper nutrition and oral care, like regular dental cleaning, can also contribute to this (68,71). This means that people that live below the poverty line are more at risk than those who have access to proper care (76). This paired with a lack of education creates problems later in life when chronic periodontitis sets in (76). This can result in often serious secondary infections (4,71). These infections can occur in parts of the world where dental care is not easy to come by, including those who cannot afford dental care (29).

Secondary infection is not just localized to the oral cavity. Once the bacteria enter the blood, bacteremia can and does occur along with the spread of odontogenic infections into the jaw and face (4,40,42,57). Other long-term sequela of periodontal disease has been linked to heart disease, stroke, and cancer such as cervical cancer (4,7,11,71). Adverse birth outcomes due to oral bacteria being found in the placenta and amniotic fluid can lead to premature births (32,71). Rheumatoid arthritis and Alzheimer's disease have also been found to stem from periodontal disease (5,7,43). These detrimental secondary conditions branch directly from chronic and aggressive periodontitis being a chronic inflammatory disease that results in years of inflammation (57,61). Treatment of these conditions depends on catching, preventing, and treating the primary infection early.

Prevention of chronic periodontitis is dependent upon hygiene management (68). Proper mechanical abrasion, such as brushing and flossing, can reduce risk along with yearly dental exams to maintain the integrity of the teeth (68). These little changes are key to offsetting the disease later in life. However, if chronic periodontal disease has already set in, or aggressive periodontal disease has been discovered, treatment by scaling and planing and debridement, or

the removal of plaque from the roots of the tooth, is the first step (38,54,68). This particular method is an attempt to control and prevent further bacterial infection (38,68). Antibiotic treatment, such as tetracycline and doxycycline, does exist; however, it is limited in its effectiveness and should be used in tandem with this initial treatment (4,38,54). Depending on how drastic the damage becomes, cutting away the gums to clean the roots and surgery for tooth repair and tooth removal may be a necessary treatment plan (4,38,54).

Between chronic and aggressive periodontitis, there are only a limited number of treatment and prevention options available (38,68). Despite being a ubiquitous disease that affects a vast portion of the population worldwide, with detailed knowledge about its progression, treatment options are not what they could be (38,68). Treatment options and therapies are expensive and invasive and, in addition, can be ineffective (4,38). Current treatment methods require more attention and development. They only serve to mitigate the damage already caused, and as stated before, there is no solid prevention for aggressive periodontitis (38). With the increased risk of a secondary complication occurring from periodontal disease, the only hope of being able to properly care for those that suffer with this disease is to study the interactions between the organisms within the oral microbiome and target them for a particular treatment plan (68).

Oral Microenvironment: Synergy and Dysbiosis

The movement from healthy to disease state within the polymicrobial microenvironment is a necessary direction to take when studying the oral microbiome (6,7,29). The oral microbiome is diverse and expansive with over 700 different species of bacteria that reside within the mouth (7,57,67). The range of temperatures, changes in pH, oxygen content, flow of

saliva and diversity of surfaces make this a dynamic location for bacteria to grow (57,60). Commensal bacterial populations out-compete pathogenic bacteria, add to homeostatic inflammation, and regulate the oral immune response (57,60). This leads to a state of chronic inflammation that leads to the destruction of supportive tissue, alveolar bone, and eventual tooth loss (57). This shift from a synergistic state to a state of dysbiosis triggers proliferation of pathogenic bacteria and initiates the production of proinflammatory cytokines and chemokines eventually leading to the activation of the host immune response (7,23,30).

Interactions between various bacterial species are complex and finely tuned in order to signal community adaptation (Figure 1-3) (6,48,67). Other pathogenic bacteria rely on these keystone pathogens to colonize specific niches within the oral cavity and helps to decrease the commensal bacteria (6,24,26). This turning point is key in the propagation of periodontal disease (67). Particular pathogenic bacteria such as *Treponema denticola, Porphyromonas gingivalis,* and *Aggregatibacter actinomycetemcomitans* have all been studied extensively and are known to make this shift occur (7,46,50,55). In addition to these classically recognized pathogens, oral microbiome studies have discovered several underappreciated pathogens like *Filifactor alocis* (5,6). An emerging pathogen that has been recently highlighted is *Selenomonas sputigena* (77).

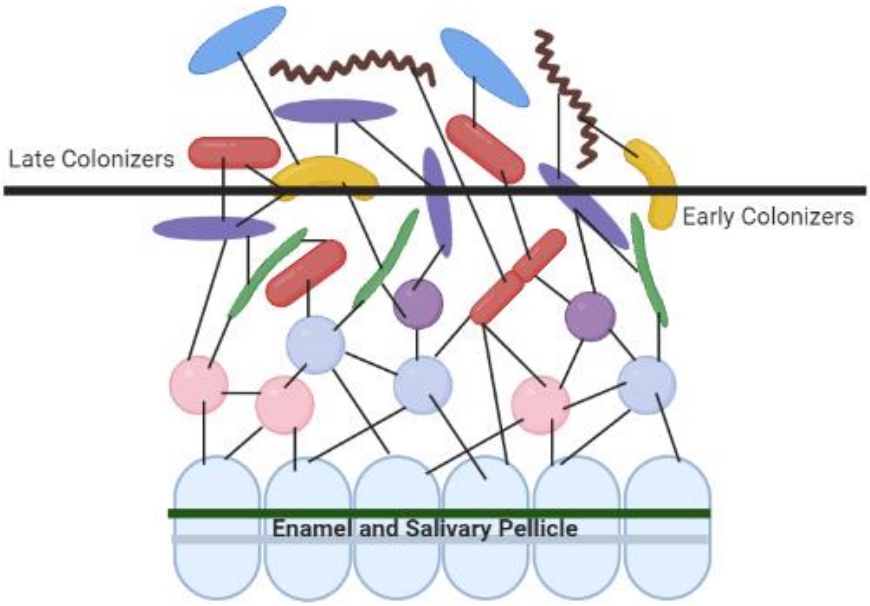


Figure 1-3 A diagram of plaque formation

This diagram of plaque formation illustrates the ability of most oral bacteria to effectively communicate and form organized aggregations. This is to either maintain synergistic interactions or facilitate dysbiosis and lead to a state of disease (6,45,51,70).

Selenomonas sputigena

S. sputigena is a Gram-negative, vibrio shaped motile bacteria with multiple flagella located in the inner crescent (47,77). The prevalence and abundance of *Selenomonas sputigena* increases from a healthy state to periodontitis (77). It was discovered in the early 1950's to be involved in the process of periodontal disease formation (41,56,77,78). Inflammation of the gingiva has also been shown to occur during its proliferation. It has also been implicated in fatal septicemia, a secondary infection, and is now being used as an indicator of disease as it was discovered throughout the oral biofilm (56,77). Through modern 16S sequencing that takes samples from both healthy and disease state, and metatranscriptomics, more pathogenic bacteria have been identified than previously known before (6,14,37). As mentioned above, *Filifactor alocis* is a newly discovered, and now verified, oral pathogen (5,6). *Selenomonas sputigena* has not been looked at as of late and is only now just being studied. It and other members of *Selenomonas* such as *Selenomonas flueggei* and *Selenomonas noxia* have been shown to interact with other oral pathogenic bacteria such as *Fusobacterium nucleatum* (21,31,78). The selenomonads do not form coaggregations with themselves; however, they do with *F. nucleatum* (21,31,78). Sequencing has also pointed to it having potential pathogenic virulence qualities that match other oral pathogens. Though evidence has been pointing to this possibility, it is unknown if it directly promotes periodontal disease and not many specifics are known about this bacterium. Defining these aspects, along with the growth conditions, is an essential part of this study as *S. sputigena* is a relatively understudied organism and its pathogenic capabilities and virulence factors are undiscovered (6,7,77). Due to its presence in the oral cavity, swift adaptation to the oral microenvironment is a necessity when it comes to oxygen concentration, pH, and temperature. It also may have the ability to sense and respond to other oral bacteria

along with biofilm formation. Cell wall and potassium homeostasis, adhesion to gingival epithelial cells, motility, and flagellar assembly are also proposed capabilities of *S. sputigena* (16,28,39,56). *S. sputigena* also has lipopolysaccharide that mirror other Gram-negative bacteria (79). Some of these responses and virulence factors are proposed to be dependent upon the understudied cyclic dinucleotide, cyclic-di-AMP: the secondary messenger that *S. sputigena* uses.

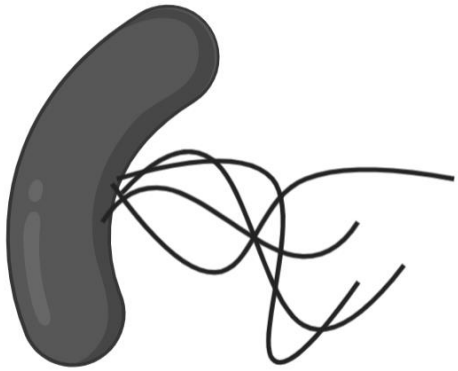


Figure 1-4 *Selenomonas sputigena* schematic

This figure exhibits the vibrio, or crescent shape, of *S. sputigena* complete with the presence of flagella coming from the inner curve.

Cyclic-di-GMP

Cyclic-di-GMP or cyclic dimeric guanosine 3',5'-monophosphate (Figure 1-5), which was originally discovered in the 1980's to regulate cellulose synthesis, in comparison to cyclic-di-AMP, is the most well-known and well-studied secondary messenger (62). Cyclic-di-GMP regulates multiple functions including cell signaling, biofilm development, and other intracellular functions including the regulation of virulence factors (28,62). Cyclic dinucleotides, such as cyclic-di-GMP, which control production of exopolysaccharides, are essential when it comes to bacteria not only responding to environmental stressors, but also when responding to each other (62). Cyclic-di-GMP, for example, helps to not only promote motility but also controls biofilm formation and maturation through regulation of fimbriae production and adhesins (28,62). This ubiquitous secondary messenger has been studied extensively in bacteria for a full 30 years. Its activities are modulated through phosphodiesterases for degradation and diguanylate cyclases for synthesis. Cyclic-di-GMP synthesis relies on two molecules of GTP and a diguanylate cyclase, which have a GGEEF/GGDEF domain, converts the GTP into cyclic-di-GMP with Mg^{2+} as the activating cation (62). When c-di-GMP is degraded by a phosphodiesterase, the two left over molecules are GMP and pGpG, which eventually converts into GMP (62,64). The domain EAL needs Mn^{2+} or Mg^{2} and results in the degradation of c-di-GMP and produces the nucleotide pGpG (62,64). HD-GYP, a third domain, is a part of the HD superfamily, or the metal-dependent phosphohydrolases (62,64). HD-GYP also acts as a c-di-GMP phosphodiesterase in addition to EAL (62,64). Along with this function, HD-GYP has also been proposed to regulate pGpG (62,64). After its synthesis, C-di-GMP interacts with effector RNA (riboswitches) or effector proteins, including PilZ domain that helps to regulate motility in *Treponema denticola* (9,36,75).

As previously stated, cyclic-di-GMP has been extensively studied; however, cyclic-di-AMP, another secondary messenger, is understudied in comparison.

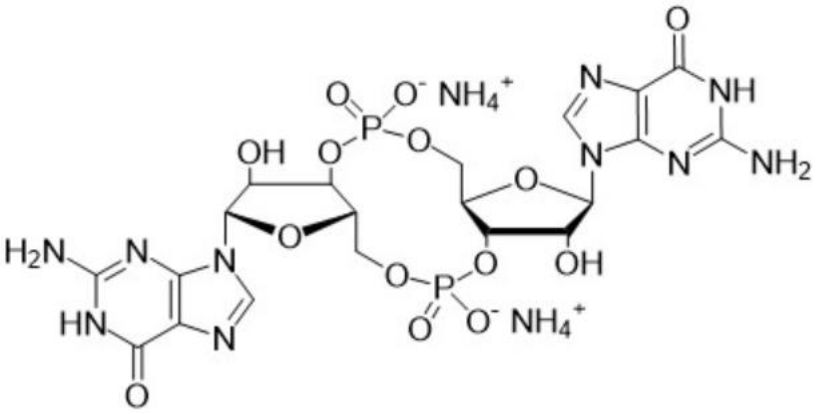


Figure 1-5 cyclic dimeric guanosine 3',5'-monophosphate

Cyclic-di-AMP

Cyclic-di-AMP has been found to be an essential secondary messenger like cyclic-di-GMP (8,10,16,17,18). It was only discovered as early as 2008 in bacteria and, in comparison to cyclic-di-GMP, it is inadequately understood (17,20,76). It, like its counterpart, is responsible for responding and adapting to the environment and other surrounding bacteria, regulates motility, and has been shown in biofilm development (17,74,76). It also facilitates potassium and cell wall homeostasis, chemotaxis, and regulates virulence factors (17,39,76). It is mostly found in Gram-positive bacteria and it is less common for Gram-negative bacteria to have this trait. It is found in Gram-negative phyla such as Chloroflexi, Spirochetes, Acidobacteria, and from the phylum Firmicutes (33). *S. sputigena*, though Gram-negative, is a member of Firmicutes and is hypothesized to use cyclic-di-AMP as a secondary messenger (17,58). *S. sputigena* comes from the phylum firmicutes, a typically Gram-positive phylum, therefore this secondary messenger might be highly conserved (17,58). Cyclic-di-AMP within *S. sputigena* has a myriad of uses as it not only regulates potential virulence factors but also aids the bacterium in adapting to environmental conditions and abrupt changes found within the mouth (3).

Cyclic-di-AMP can be synthesized from two molecules of ATP by several different types of diadenylate cyclases, or DAC, like DacA in *S. aureus* and DacB in *B. subtlis* (Figure 1-7) (17,20,22,25,33). Within *S. sputigena*, the gene coding for this particular diadenylate cyclase, and the focus of this thesis, is hypothesized to be SELSP_1610 (Figure 1-6). In terms of potential receptors for cyclic-di-AMP, in comparison to cyclic-di-GMP, which has multiple different binding molecules identified, very little have been discovered yet (25,33). Though it does not have a PilZ equivalency, cyclic-di-AMP has its own set of receptor types such as transcriptional regulators, enzymes, and transporters that bind to it (25,33,73). These regulator proteins bind to

cyclic-di-AMP and allosterically change when bound to in order to specifically regulate pathways (17,25,33,66). For example, to help regulate osmotic pressure through K+ transport, RCK_C is a domain used by *Staphylococcus aureus* (25,33,39,69). TetR-family transcriptional regulators are another group of receptor proteins that were first found in *M. smegmatis* that regulate stress response and membrane homeostasis (25,33). TetR-family transcriptional regulators bind to DNA through a DNA-binding domain, an N-terminal TetR-like helix-turn-helix domain (25,33). This occurs after a structural change facilitated by stimulation by cyclic-di-AMP.

S. sputigena is hypothesized to have several TetR-family transcriptional regulators such as SELSP_1770 and SELSP_1836 that interact and bind to cyclic-di-AMP. These are not the only two possible TetR-family transcriptional regulators or effectors; however, they are the ones that are being focused on. Finally, cyclic-di-AMP is degraded by phosphodiesterases, or PDEs, into pApA (5'-phosphadenylyl-adenosine) (Figure 1-17) (25,33,65). GGDEF domain protein containing PDE, or GdpP, has a DHH domain, a DHH associated DHHA1 domain, a PAS sensory domain and a GGDEF domain (17,25,33). The PDE activity is contained within the DHH/DHHA1 domains (17,25,33). The PAS sensory domain, when bound to heme, leads to suppressed PDE activity, giving it a regulatory role (17,25,33). GdpP overall can hydrolyze cyclic-di-AMP, for example, in *B. subtilis* (25,33,49). When DHH-DHHA1 domain, known for its conserved DHH motif, and the PAS domain, known for its conserved Per-ARNT-Sim proteins, are together in GdpP-type proteins, they are mainly found in the phylum Firmicutes which *S. sputigena* is a part of (17,25,33,58). SELSP_2051 is the proposed phosphodiesterase within *S. sputigena* that has DHH/DHHA1/PAS domains (17,25,33).

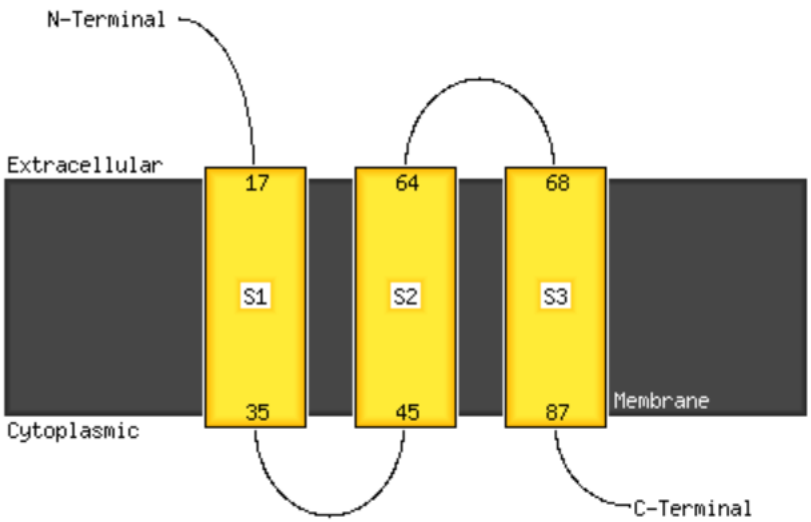


Figure 1-6 Model of SELSP_1610 Full length Membrane Imbedded Domain

This is the predicted picture of the membrane-bound protein SELSP_1610, the projected diadenylate cyclase, by PHYRE.

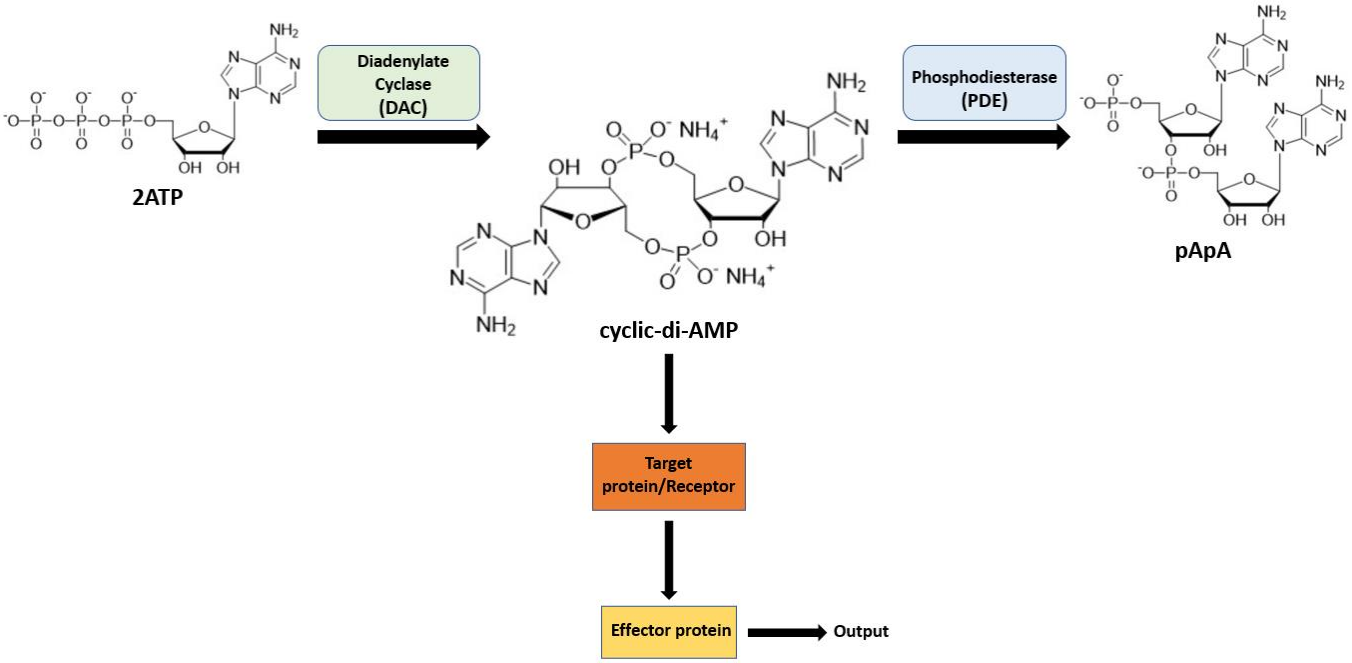


Figure 1-7 Synthesis and Degradation of cyclic-di-AMP

This figure exhibits the synthesis of the secondary signaling molecule, cyclic-di-AMP, from diadenylate cyclase, its interaction with target proteins, and finally its degradation by phosphodiesterase with a DHH-DHHA1 domain into pApA.

***Selenomonas sputigena* and cyclic-di-AMP**

By studying *Selenomonas sputigena*, the hope is to be able to not only find a marker for periodontal disease, but also find out if *S. sputigena* causes periodontal disease through virulence factors that are regulated by cyclic-di-AMP. Based on bioinformatic analysis, we can make the prediction that *S. sputigena* possesses a functional cyclic-di-AMP system that regulates these pathogenic and physiological functions (3). The proposed genes listed above include SELSP_2051, a phosphodiesterase, SELSP_1770 and SELSP_1836, TetR-family transcriptional regulators, and SELSP_1610, a diadenylate cyclase, are the focus with a particular emphasis on SELSP_1610. In addition, focus on potential virulence factors such as hemagglutination, biofilm formation, and adhesion to gingival epithelial cells are also explored along with previously unknown growth factors. These studies provide significant and new insights into this emerging periodontal pathogen. This may highlight future strategies for the prevention and treatment of periodontal disease.

Chapter 2: Methods and Protocols

Standard Growth curve

A standard growth curve was started by inoculating an overnight 10ml culture of *Selenomonas sputigena* in tryptone-yeast extract-gelatin-volatile fatty acids-serum (TYGVS) media from frozen stocks. This starter culture was used to inoculate a secondary culture overnight the following day. On the third day, two cultures were inoculated of 10ml TYGVS media and two cultures of *S. sputigena* media simultaneously at a starting OD_{600} of .05. All media was pre-reduced for at least 8 hours in the anaerobic chamber prior to use. The culture was incubated and measurements of optical density at OD_{600} were taken every hour for ten hours to establish a growth curve.

Plate Count

An overnight culture was grown from either a frozen stock or a maintained culture of *Selenomonas sputigena*. The OD_{600} was checked the following day and the culture was brought to an OD_{600} of 1. A 1:10 serial dilution of this culture was completed ten-fold and 100µl of each dilution, 10^{-5} to 10^{-9}, was plated in triplicate on pre-reduced TYGVS plates using proper aseptic technique. The plates were incubated at 37°C for 4 days and the colonies were counted and recorded at the end of the incubation.

Oxygen resistance

An overnight culture of *S. sputigena* was grown, brought to an OD_{600} of 1, and allowed to incubate on a shaker in either anaerobic conditions or aerobic conditions for 4hrs prior to being

plated. As aligned with the plate count protocol, serial dilutions were performed and 10^{-3}, 10^{-4}, and 10^{-5} were plated on TYGVS plates.

Hemagglutination/Hemolysis assay

In order to check if *S. sputigena* agglutinated red blood cells, a hemagglutination assay was performed. An OD_{600} of 1 is equal to $1.0x10^9$ cells per ml, which was found previously through colony counts, allowed for the starting calculation of an OD_{600} of .470, or $4.70x10^8$ cells per ml. Erythrocyte suspension prep was completed by gently mixing blood to suspend erythrocytes and transfer .2 ml of blood to 4.8 ml 0.05M Tris-HCL buffer pH 7.5 containing 0.15M NaCl (TBS). This results in a 2% suspension of human erythrocyte based on a hematocrit of about 50%. The gently mixed 2% suspension was centrifuged at 2000 rpm for 10 minutes at 4 °C. Two washes were performed in 5 ml of TBS and centrifuged at 500 g for 8 minutes. A 1:2 serial dilution was performed with a total of 50 µl within each of 12 wells of the 96 well V shaped microtiter plate. The 12^{th} well was blank with just erythrocytes and each dilution is completed with TBS. The plates were left at 4°C to equilibrate for at least an hour and then 50µl of the washed 2% erythrocyte suspension was added to each well. The plates were tapped to mix the suspension. Plate 1 was left at 4°C to measure hemagglutination and plate 2 was left at 37°C to measure hemolysis for four hours. The plate was covered to prevent evaporation.

Biofilm assay

In order to see if *S. sputigena* forms a monospecies biofilm, a culture of 5mls was grown overnight. In addition, *P. gingivalis* was also grown as well as a positive control. It was diluted from an OD_{600} of 1, 1:2 fold in a 96 well plate with a total of 100µl per well with 4 replicates. The plate was incubated in the anaerobic chamber at 37°C for 4 hours, 24 hours, and 48 hours.

After the timepoints were met, the plates were dumped out and submerged in water to wash twice. 125µl of 0.1% solution of crystal violet in water were placed into each well of the plate and incubated for 15minutes covered at room temperature. The plate was submerged again in water 3 times, blotted on paper towels, and dried overnight. 30% of acetic acid in water was added to each of the wells with a total of 125µl per well and incubated for 15 minutes. It was then transferred to another flat bottomed 96 well plate. The absorbance was to be read at 550nm with a blank. It was repeated once with paraformaldehyde in an attempt to fix the cells prior to the washes.

4% paraformaldehyde solution in 1xPBS was added to the wells after the initial media was carefully removed. The plate was incubated for 15minutes at room temperature. The plate was submerged and washed twice prior to the addition of crystal violet to the wells. Another 15-minute incubation occurred, the plate was blotted and dried overnight.

Epithelial cell Adhesion assay: TIGK cells

S. sputigena adhesion to oral epithelial cells is yet unknown, so an adhesion assay was performed in order to test this. Telomerase-immortalized gingival keratinocyte (TIGK) cells were seeded in half of a black 96 well plate and the other half of the plate was left empty. *S. sputigena* was grown up in a 10ml culture in TYGVS media in the anaerobic chamber the night before. The culture was spun down at 5000g for 5 minutes and adjusted to 2 ml per $1OD_{600}$ in 2mls of PBS. A working concentration of BacLight Green was used to stain, as per the manufacturer's protocol. The cells were spun down and washed with 2mls of PBS to remove unbound BacLight. A serial dilution of *S. sputigena* cells were performed into PBS and 100µl of cells were added per well. The plate was incubated at room temperature, covered in foil, and

rocked for 1 hour. The remaining bacteria is removed by washing the wells 5 times with 100mls of PBS per well leaving the final wash in the wells. It was then imaged using a fluorescence plate reader.

Antibiotic Sensitivity

In order to establish bacterial OD_{600} and be able to figure out when *S. sputigena* is suppressed in the presence of particular antibiotics, bacterial resistance to different antibiotics at different concentrations was a necessity. Ampicillin, Erythromycin, and Chloramphenicol were chosen first. A starting culture of *S. sputigena* was grown in TYGVS media overnight and was used to inoculate another overnight culture. 1ml of this overnight culture was used to inoculate the tube. Stock solutions of each antibiotic were created at 50mg/ml and 10µl, 5µl, 2.5µl, 1µl were each added to their corresponding 10ml culture. One culture was left without any antibiotics. Each OD_{600} was measured at a 4hr timepoint.

This was repeated in duplicate with the two most promising antibiotics: Ampicillin and Erythromycin. A stock solution of 100mg/ml was made for both. Of the stock, 10µl and 5µl of Ampicillin and 75µl, 50µl, and 25µl of Erythromycin were added, respectively. The 10ml cultures grew from an OD_{600} of .05 and final OD_{600} was recorded at 4 hours.

Cloning

Gene	Product domains and Description	Sequence size	Protein size
SELSP_1610 full length	Diadenylate Cyclase	834 nucleotides	30.86 kilodaltons
SELSP_1610 Truncated	Diadenylate Cyclase missing membrane domain	537 nucleotides	19.49 kilodaltons
SELSP_2051	DHH/DHHA1/PAS Phosphodiesterase	2034 nucleotides	75.18 kilodaltons
SELSP_1770	TetR-family transcriptional regulator	624 nucleotides	23.46 kilodaltons
SELSP_1836	TetR-family transcriptional regulator	561 nucleotides	21.24 kilodaltons

Table 1. *Selenomonas sputigena* genes

This table shows the genes, SELSP_1610, SELSP_2051, SELSP_1770, and SELSP_1836, that were successfully cloned from *Selenomonas sputigena*. SELSP_1610 was truncated and cloned again, as seen above. Each are listed with their domains and descriptions, their sequence size, and their protein size as a reference.

Primer design and PCR

The cloning process was started when the original sequence for *S. sputigena*, found on the Kyoto Encyclopedia of Genes and Genomes (KEGG), was used to create primers for the desired protein sequences. The chosen sequences: SELSP_1836 and SELSP_1770, both TetR-family transcriptional regulators, SELSP_2051, DHH/DHHA1/PAS domain, and SELSP_1610, a Diadenylate cyclase, from *Selenomonas sputigena*. Primers were designed an annealing temperature of 55°C. The forward primer was designed with an overhang from the pLATE52 vector on the 5' end, and the reverse compliment was taken for the reverse primer and the reverse primer was added on the respective 5' end.

Primers	Forward	Reverse
pLATE52 Vector overhangs	5'-**GGT TGG GAA TTG CAA**-insert sequence- 3'	5'-**GGA GAT GGG AAG TCA TTA**-insert sequence-3'
LIC Sequencing primer	TAATACGACTCACTATAGGG-	GAGCGGATAACAATTTCACACAGG-
SELSP_1836	**GGTTGGGAATTGCAA**ATGGACAGACGACAGAAAAAGAC-	**GGAGATGGGAAGTCATTA**GAGAATCGGCTCGATCACG-
SELSP_1770	**GGTTGGGAATTGCAA**ATGGAAGAAGAGAAGATAAGCCGA-	**GGAGATGGGAAGTCATTA**TTCCTTTTGTTCTTCCTGCTTTTC-
SELSP_2051	**GGTTGGGAATTGCAA**TTGCCCCGAAATCTGTCG-	**GGAGATGGGAAGTCATTA**CCCTTGCTTCCGACCTTC-
SELSP_1610	**GGTTGGGAATTGCAA**ATGCCGTTCCATATTCCGATTC-	**GGAGATGGGAAGTCATTA**TTTCCCCTTCCTCCAATTCAG-
SELSP_1610 Truncated	**GGTTGGGAATTGCAA**CAGGGACGCTTCTTCGG-	**GGAGATGGGAAGTCATTA**TTTCCCCTTCCTCCAATTCAG-

Table 2. Primer design

This table shows the design of both the forward and the reverse primer pairs for each of the genes that were cloned from *S. sputigena.* The first shows the forward and reverse overhang for the pLATE52 vector. The second exhibits the primers used during sequencing.

The overhang forward primer and the overhang reverse primer can be seen in Table 2. *S. sputigena* was used as template DNA. Template gDNA was prepared by boiling *S. sputigena* in water for 10minutes. PCR protocol for Phusion, high-fidelity DNA polymerase, was run for all constructs with their respective forward and reverse primers. SELSP_2051 only worked with Touchdown PCR program. This program relies on the gradual decrease of annealing temperature until the desired annealing temperature is reached. It is decreased by 1-2°C each second until the annealing temperature of the primers has been reached. All products were run on an 8% agarose gel and photographed with the correct ladder: either 100kb DNA ladder from New England Biolabs (NEB) or 1000kb DNA ladder from (NEB). Each DNA band was compared to its known size respectively to make sure they were correct (Table 2).

Each band from the gel was imaged, cut out, and purified twice using Monarch Kit for gel extraction and purification (NEB). The concentration was found using a nanodrop. The resulting product was then ligated into the pLATE52 vector using the recommended amount of purified product from aLICator ligation independent cloning and expression system kit from Thermo scientific with an N-terminal His-tag. The transformation following the ligation was performed into competent cells: *E. coli*: Nova Blue (DE3) cells. Within 24 hrs, the colonies were taken, plated, and labeled in a grid on ampicillin plates to keep each colony organized. The colonies were grown for 14 to 16 hours and standard minipreps were performed the following morning. The plasmids were isolated and purified. The plasmids were sequenced by Genewiz using the LIC forward and reverse primers (Table 2). It was then compared with the original sequence found on KEGG to confirm their accuracy.

Protein Induction

Protein induction followed. Colonies were chosen from the corresponding correct sequences and were added to an overnight culture (LB ampicillin). It was grown at 37°C in a shaker (14 and 16 hours). The OD_{600} was checked at the end of the incubation. A 500 ml culture of LB broth with (1/1000) ampicillin from a 100 mg/ml stock was brought up to an OD_{600} of .05 and grown until the OD_{600} was between .400 and .600. A sample was taken and resuspended with SDS page buffer. 500μM of IPTG (isopropyl-beta-D-thiogalactopyranoside) was then added to the culture to induce protein production for four hours. Cells were harvested at the four-hour time point and another sample was taken and resuspended using SDS page buffer. This process was completed for each respective protein. A control was used at the end of each protein induction in the form of a Coomassie gel. Each sample of competent cells, pre and post IPTG, were boiled and fractioned by SDS-PAGE. The gel was stained using Coomassie blue, and the protein sizes were compared to the duel color standard protein ladder from Bio-Rad and known sizes were found from the sequence (Table 1).

SELSP_1610 had a membrane bound domain that prevented the protein from leaving the cell. This membrane bound portion reduced OD_{600} and resulted in no protein production. This protein was modified to remove the membrane-bound domain in order to facilitate the expression of the protein. The forward primer was redesigned (Table 2) to exclude the membrane-bound domain. PCR was repeated, as stated above, and SELSP_1610 truncated was sequenced. With the sequence confirmed, the truncated protein was induced again using IPTG. The Coomassie gel run with the pre and post induction samples confirmed the protein expressed. The size was also confirmed (Table 1).

Homogenization and FPLC

All the pellets were spun down, the supernatant discarded, and the pellets were collected and stored at -80°C until they were homogenized. The homogenizer used: 20mM imidazole, 20mM NaH_2PO_4, 500mM NaCl, and 1mg/ml lysozyme. The pellet was vortexed and pushed through a high-pressure homogenizer. The protein was purified by metal affinity chromatography using a Ni-NTA column. The crude lysate was washed out with the buffer and high purity protein was eluted with imidazole. The purified protein was eluted into PBS with 50 mM glutamic acid and arginine.

Reverse Phase Chromatography
SELSP_1610 Enzymatic Assay

Both buffer A and buffer B were made the same week the reaction was going to be run. Buffer A included 100mM KH_2PO_4, 4mM tetrabutylammonium hydrogen sulfate and the pH was equalized to 5.9, and Buffer B included 70% Buffer A and 30% methanol (MeOH) , was used to run the HPLC machine. Diguanylate cyclase buffer (DGC buffer) needed to be made within two days of running the reaction. This buffer contained 10mM of an activating cation chosen for the reaction, and the remaining compounds remained unchanging. The DGC buffer contained 50mM Tris-HCL (pH 7.6), 0.5mM EDTA, 50mM NaCl. The suggested cations to use in the literature were Magnesium, Manganese, and Cobalt (34). The conversion of ATP to cyclic-di-AMP was to be tested using a reaction of the enzyme, truncated SELSP_1610, the chosen cation within the DGC buffer, plus an excess of ATP incubated at 37°C for an hour. 5µM of SELSP_1610 was added to the DGC buffer. 150µM ATP of a 10mM stock was used. 58.5µg was needed of SELSP_1610. Overall, the total reaction volume was 600µl and of that, 97.5µl was needed of SELSP_1610 along with 9µl of ATP. The reaction was incubated 37°C for an

hour, boiled for 3 minutes to stop the reaction, and then centrifuged at 15,000g for 2minutes. The sample was then filtered through a .22µM syringe filter and 20µl was loaded into the column. Prior to the reaction being run through the HPLC column, a 600µl reaction of 9ul of ATP and another 9µl of cyclic-di-amp should be run as controls in order to determine the elution profile of each control molecule. The two controls that were initially used were ATP alone and cyclic-di-AMP alone. The controls were to be run each time the reaction was run as they shift minutely per run through the HPLC column and need to be compared to each respective reaction and buffer used. It has also been suggested to run the c-di-AMP control post reaction as to mitigate any false positives.

Reaction volume	Protein run SELSP_1610	Incubation Time	Amount of ATP added	Cation	Buffer used
Reaction set 1 600µl	Original protein 600µg/ml	1 hour	9µl ATP	10mM $MgCl_2$	1x DGC buffer, Elution 30% MeOH
Reaction set 2 600µl	Original protein 600µg/ml	1 hour	9µl ATP	10mM $MgCl_2$ $MnCl_2$	1x DGC buffer, Elution 50% MeOH
Reaction set 3 600µl, 300µl 200µl	Dialyzed protein 137µg/ml Original protein 600µg/ml Dialyzed protein 137µg/ml	1 hour, 1 hour, 2.5 hours	9µl ATP, 3µl ATP 3µl ATP	10mM $MgCl_2$ $MnCl_2$	10x DGC buffer, 1x DGC buffer, Elution 50% MeOH
Reaction set 4 New column 600µl	Original protein 600µg/ml Dialyzed protein 130µg/ml	2 hours 1.5hours	9µl ATP	10mM $MnCl_2$	1x DGC buffer, Elution 50% MeOH

Table 3 Enzymatic Assay Variables
This table is a visual representation of all of the variables changed to improve this assay: kinetics, stoichiometry, cations, and buffer concentrations with focus on the reaction volume, the protein concentration, dialyzed protein vs original protein, incubation time, the amount of ATP added to the reaction, and the cation of choice.

This enzymatic assay began with the reaction run in 1xDGC buffer (table 3). Based on the literature, the first activating cation was chosen (magnesium chloride at 10mM). This reaction took the purified protein, truncated SELSP_1610, and added 58.5 µg (97.5µl), and an excess of ATP at 150µM (9ul added) then brought up to a total volume of 600 µl with 1xDGC buffer. The reaction incubated for an hour at 37°C, was boiled at 99°C and was processed as previously described above. The two controls, ATP and c-di-AMP, were both run prior to the 20µl injection of the reaction run through the HPLC column and eluted with 30% MeOH. Due to the fact that ATP was eluted and compared to the ATP peak, the experiment was repeated to improve the assay (table 3). The controls were run every time to compare the products. Though the baseline assay remained the same, following the protocol described above, different variables were adjusted such as different choice in cation, change in stoichiometry, variation in kinetics, and changes in other minute details to improve the assay.

. The first variable changed was the cation added to the DGC buffer. Initially magnesium chloride was used. The buffer was remade again using two cations instead of one, magnesium chloride, and manganese chloride (10mM), and was repeated again. The last buffer was made only including manganese chloride and excluding magnesium as the cation of choice based on findings in the literature. In addition, dialysis was completed using the original protein over the weekend at 4°C into 1xDGC buffer with magnesium and manganese, then overnight with manganese alone. These reactions with the dialyzed protein were completed in addition to the non-dialyzed original protein (table 3) to prevent phosphate inhibition from the original buffer.

More stoichiometric changes were made by reducing the total volume size of the reaction from 600µl, to 300µl, to 200µl using both the dialyzed protein at both full volume and reduced volume size (table 3). The addition of ATP was reduced along with the reaction size. The total

concentration of the DGC buffer was increased to 10x, instead of 1x, as well (table 3). This was only performed in one reaction. The next condition to be adjusted, in terms of kinetics, was the reaction time.

The reaction time (incubation) started with, was 1hr. Over the course of these reaction sets, the time was increased to 1.5hrs, 2hrs, and last 2.5hrs (table 3). In total, the combination of changes in condition included reaction time, change in reaction buffer concentration, change in protein concentration and reaction size, change in protein buffer, and change in cation of choice. These were tried together in various combinations to reach a different outcome and improve the assay (table 3). In addition, small changes were made to the assay such as thawing the protein in water to preserve the integrity of the enzyme, changing the methanol elution from 30% to 50%, running blanks to screen for background peaks, and using a new column. More time is needed to change any other variable that may need to be adjusted to improve the assay.

Chapter 3: Results

Basic Characterization of *Selenomonas sputigena*

Though *Selenomonas sputigena* had been discovered a little over 6 decades ago to be associated with fatal septicemia and found within the oral biofilm as a marker for periodontal disease, it is considered to be an emerging oral pathogen. Its growth patterns in the laboratory, let alone virulence factors as a potential pathogen, have yet to be truly defined within available literature. There has been little to no basic background information or supplemental information published on this organism. As it has not been looked at in detail to date, there is very little starting material on *S. sputigena*. Due to this fact, the initial step was to begin with establishing basic information on this anaerobic organism. Without defining the parameters of *S. sputigena*

growth within the laboratory, there would be no solid foundation on which to begin this project. In order to determine this, the project began with defining basic growth characteristics such as proper growth media, a bacterial growth curve, plate counts, oxygen resistance, and finding antibiotic sensitivity. Hemagglutination, hemolysis, monospecies biofilm development, and adhesion to epithelial cells was also tested in addition.

Selenomonas sputigena Growth Characteristics

Growth Curve

The growth curve was meant to test two different types of growth media over the course of 10 hours with measurement of OD_{600} every hour. The growth for *S. sputigena* media never met the proper parameters for a growth curve and was excluded from all future attempts to grow *S. sputigena* (Figure 3-1). The rate of growth was too low to use it as a viable option; however, TYGVS media exhibited a classic growth curve. Exponential phase was reached between three and six hours and the stationary phase followed at approximately seven hours. The doubling time was calculated to be between 25-30 minutes (Figure 3-1) during the Log phase. The growth curve, when repeated, should be extended past the ten-hour mark for a more complete picture of bacterial growth. The stationary phase never entered the death phase; therefore, the time should have been extended another 4 to 6 hours.

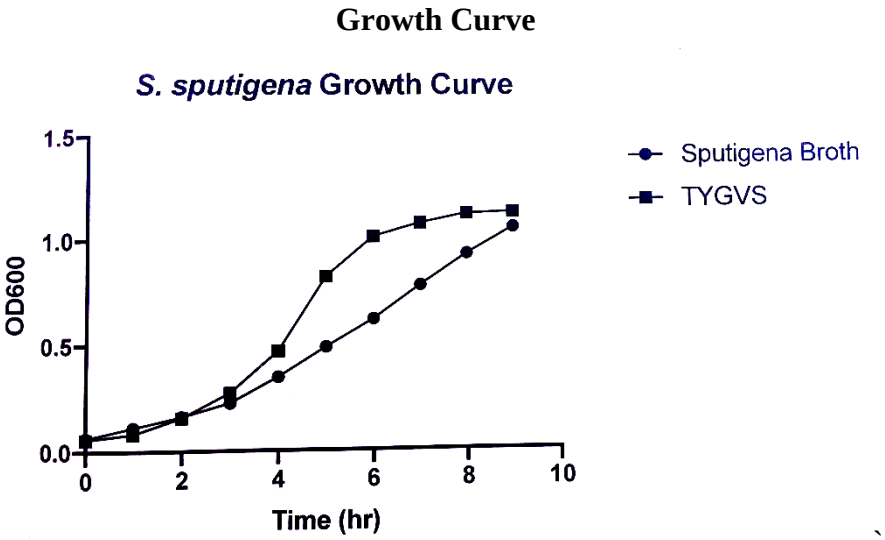


Figure 3-1 Growth Curve for *Selenomonas sputigena*

The growth curve began with an OD_{600} of .05. The growth curve was completed over the course of 10 hours and was checked every hour. The lag phase happened between 0 and 3 hours. From 3 to 6 hours the exponential phase occurs and this leads into the stationary phase. The time needs to be extended in order to show the death phase. The equation: $N_t = N_0*(1+r)^t$ was used to calculate the doubling time, which was discovered to be 25-30minutes.

Plate counts

The plate counts were completed several times and have not been able to be replicated successfully without some level of contamination on the plates, or inconsistencies with the number of colony forming units. Consistent counts have been unexpectedly difficult to achieve because of this. The most consistent results were the initial counts, which showed approximately $1.0x10^9$ cells per ml, (used in the hemagglutination assay) and the last plate count which is seen below in Figure 3-2. This experiment needs to be repeated three times with consistent counts on each plate, and no contamination, to find a more accurate plate count and cells/ml. This, however, has given us a start in order to complete more assays like the hemagglutination assay.

Plate count

Dilution Plated	Number of Colonies	Average Colony Count	Cells/ml
10^{-4}	2320, 2595, 2363	2426 cfu	2.4×10^8
10^{-5}	367, 356, 266	330 cfu	3.3×10^8
10^{-6}	35, 16, 15	22 cfu	2.2×10^8
10^{-7}	-,2,4	3 cfu	3.0×10^8
10^{-8}	-,1,-	1 cfu	1.0×10^8

Figure 3-2 Plate counts

This table shows the plate counts for *Selenomonas sputigena* grown over the course of four days. Each plate was counted and the cells per ml were calculated from the known colony forming units and starting OD_{600} of 1. The average calculated for cells/ml was 2.4×10^8.

Oxygen Resistance

Oxygen resistance was repeated by another member of the lab. *S. sputigena* plated in anaerobic conditions mirrored the plate counts grown previously (Figure 3-3) with 1.7 x 10^8 cells/ml calculated. The aerobic conditions exhibited no colony formation on the plates. This indicates that *Selenomonas sputigena* has little to no oxygen resistance and cannot grow in an oxygen rich environment. The question is: at what point does growth slow, stop, and result in the death of the *S. sputigena* colony. A more detailed test would be needed, perhaps with more timepoints for plating.

Oxygen Resistance Plate Counts

Dilution Plated	Number of Colonies	Average Colony Count
Anaerobic		
10^{-3}	TNTC	-
10^{-4}	TNTC	-
10^{-5}	206, 168, 137	170
Aerobic		
10^{-3}	No Colonies	-
10^{-4}	No Colonies	-
10^{-5}	No Colonies	-

Figure 3-3 Plate Counts for Oxygen Resistance

This table exhibits the data for the plate counts done in both anaerobic conditions and aerobic conditions. The anaerobic conditions mirror the previous plate counts with an average cells/ml of 1.7 x 10^8 cells/ml and too many colonies to count for the first two dilutions. The aerobic conditions resulted in no colonies plated, indicating *S. sputigena* is not resistant to the presence of oxygen.

Antibiotic Sensitivity

Antibiotic sensitivity needed to be studied in order to begin the cloning process. Without knowing what type of antibiotic to use, choosing the cloning vector would not be possible. Three types of antibiotics were used: ampicillin, erythromycin, and chloramphenicol. Initially, because little to no information exists on antibiotic sensitivity with *Selenomonas sputigena*, a broad spectrum of types and concentrations were used. 50µg/ml, 25µg/ml, 12.5µg/ml, and 5µg/ml of each were added, complete with two blanks and two cultures grown without (Figure 3-4). This was just an initial test to see where to begin. It was then narrowed down to erythromycin and ampicillin and was repeated. The bacterial culture was started at an OD_{600} of 0.05 this time and allowed to grow for 4hrs. The amount of erythromycin added was increased to 750µg/ml, 500µg/ml, 250µg/ml and ampicillin with 100µg/ml and 50µg/ml (Figure 3-5). The blank and the culture without antibiotics were also run again.

The initial round was used as a starting point to narrow down the choice of antibiotics. It was evident that ampicillin reduced the OD_{600} greater than any other of the chosen antibiotics. It was then replicated, and the second round was more specific and focused on the last two antibiotics that would be a viable option. This was repeated one last time before ampicillin was the chosen antibiotic, as it was shown to reduce OD_{600} when compared to the culture without antibiotics. Ampicillin, at the concentration of 100µg/ml, was found to be the most effective antibiotic as *Selenomonas sputigena* showed sensitivity to it and the total OD_{600} was decreased (Figure 3-5). This was the chosen concentration that was used throughout the cloning process.

Initial Antibiotic Sensitivity

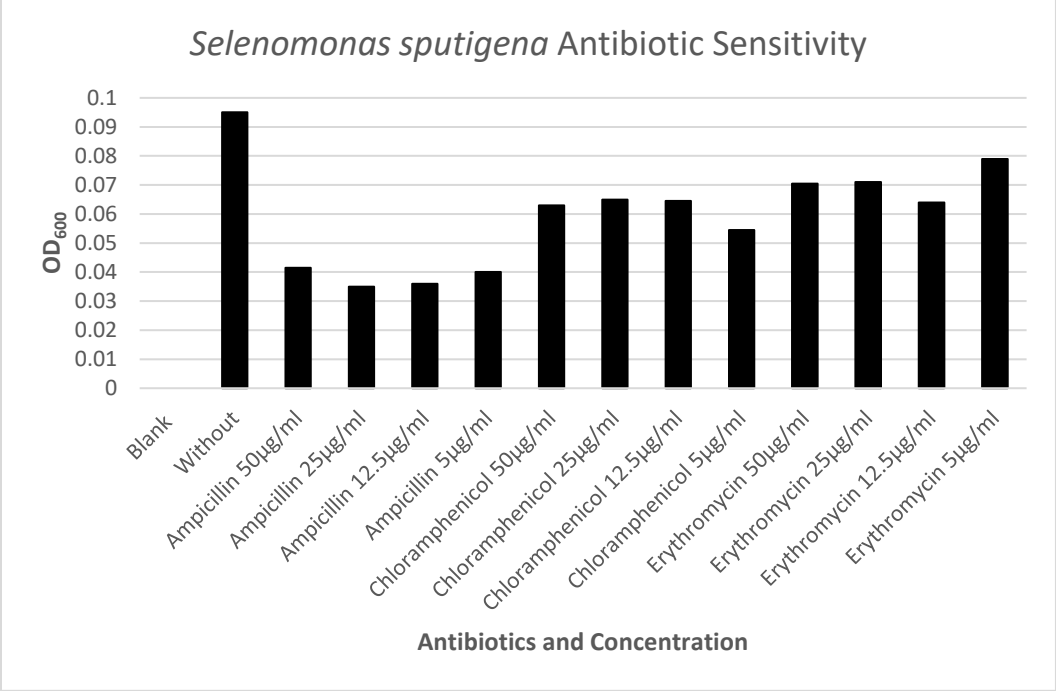


Figure 3-4 Antibiotic Sensitivity Initial

This bar graph exhibits the initial antibiotic sensitivity panel chosen with concentrations of 50µg/ml, 25µg/ml, 12.5µg/ml, and 5µg/ml for ampicillin, chloramphenicol, and erythromycin. The optical density was recorded after 4 hours and ampicillin appears to have reduced the OD_{600}.

Antibiotic Sensitivity Replicate

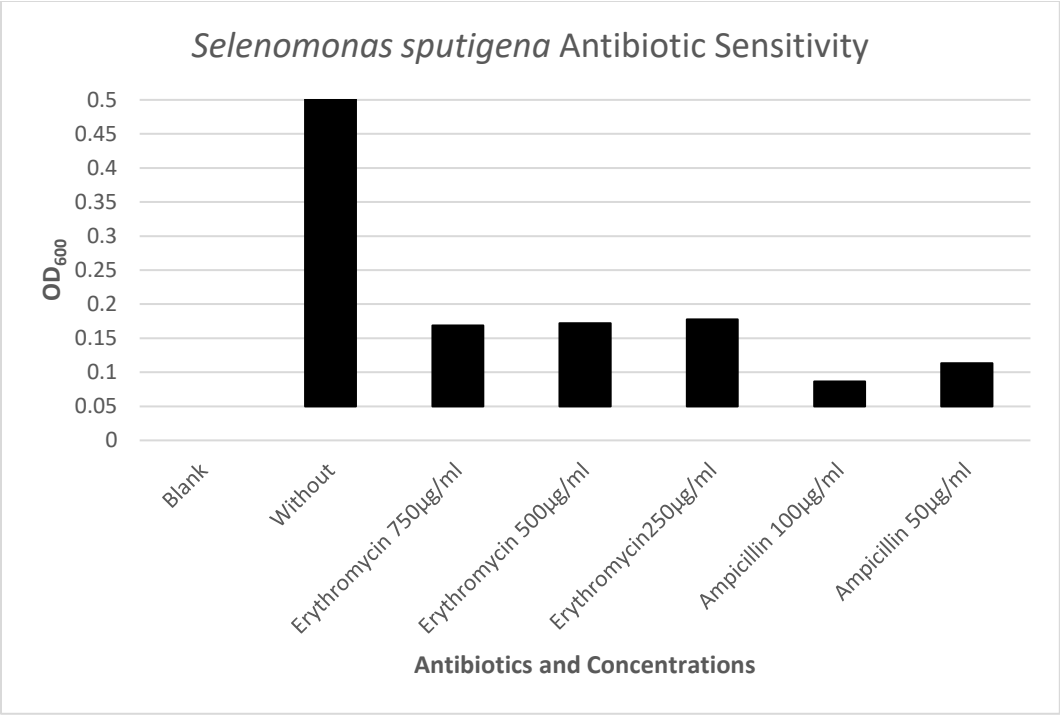


Figure 3-5 Antibiotic Sensitivity

This bar graph shows the application of 750µg/ml, 500µg/ml, 250µg/ml of erythromycin and 100µg/ml and 50µg/ml of ampicillin. For this experiment, the cultures were started with an OD_{600} of 0.05. The optical density was taken again at four hours. Ampicillin, at the concentration of 100µg/ml, was found to be the most effective and was used throughout the cloning process.

Selenomonas sputigena Virulence Factors

Hemagglutination Assay

Hemagglutination, or the ability to bind to erythrocytes, and hemolysis, the lysing of erythrocytes, was the next thing that was tested with the help of the Jefferson lab. As *Selenomonas sputigena* is considered to be an emerging oral pathogen, its virulence factors also have yet to be defined as much as its growth capabilities. This was the first concrete step taken to find *S. sputigena* virulence factors in the lab. This assay would indicate if *S. sputigena* has the capability to bind to and lyse human erythrocytes. If hemagglutination and hemolysis of erythrocytes occurs, it is a good indication that *Selenomonas sputigena* not only has pathogenic capabilities but also have virulence factors that facilitate them as well. Checking if *S. sputigena* can agglutinate and lyse red blood cells would have allowed for more specific studies of this virulence factor. Human erythrocytes were used and *S. sputigena* was grown in an overnight culture. A 1:2 dilution was performed on a 12 well plate with a control with only erythrocytes in well 12 (Figure 3-6). Both incubation at 4°C, to study agglutination, and 37°C, to study hemolysis, were chosen. Had the results been positive for hemagglutination, *S. sputigena* would have bound the erythrocytes in a lattice, and the blood would have been uniform in the well. Had the results been positive for hemolysis, the buffer would have become red with a pellet in the bottom. However, the wells were negative for all the dilutions. The condensed pellet at the bottom indicating negative results corresponded with the negative control in the last well (Figure 3-6). This indicates that *Selenomonas sputigena* does not agglutinate or lyse red blood cells. This could be repeated with different conditions: aerobic vs anaerobic.

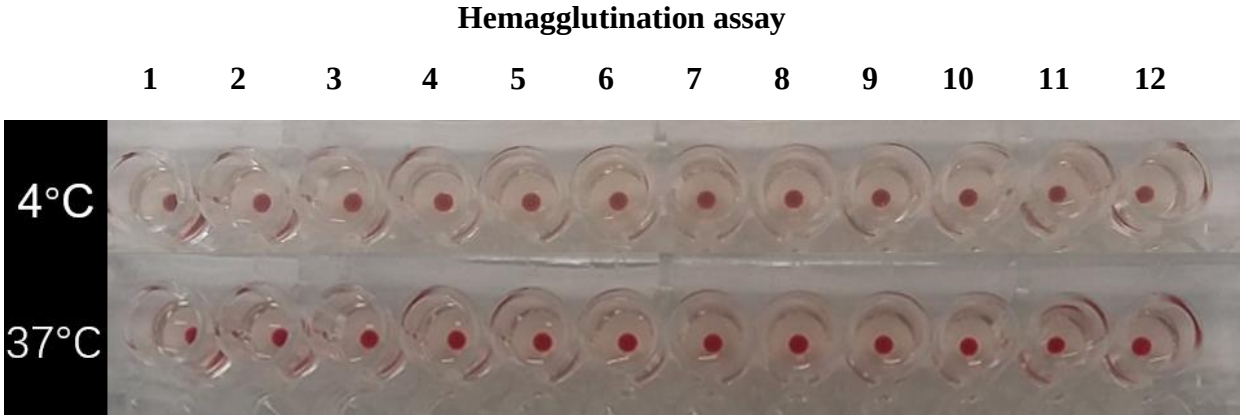


Figure 3-6 Hemagglutination/Hemolysis Assay

This figure is a picture taken of the first and second 96 well plate at the 4-hour time point. Well 12 is the negative control used for both incubation at 4°C (Hemagglutination) and 37°C (Hemolysis). This indicates that neither hemagglutination nor hemolysis has occurred.

Biofilm Development

Selenomonas sputigena monospecies biofilm development would indicate the presence and use of this as a virulence factor. Pathogenic bacteria in the oral cavity form an intricate overlay, interacting not only with other pathogens, but also commensal bacteria as well. Many species form both monospecies biofilms as well as polymicrobial biofilms to incite disease. They can interact with each other and can increase other virulence factors to cause disease. *Porphyromonas gingivalis* is a known oral pathogen that forms a biofilm, not only with itself, but with other oral pathogens like *F. alocis* (5). It is unknown if *Selenomonas sputigena* forms a biofilm with other pathogens as well as if it forms a monospecies biofilm. This assay performed was to study a monospecies biofilm for *S. sputigena.*

The assay was performed four times, and each time it resulted in an empty plate. *P. gingivalis* was used as the positive control. It was probable that the harsh washes cleared the cell layer completely, even though one plate was fixed first. The supernatant should have been taken off, and the wells washed gently. Another possibility is that *S. sputigena* should have been stressed first. Biofilms can and do also form under stressed conditions as well. This assay could be performed again by stressing both the positive control and *S. sputigena*. Either *S. sputigena* does not form a biofilm with itself, or the assay was incomplete and needs modification. The latter seems to be the case. As *P. gingivalis* did not appear on the plate either, this indicates that the assay needs to be developed more before it can work. It could also be the fact that *Selenomonas sputigena* does not form a biofilm with itself, but it interacts with other pathogens to form biofilms. The monospecies biofilm assay should be perfected and tried again with another protocol and it should be tried with other known oral pathogens to see if it interacts and forms polymicrobial biofilms.

TIGK Adhesion Assay

Selenomonas sputigena adhesion to gingival epithelial cells was looked at in this assay and has been replicated successfully. The graph illustrates the adhesion of *S. sputigena* to telomerase-immortalized gingival keratinocytes, or TIGK cells (Figure 3-7). *S. sputigena* was added in a series of dilutions to the first four rows. The last four rows were TIGK cells alone. The average background fluorescence in the non-infected wells was subtracted from the average fluorescence of the infected wells. This allowed for the fluorescence to be analyzed without the possibility of background changing the data. The data in Figure 3-7 indicates that *S. sputigena* does adhere to TIGK cells. Adhesion to epithelial cells is another indicator that *S. sputigena* possesses virulence factors that contribute to bacterial pathogenesis. This not only allows for the colonization of this bacteria through adhesion to the host cells but can also contribute to biofilm development. This is not only an indication that *S. sputigena* possesses this virulence factor and provides a direction to study adhesion, but also indicates that the biofilm assay should be revised and repeated.

***Selenomonas sputigena* Adhesion Assay: TIGK cells**

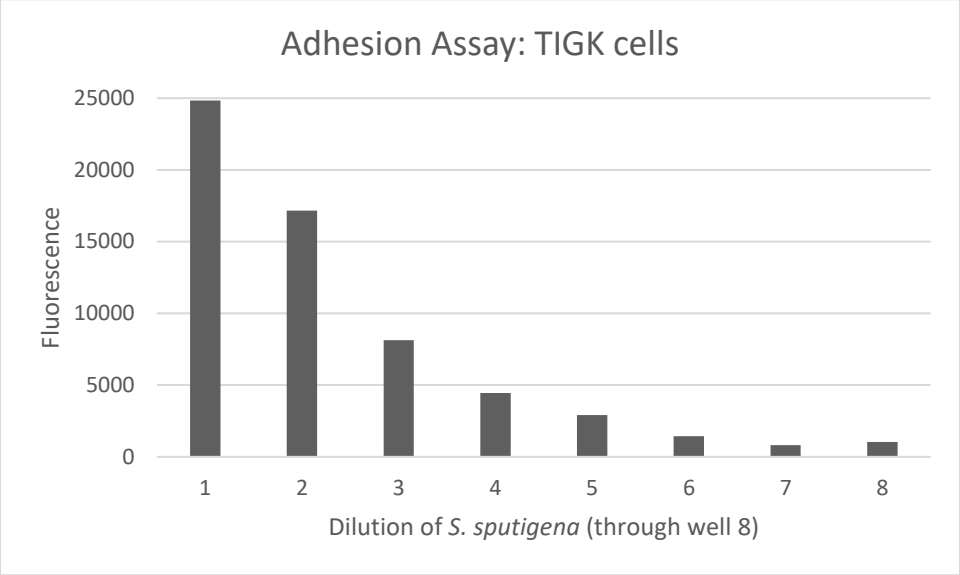


Figure 3-7 *Selenomonas sputigena* Adhesion Assay: TIGK cells

Once the background fluorescence from the TIGK (telomerase-immortalized gingival keratinocyte) cells was removed from the average, the remaining fluorescence was noted in this graph. The wavelength with which it was measured was 485nm/535nm. This indicates that *Selenomonas sputigena* does adhere to epithelial cells showing that it has potential virulence factors that lead to adhesion and interaction with the gingival epithelium.

Cyclic-di-AMP Metabolism in *Selenomonas Sputigena*

The focus was eventually shifted from microbial analysis in a laboratory environment to molecular and biochemical analysis of its potential synthesis, use, and degradation of the secondary messenger cyclic-di-AMP. All PCR products were run on agarose gels and the size was checked (Figure 3-8). SELSP_1836 and SELSP_1610 full length were easily PCR amplified. SELSP_1770 did not work until after the forward primer was redesigned and SELSP_2051 did not work with conventional PCR and only worked with touchdown PCR. This factor may have been contingent upon the size of the amplified product. The cloning process was completed, and the proteins successfully induced and purified (Figure3-9). The genome of *S. sputigena* was already sequenced and added to databases; however, certain genes have not been established in terms of their function. They can only be surmised by comparison to other known genes from other established microorganisms. The functions of SELSP_1810 and SELSP_1770 were hypothesized to be TetR-family transcriptional regulators, the function of SELSP_2051 is hypothesized to have DHH/DHHA1/PAS domains and has phosphodiesterase activity, and the main focus of this section is SELSP_1610, a hypothesized diadenylate cyclase.

PCR products

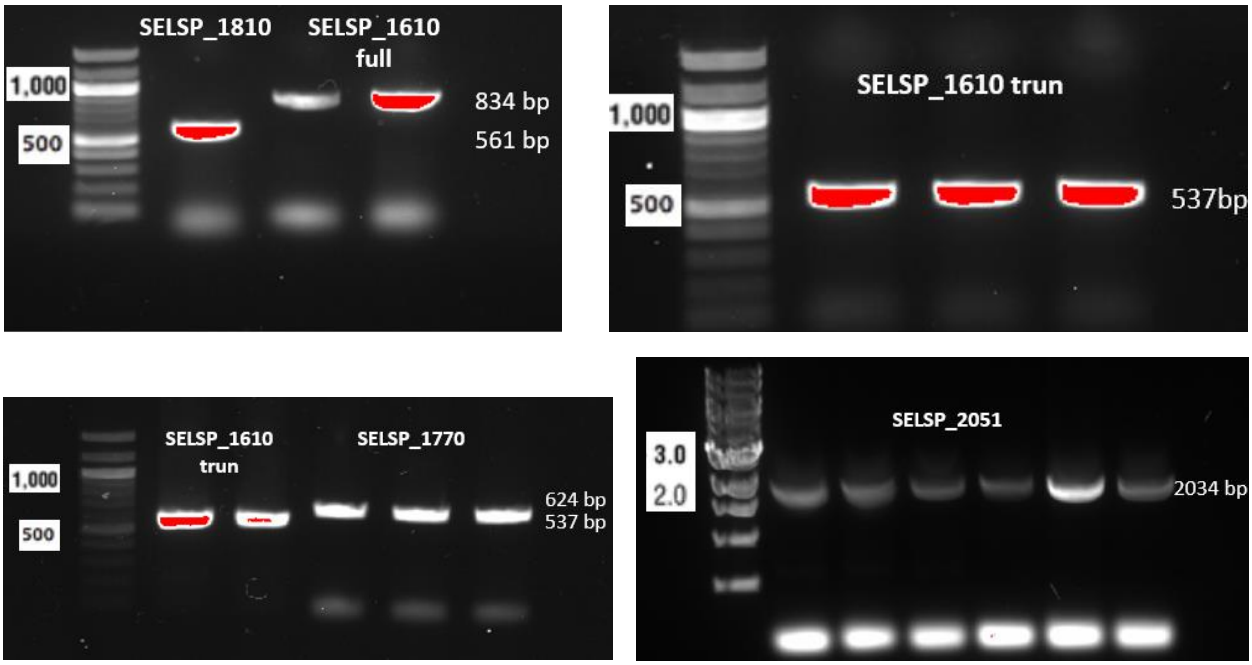


Figure 3-8 PCR Products

The bands for each group that are side-by-side are replicates of the same reaction. SELSP_1836 at 561bp, SELSP_1610 full length at 834bp and truncated at 537bp, and SELSP_1770 at 537bp were all strong bands. SELSP_2051 at 2034bp included intense primer dimer bands and was eventually amplified with Touchdown PCR as seen in lane 5.

SDS Page gel

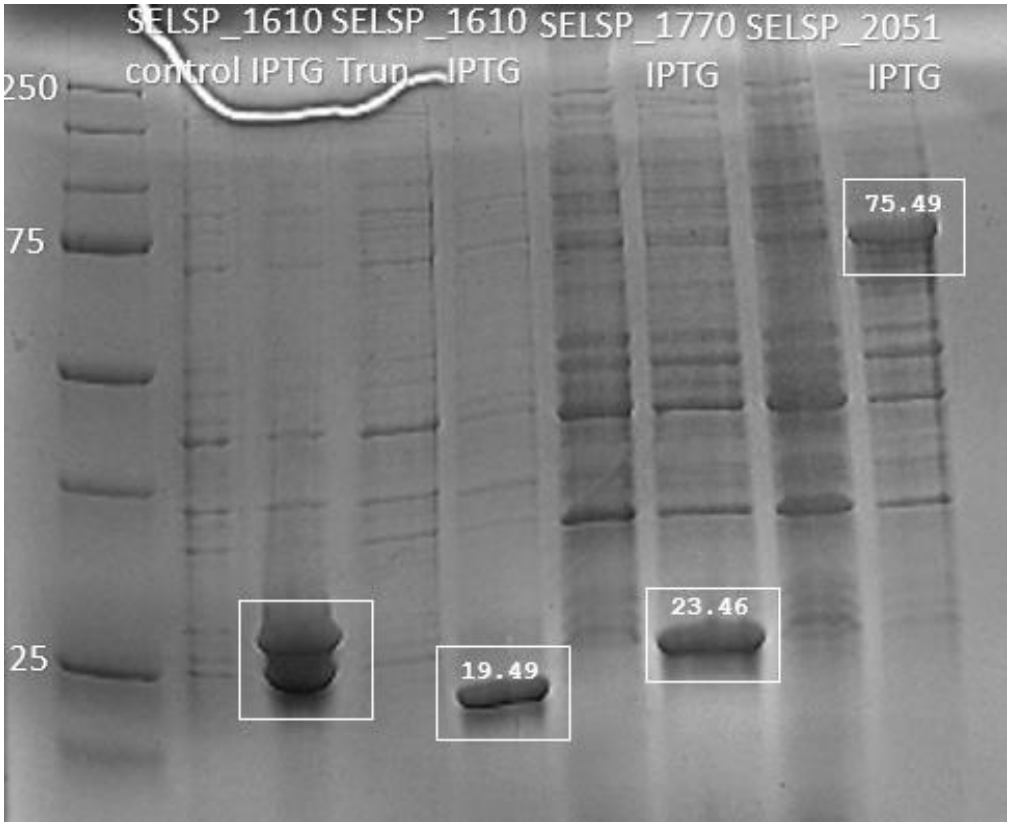


Figure 3-9 Coomassie Stain Protein Induction

This Coomassie gel shows the remaining induction of SELSP_2051 at 75.49 kilodaltons and SELSP_1770 at 23.46 kilodaltons. SELSP_1610 truncated was induced again to isolate more protein. SELSP_1836 can be viewed in Figure 3-10.

.

Enzyme Assay: SELSP_1610 cyclic-di-AMP

In addition to establishing basic growth conditions and beginning to find possible virulence factors for *Selenomonas sputigena*, an assay to study synthesis of cyclic-di-AMP in *S. sputigena* was developed using HPLC. We can make the prediction that *S. sputigena* possesses a functional cyclic-di-AMP system based on bioinformatic analysis. This suggested pathway regulates pathogenic and physiological functions within *S. sputigena*. The proposed diadenylate cyclase that was the focus of this assay was SELSP_1610. During the cloning process, SELSP_1610 had to be truncated due to prevention of protein induction due to the membrane-bound domain (Figure 3-10). Once truncated SELSP_1610 was successfully induced and purified, the next step was to find evidence to support that the purified protein acts as a diadenylate cyclase. SELSP_1610 was a match with other diadenylate cyclases for protein structure and, because of this, it was thought to have potential DAC activity and was hypothesized to convert ATP into cyclic-di-AMP.

IPTG protien indiuction of SELSP_1610

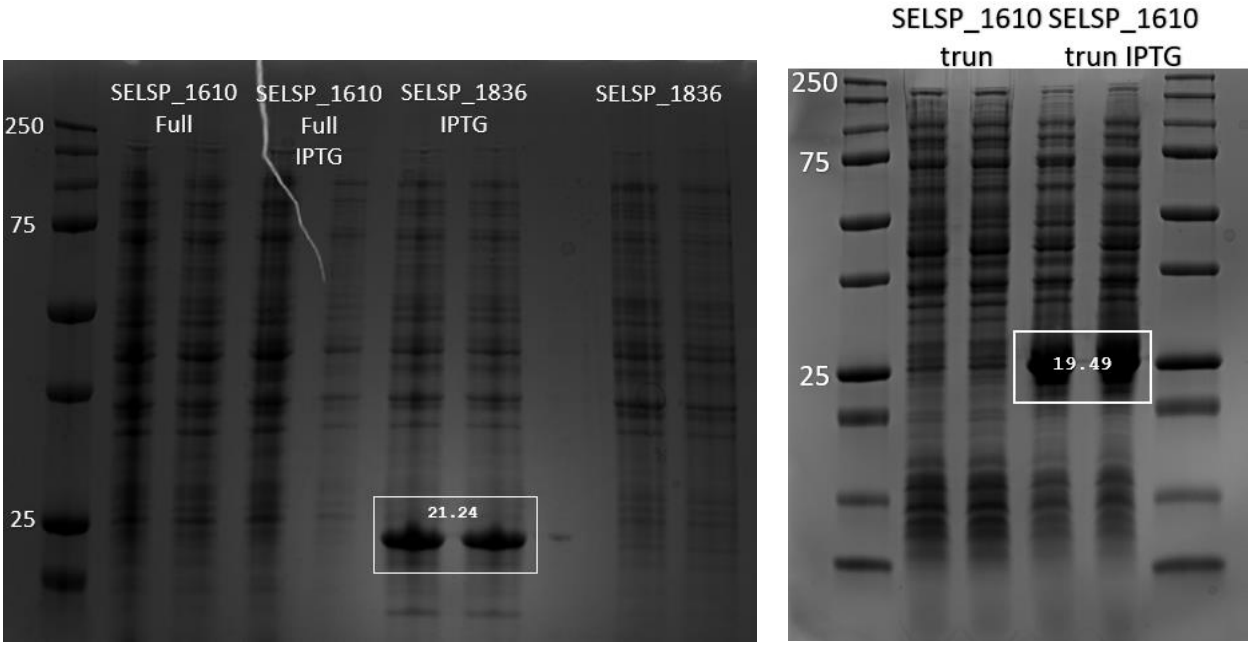


Figure 3-10

The Coomassie gel to the left shows the induction of SELSP_1610 side by side with the induction of SELSP_1836. SELSP_1836 was expressed with IPTG and appeared at 21.24 kilodaltons. When the membrane domain of SELSP_1610 was removed, the protein was induced and appeared on the gel to the right at 19.49 kilodaltons.

The amino acid sequence that was found on KEGG, matched with other transferases, and fit in the category of diadenylate cyclase within the KEGG orthology as a conserved hypothetical protein, CHP00159 (DacA diadenylate cyclase). BRITE hierarchy also confirmed this enzyme as a potential DacA diadenylate cyclase within *Selenomonas sputigena*. The amino acid sequence was run through NCBI blast and came back with similarity to diadenylate cyclase and within *S. sputigena* as a TIGR00159 family protein. The other sole fully sequenced selenomonad, *Selenomonas ruminantium* also has diadenylate cyclase (SFH94163.1) that has a similar structure to SELSP_1610 within *S. sputigena*. In addition, a model of the protein needed to be constructed and compared to other known sequences to further build evidence that SELSP_1610 had potential DAC activity and can be considered to be a diadenylate cyclase.

The known amino acid sequence from KEGG was run through both PHYRE and SWISS-model with both the truncated version and the full-length version. The focus, as the truncated version was what was used for the reaction, was the truncated version of SELSP_1610 that began with the membrane domain removed. The amino acid sequence was run through both modeling programs and the monomers from both were compared and found, not only to have similar structure, but were both considered to be a diadenylate cyclases when compared to other templates (Figure 3-11).

Truncated SELSP_1610 Model

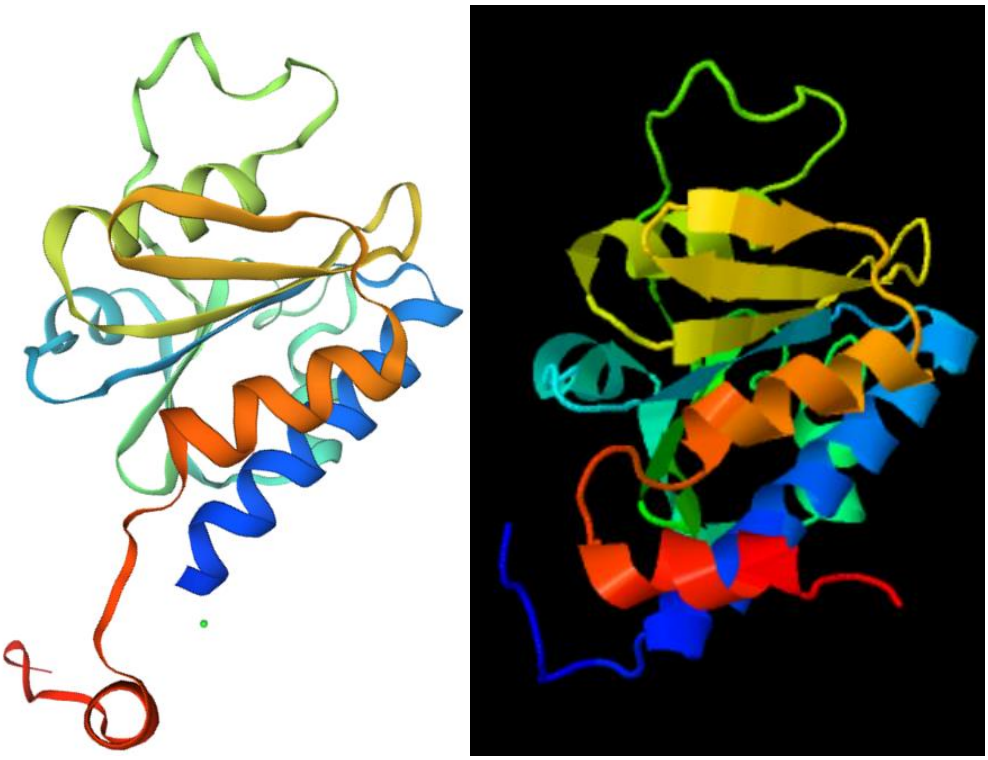


Figure 3-11 Truncated SELSP_1610 SWISS-model vs PHYRE model

The protein structure on the left is a model created by SWISS-model of the potential conformation of truncated SELSP_1610. The other model to the right was created by a similar protein modeling database called PHYRE which also created a potential model for truncated SELSP_1610. As seen comparatively, both models are similar to each other. This denotes the fact that, as it matches up to other enzymes that contain DAC activity, this protein could be considered a diadenylate cyclase.

Confidence Model of Truncated SELSP_1610 PHYRE

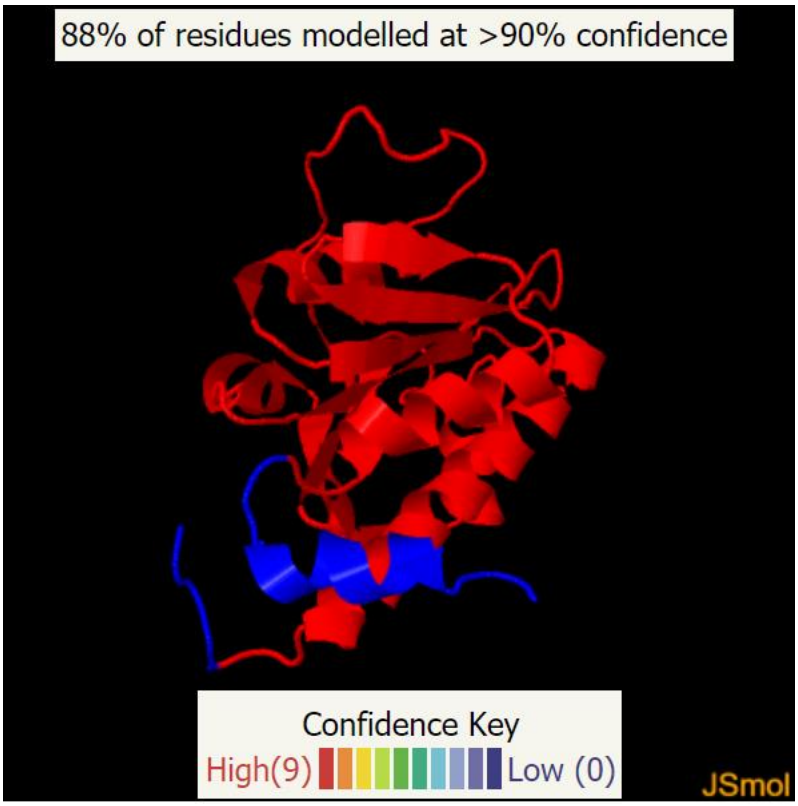


Figure 3-12 Confidence Model of Truncated SELSP_1610 PHYRE

This model of truncated SELSP_1610, the potential diadenylate cyclase, shows the confidence score when it comes to the structure created by PHYRE. This model was created with 88% of the residues modelled and greater than 90% confidence. The removed membrane portion seems to be the area of least confidence.

Confidence Model of Truncated SELSP_1610 SWISS-model

Figure 3-13 Confidence Model of Truncated SELSP_1610 SWISS-model

This homodimer model of truncated SELSP_1610, the possible diadenylate cyclase, was created by the SWISS-model. This model also shows the areas of most confidence in blue to least confidence in red. As with the model from PHYRE, the areas of least confidence were the removed membrane portion. When compared to other templates, this protein was suggested to have DAC activity. The active site and interaction with cations are in the middle.

PHYRE was found to have 88% total coverage with greater than 90% confidence (Figure 3-12). The SWISS-model had a positive QMEAN score of .29 with a loss of confidence near the cutoff point of the protein where the membrane domain had been removed (Figure 3-13). Both models together, when compared to other templates, point to SELSP_1610 being a membrane-bound enzyme that falls under the category of nucleotide transferases and matches other diadenylate cyclases from other bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes* (35,58,69,72). Though there are different types of diadenylate cyclase, SWISS-model suggested that the top two types of diadenylate cyclase that SELSP_1610 truncated mirrored, were CdaA, cyclic di-AMP synthase A, in *Listeria monocytogenes* and DacA, diadenylate cyclase A, in *Staphylococcus aureus* (35,58,72). Both have different nomenclature for the same enzyme: diadenylate cyclase. It is also the type that is conserved in *Firmicutes*, the phylum that *S. sputigena* is a part of. It can then be hypothesized that the function would be the same as well.

A detailed matching of suggested motifs within SELSP_1610 was also run as well to look for activation sites and binding motifs. A glycosylation site, protein kinase phosphorylation site, and myristoylation sites were all found. SELSP_1610 also has conserved RHR and DGA motifs which are responsible for ATP binding (59). To test the function of SELSP_1610, HPLC for Reverse Phase Chromatography was used. It had previously been used to study diguanylate cyclase in detail, but not diadenylate cyclase. Due to this fact, developing an assay through reverse phase chromatography using HPLC (High Performance Liquid Chromatography) was the concentration of this experiment and would be the chosen method to test this particular hypothesis.

Initially, primers for the original sequence for SELSP_1610 in its entirety were designed and an attempt at induction of the full-length protein occurred (Figure 3-10). This resulted in no production of SELSP_1610 as the protein is a membrane-bound protein. The membrane-bound portion did not allow for the production of this protein and only resulted in the drop of OD_{600} as the competent cells died instead. A new approach was taken, and the primers were redesigned to remove the first portion of the protein sequence, effectively removing the membrane-bound domain. This was then used as the protein, a truncated SELSP_1610, that was able to be expressed during protein induction. The hypothesis was that the truncated SLESP_1610 would have DAC activity, and within the literature, there were several suggested activating cations in order to facilitate the reaction with ATP that forms cyclic-di-AMP. The suggested cations were Magnesium, Manganese, and Cobalt (34). Through the help of the Marconi lab, a protocol that was previously used for looking at diguanylate cyclase and converting GTP to cyclic-di-GMP was already provided. This protocol however, had not been used to study diadenylate cyclase activity.

The initial reaction began with building the potential assay by choosing magnesium chloride as the first cation that was added to the 1x DGC buffer. Magnesium was chosen as both one of the cations recommended in the literature and one that had been used successfully in the cyclic-di-GMP study. The protein was thawed on ice prior to setting up the reaction that was run for one hour with 9µl of ATP. The controls, both ATP and c-di-AMP, were both run before the reaction was run and the recommended elution percentage of methanol used was 30%. The peak for ATP was eluted at 16 minutes and cyclic-di-AMP was eluted at 18 minutes with some background. When 20µl of the 600µl reaction was run through the HPLC machine, there was more background and an unknown peak. In addition, there was a peak that was eluted at 16

minutes indicating that ATP was eluted and not cyclic-di-AMP (Figure 3-14). The next reaction needed to be adapted as the primary reaction set did not work.

Reaction Set 1

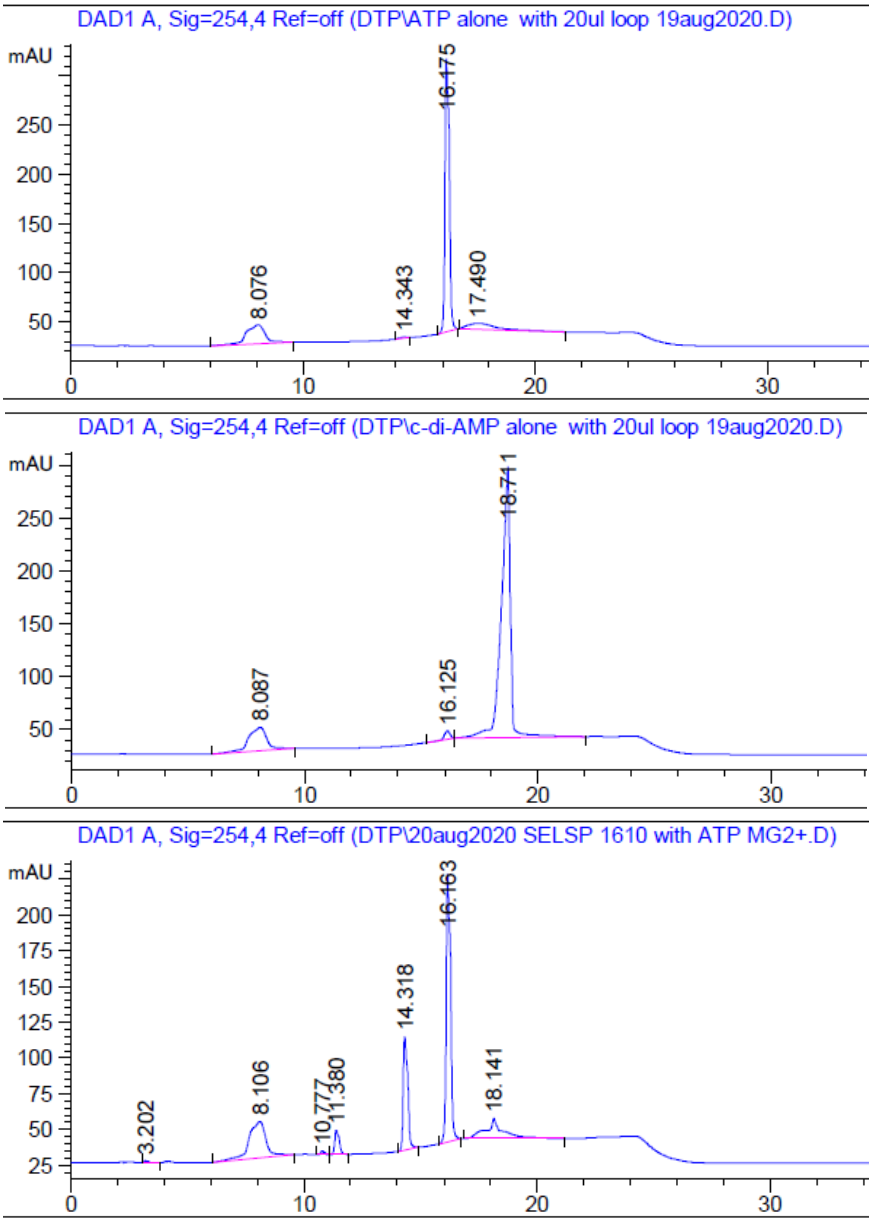
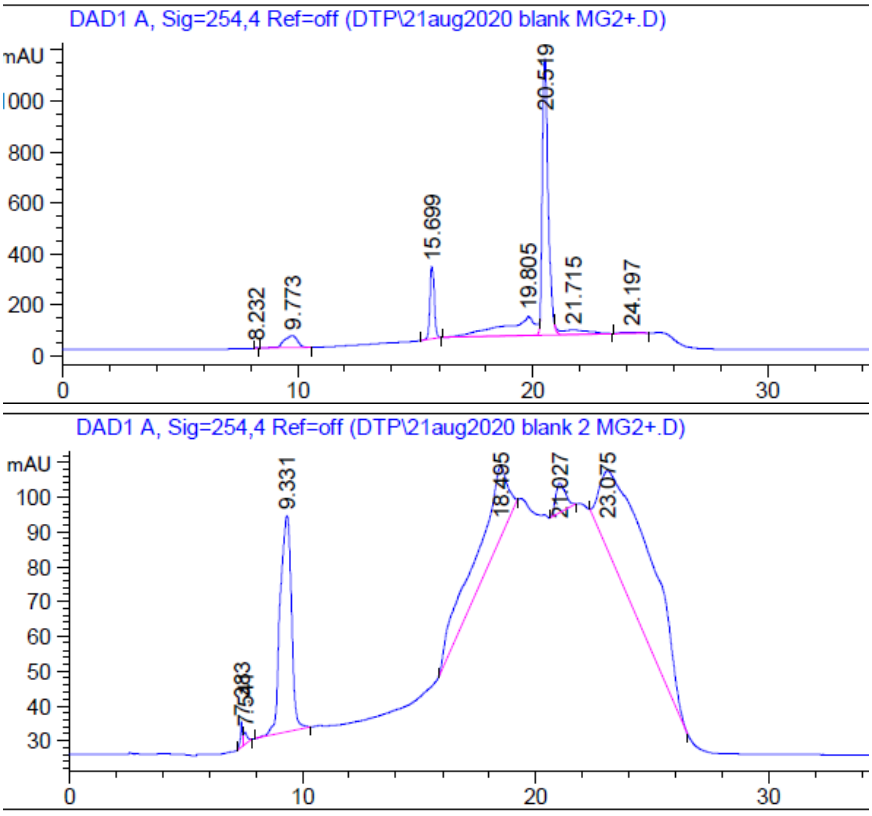


Figure 3-14 Reaction Set 1 for SELSP_1610: Magnesium

Reverse Phase Chromatography was run on an HPLC column. The first two graphs are controls featuring ATP alone with a peak eluted at 16 minutes in the first and cyclic-di-AMP run alone with a peak eluted at 18 minutes on the second. The third following was the first reaction set up with SELSP_1610 truncated, an excess of ATP, incubated for 1hr with 1xDGC buffer containing magnesium as the cation. The peak was eluted at the ATP mark: 16 minutes.

The second reaction set was changed so that it included two of the known activating cations found within the literature: magnesium chloride and manganese chloride. Every other aspect remained the same other than the elution percentage of methanol which was increased to 50% because it was suggested in another reverse phase chromatography protocol. Two blanks were run prior to running the control, cyclic-di-AMP, through the column in order to clear any potential background. Both blanks had background peaks, and when both the control and the reaction were run, they too had background peaks. Cyclic-di-AMP eluted at 16.5 minutes and the reaction that was run with the same conditions as reaction set 1 and was eluted at 14.5 minutes (Figure 3-15). This indicates that the product that was eluted was ATP and not cyclic-di-AMP. For the third reaction set, several variables were changed.

Reaction Set 2



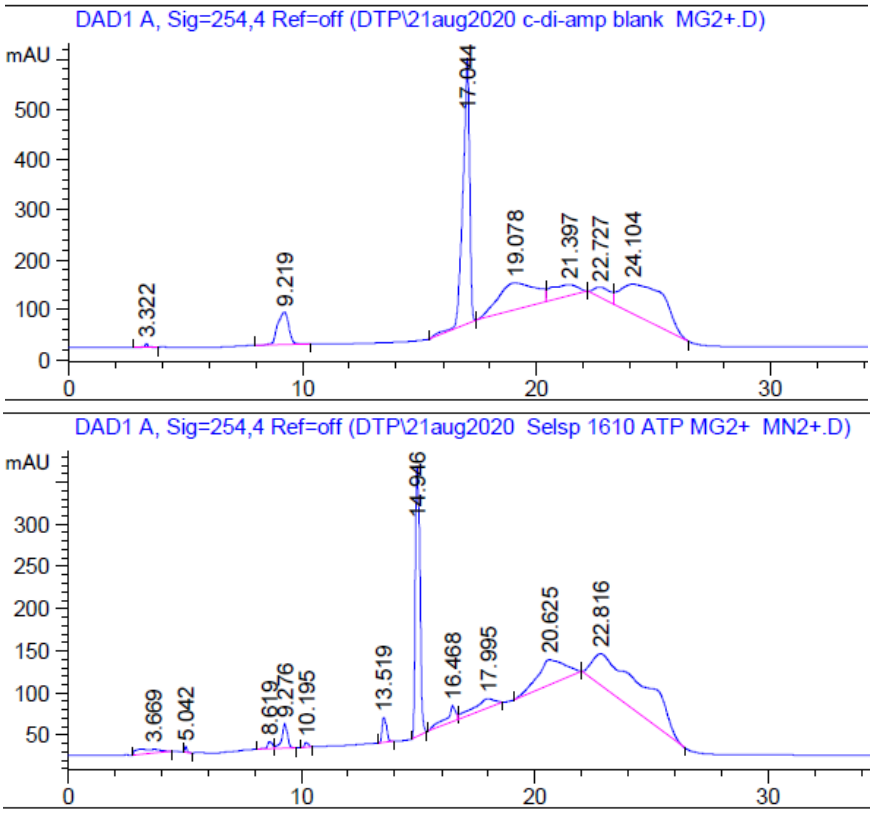
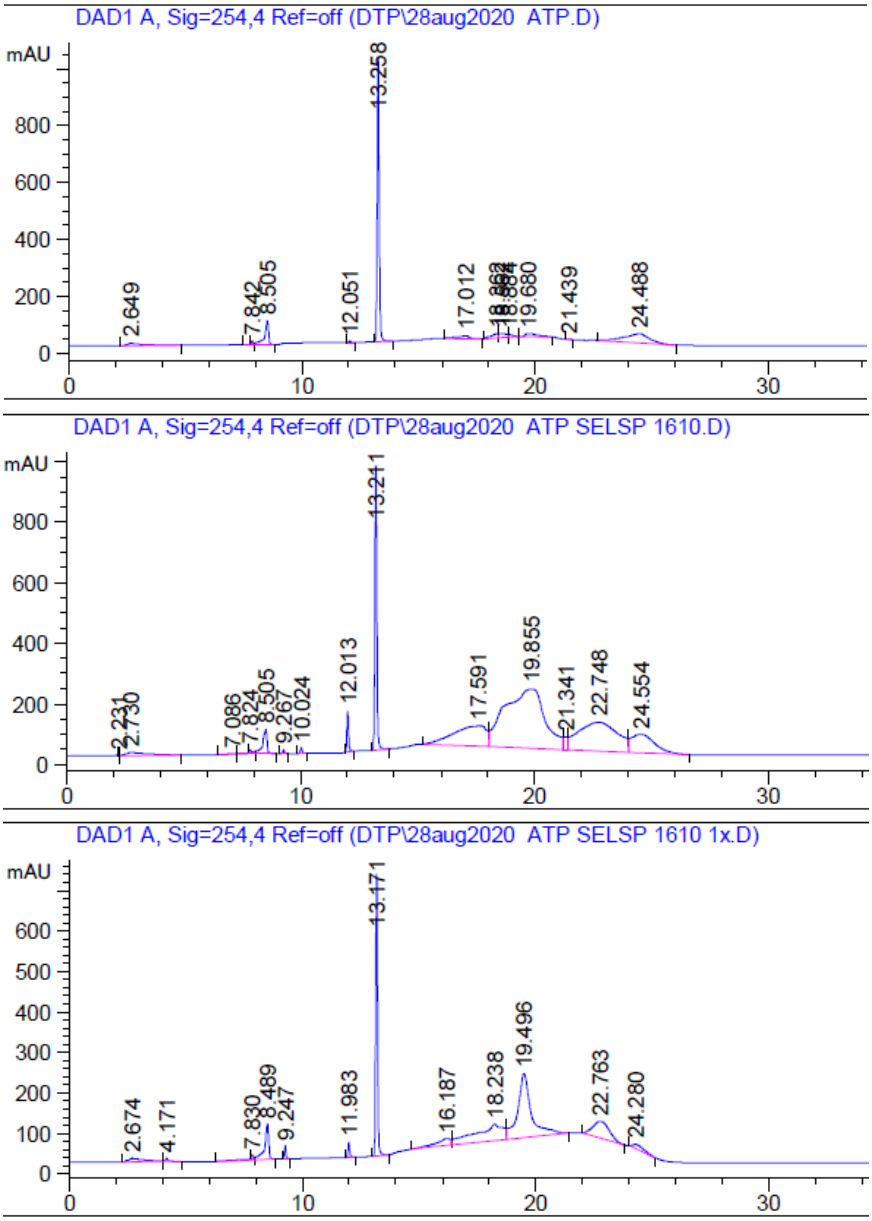


Figure 3-15 Reaction Set 2 for SELSP_1610: Magnesium and Manganese

Reverse Phase Chromatography was run on an HPLC column. The first two graphs are blanks to clear the column. The third is a cyclic-di-AMP control run alone with a peak eluted at 16.5minutes. The fourth following was the first reaction set up with SELSP_1610 truncated, an excess of ATP, incubated for 1hr with 1xDGC buffer containing magnesium and manganese as the cation. The peak was eluted at the ATP mark: 14.5minutes.

The cations chosen for the third reaction set were the same as reaction set two: magnesium chloride and manganese chloride. The original purified protein, truncated SELSP_1610, was dialyzed with the 1xDGC buffer containing both cations. This replaced the original buffer with the reaction buffer in an attempt to prevent phosphate inhibition of the reaction. After the protein was left in the buffer over the weekend, crashed out of solution, and resuspended, the new concentration was found. This may be why this reaction did not work. ATP was run as a control before the rest of the reactions were run with an elution peak at 12.5. The dialyzed protein reaction was run for 1 hour with ATP with a 600µl reaction volume and a 10xDGC buffer that was made earlier that week. The peak that was eluted denoted ATP elution. For the next reaction, the reaction volume size was changed to 300µl and the original protein, that was thawed on ice, was used along with 1xDGC. ATP was eluted again at 12.5 minutes. Cyclic-di-AMP was run as a second control after this reaction was run and eluted at 14.5 minutes, then a blank was run to make sure it had cleared the HPLC column. The last reaction of the day used the last of the dialyzed protein and a reduced reaction volume size, 200µl in total, with 1xDGC buffer and 3µl of ATP was used. The reaction incubation time for this run was increased to 2.5hours. ATP was eluted again at 12.5 minutes (Figure 3-16).

Reaction set 3



Reaction set 3 continued

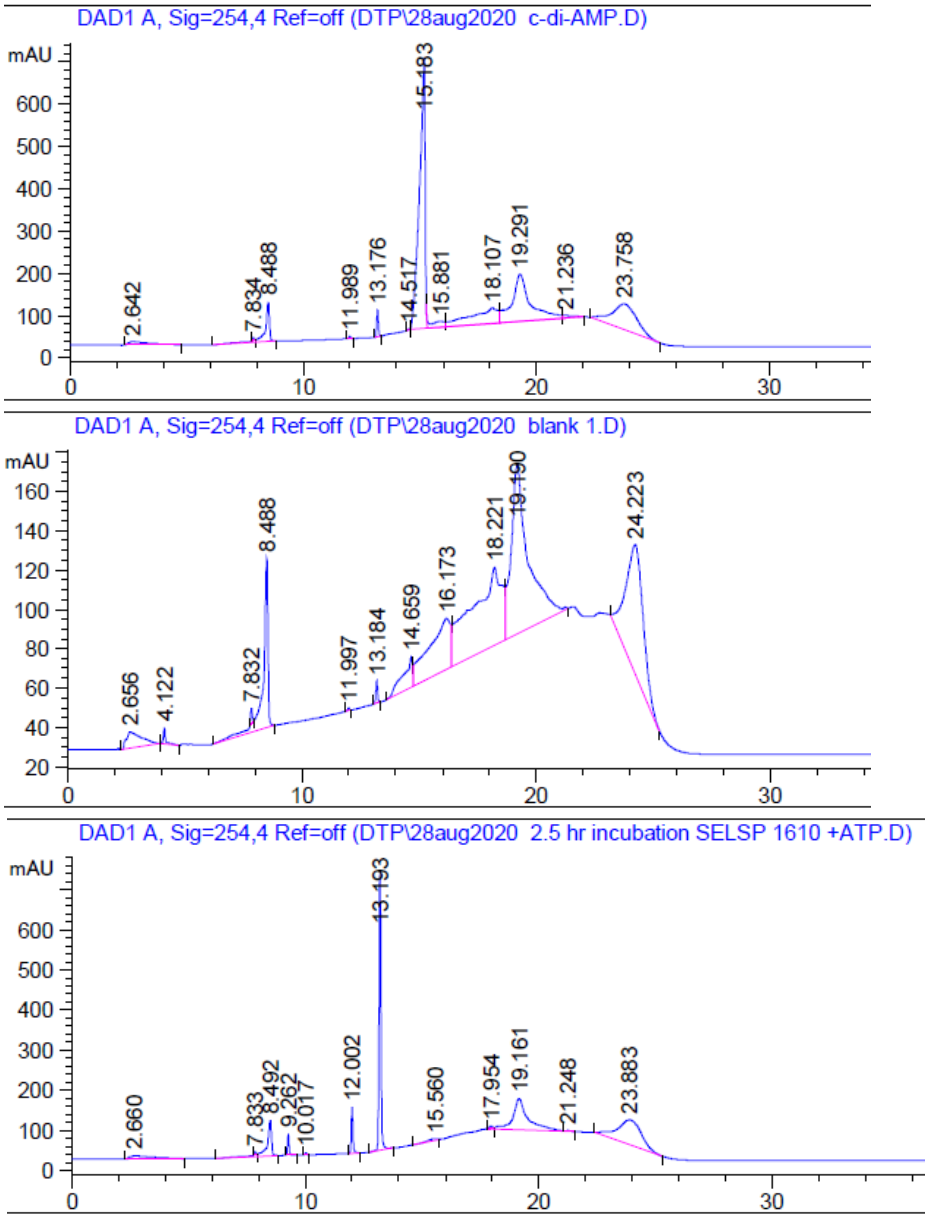


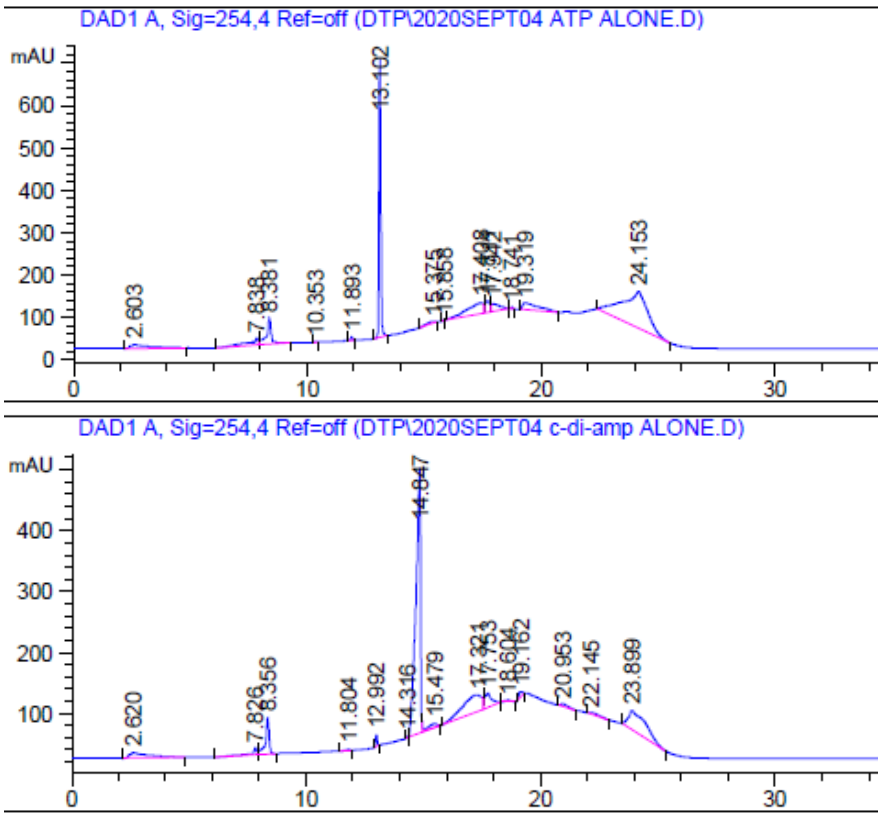
Figure 3-16 Reaction Set 3 for SELSP_1610: Magnesium and Manganese

Reverse Phase Chromatography was run on an HPLC column. The first graph is a control featuring ATP alone with a peak eluted at 12.5 minutes. The second and third exhibit the reaction of dialyzed protein at 600µl in 10xDGC buffer and the original protein in the original buffer but reduced to 300µl. Cyclic-di-AMP run fourth alone with a peak eluted at 14.5minutes. The following was a blank and the last run is 200µl with dialyzed protein for 2.5 hours 1xDGC buffer containing magnesium and manganese as the cation was used. The reaction peaks were all eluted at the ATP mark: 12.5 minutes.

For the fourth reaction set, the other variables for each of the other reactions were taken into consideration. The activating cation was changed, the methanol elution concentration was increased, and then dialysis was performed on the original protein into the new buffer. After this, the buffer concentration was varied between 1x and 10x DGC, the reaction volume size was decreased for both the original protein and the dialyzed protein along with ATP added, and the kinetics were changed as well by increasing the incubation time. In addition, a new column was used on the HPLC machine. For the fourth reaction set, the two variables that were changed were the chosen cation and the incubation time. Manganese was chosen as the only cation and the new incubation times chosen were 2 hours and 1.5 hours. After further literary analysis, it had been suggested in the literature that some DAC activity was inhibited by magnesium (35,58). This applied to CdaA, cyclic di-AMP synthase A, in *Listeria monocytogenes* and DacA, diadenylate cyclase A, in *Staphylococcus aureus* (35,58). As SELSP_1610 mirrors both of these proteins, magnesium was eliminated from the buffer and a new buffer was made.

The buffer was remade with manganese as the sole cation and the dialysis of SELSP_1610 was completed again overnight into the new buffer. The protein crashed out of solution again and the concentration was found again. The original protein was thawed in room temperature water to facilitate thawing all at once so the buffer would not affect the enzyme, then it was stored on ice prior to the reaction. Both ATP and cyclic-di-AMP were used as controls prior to the 2hour reaction that was run. ATP eluted at 12.5 minutes and cyclic-di-AMP eluted at 14.5 minutes. The two-hour reaction that was incubated with the original protein thawed in room temperature water was eluted with an ATP peak. The second reaction was incubated for 1.5hours with the newly dialyzed protein (Figure 3-17). This product was also eluted at the ATP peak.

Reaction set 4



Reaction set 4 continued

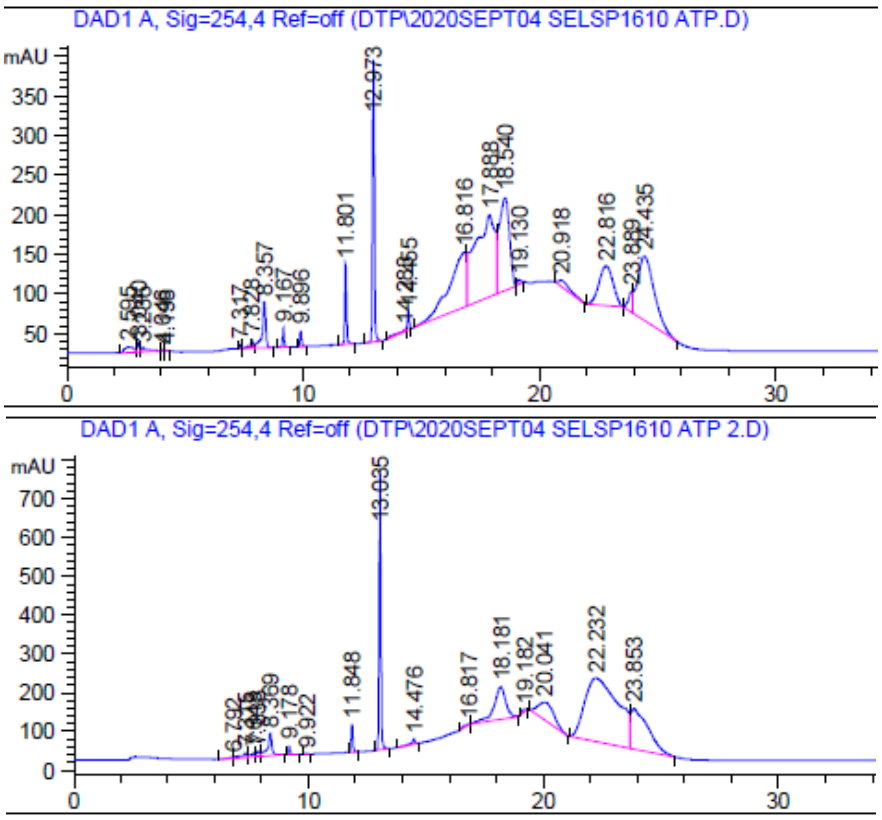


Figure 3-17 Reaction Set 1 for SELSP_1610: Manganese

Reverse Phase Chromatography was run on an HPLC column. The first two graphs are controls featuring ATP alone with a peak eluted at 12.5minutes in the first and cyclic-di-AMP run alone with a peak eluted at 14.5 minutes on the second. The third following was the first reaction set up with SELSP_1610 truncated original protein, an excess of ATP, incubated for 2.5hrs with 1xDGC buffer containing manganese as the cation. The final reaction with SELSP_1610 truncated dialyzed protein, an excess of ATP, incubated for 1.5hrs with 1xDGC buffer containing manganese as the cation. The peaks were eluted at the ATP mark: 12.5 minutes.

Chapter 4: Discussion

Over the course of this project, *Selenomonas sputigena* has been studied and evaluated as an emerging oral pathogen in a novel way. Prior to this study, it has not been thoroughly explored within the literature. Within this project, the growth factors for *S. sputigena* have been fleshed out. Conditions for both broth and plates have been discovered through the process of plate counts and growth curves. Oxygen sensitivity and antibiotic sensitivity were also explored. This background is extremely important to continue studying this emerging oral pathogen. This knowledge allows for smooth laboratory research and can act as a foundation to study this organism. For example, the data from the plate counts allowed for the completion of the Hemagglutination/Hemolysis assay. This information was firmly used as the building blocks of studying this oral bacterium. Without this essential process of collecting background information on *S. sputigena*, there would be no direction to go in.

In addition to the established growth factors that were found in this study, virulence factors were also looked at. The hypothesized virulence factors that were tested began with Hemagglutination/Hemolysis assay and progressed to monospecies biofilm formation and adhesion to oral epithelial cells. Hemagglutination/hemolysis showed a definite negative for that virulence capability for *S. sputigena.* There is a possibility that it does form biofilms, both with itself and with other oral pathogens, as the biofilm assay was lacking and needed significant improvement. The adhesion assay, however, showed the capability of *S. sputigena* to bind to oral epithelial cells which contributes to biofilm formation and colonization of the mouth. This further results in the state of chronic inflammation, or periodontal disease. As more virulence factors are discovered, this knowledge aids in moving forward while studying host/pathogen

interaction, inflammation, colonization, and biofilm development during periodontal disease. This can point further studies in the correct direction as we now know what to look for.

The last part of this project was studying the metabolism of cyclic-di-AMP by *S. sputigena.* This secondary messenger can carry out many pathogenic and physiological functions to both sustain homeostasis and cause disease. As the assay is still being developed, evidence for *S. sputigena* having a diadenylate cyclase has been based, not only on the predicted protein model by both SWISS-model and PHYRE, but on the literature as well. Due to bioinformatic analysis it is probable that enzyme SELSP_1610, found in *S. sputigena*, acts as a diadenylate cyclase. It appears to have DAC activity when compared to other structures from other organisms like DacA in *S. aureus* (35,58). When studied in the enzymatic reaction run through HPLC, several variables were changed including kinetics, such as reaction time, stoichiometry, with protein concentration, cation of choice changed based off of the literary references and predicted models, and change in buffer concentration (15,22). These meticulously chosen variables help to point us in the correct direction for further assay development. More changes to the assay are needed to continue building evidence that supports the bioinformatic analysis of SELSP_1610.

Limitations and future directions

In terms of setbacks and future directions, all experiments for the growth factors need to be replicated again with higher consistency now that a direction is established. This is especially true for the plate counts that had high inconsistency and some contamination whenever attempted. The biofilm assay needs to be refined with another reference protocol than the one that was used in this study. A polymicrobial biofilm study should also be completed with *S. sputigena.* The adhesion assay to TIGK cells needs to be pursued as well as a potential virulence

factor that *S. sputigena* possesses. In addition to the growth and virulence factor study, the remaining components of cyclic-di-AMP receptor proteins (SELSP_1770, SELSP_1836), and degradation (SELSP_2051) need to be explored as well.

Each one of their potential activity, like the process for SELSP_1610, needs to be analyzed through bioinformatics. Reverse phase chromatography can be used again to create an assay with SELSP_2051 that contains possible PDE activity. Cyclic-di-AMP would be degraded into pApA and this could be measured by running it through an HPLC column. If it does have a PAS domain, heme can bind to this area and this can also be measured in an assay as well. An assay can also be established to see if SELSP_1770 and SELSP_1836, possible TetR-family transcriptional regulators, can bind to cyclic-di-AMP. As for SELSP_1610, future directions and setbacks hinge on the assay discussed in this study. There are a few questions that might aid in its development as well.

Diadenylate cyclases need to form a dimer in order to interact with ATP. Further research would be needed to see if it naturally exists as a homodimer or it becomes one when it binds to ATP (59). Another factor may be needed to assist in dimerization. Size exclusion chromatography can be used to study this. A tyrosine “lock” was also mentioned in the literature that slides the ATP into place (27,66). A known protein structure may aid in knowing if this portion exists in the correct place. In addition to looking at structural changes in the enzyme, cobalt was suggested to have the highest affinity in the reaction: even greater than the suggested manganese (34). This would be another direction to go in as well. The truncated protein, according to the literature, does not lose function due to the missing membrane domain so this is not a possible reason as to why it is not working; however, the reason why it is crashing out of solution may be a key to assay development (34). If the assay does not work, an ELISA is being

developed as well in the lab to test for reduction in ATP presence using a known positive control for diadenylate cyclase. This can further support the bioinformatics findings though, like the initial assay, it needs to be refined. Coralyne, a molecule that binds to cyclic-di-AMP and fluoresces, was the original proposed way of testing if SELSP_1610 is a diadenylate cyclase (27,34). The assay had been replicated multiple times successfully in the literature; however, coralyne is no longer commercially available. This led to more difficult and creative assay development through reverse phase chromatography and ELISA to test this particular function.

Overall, periodontal disease is a detrimental disease with very little to no proper treatment and preventative methods. This results in an inflammatory disease that can lead to life threatening sequela. The treatment that does exist is invasive, dangerous, and is expensive and requires better methods of care. This is contingent upon knowing and treating based upon knowledge of the oral microbial community and the shift from healthy state to disease state. *Selenomonas sputigena* is an emerging oral pathogen, and by seeking to study this organism, better treatment options and tests can be provided for those suffering from periodontal disease.

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