

The Investigation of SliP in *Campylobacter jejuni* as an Intracellularly Secreted Protein

Chancellor's Honors Program Capstone Project

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Abstract

Campylobacter jejuni is the leading cause of bacterial-borne gastroenteritis worldwide and is one of the most common food-borne illnesses in the United States (Man, 2011). It is a causative agent of Guillian-Barré syndrome, and is also associated with other post-infection disorders such as irritable bowel syndrome and reactive arthritis (Nachamkin et.al, 1998) (Reti et.al, 2015). Because of its prevalence, the study of *C. jejuni*, and its interactions with the human host, can have far-reaching implications by revealing the machinery by which harmful intestinal pathogens like *C. jejuni* can promote infection. One system in which *C. jejuni* is predicted to exploit the host immune system is through its interaction with neutrophils. Therefore, understanding *C. jejuni* interactions with neutrophils is crucial to reducing the burden of disease caused by *C. jejuni* infection. While screening a transposon library of *C. jejuni*, a mutant was identified that lacked the ability to provoke NET extrusion characteristic of the response of a neutrophil. The transposon in this mutant was found to have interrupted a gene that encodes a protein that shares secondary homology with sirtuins and deacetylases in other bacterial and eukaryotic species. Accordingly, the uncharacterized protein was named SliP (Sirtuin-like Protein). Preliminary studies indicate that SliP is associated with a number of virulence factors including lysine deacetylation, neutrophil activation, and NET formation when compared to wild-type *C. jejuni*. It remains unknown the exact process by which SliP interacts with the neutrophil, including whether the protein is translocated from the bacterium into the host cell. This paper will discuss the potential intracellular secretion of SliP into the neutrophil as a mechanism by which *C. jejuni* induces the host immune system to increase pathogenicity and promote human disease.

Background

Campylobacter jejuni is a Gram-negative Epsilonproteobacteria that is the leading cause of bacterial-derived gastroenteritis worldwide (Man, 2011). It is an incredibly widespread pathogen and is projected to infect nearly ninety-six million people annually (WHO, 2020). *C. jejuni* has diverse environmental sources. Due to the fact that it is a common commensal member of the gastrointestinal tracts of chickens and cattle, *C. jejuni* infection is largely foodborne (WHO, 2020). Because of this, infection in the developed world is most commonly acquired through the ingestion of undercooked, contaminated meat, primarily poultry (Platts-Mills et.al, 2014). *C. jejuni* can also contaminate water sources. Because of this ability, *C. jejuni* infections are particularly pervasive in the developing world (Platts-Mills et.al 2014). Symptoms of acute gastrointestinal disease during *C. jejuni* include bloody diarrhea, fever, nausea, vomiting, and stomach pain (WHO, 2020). Although gastrointestinal symptoms generally resolve after three to six days (WHO, 2020), infection of children under the age of two in developing countries are frequent and can lead to persistent infection and the development of enteric dysfunction (WHO, 2020).

C. jejuni infection is linked to long term complications in the developed world as well. The most notable of these is the development of Guillain-Barré syndrome, which is a neurological autoimmune disorder. In patients with Guillain-Barré, *C. jejuni* has been isolated from stool samples in up to 50% of patients and was detected in up to 76% of serum samples isolated from Guillain-Barré patients (Nachamkin et.al, 1998). There is also a strong association between *C. jejuni* and the development of other inflammatory conditions. For example, patients who have previously had *C. jejuni* infection are at an increased risk for developing irritable bowel syndrome (IBS) (Gradel et.al, 2009) and may also be at increased risk for developing

reactive arthritis, which causes joint pain and limited motion in the joints. For example, up to 15% of patients infected with *C. jejuni* went on to develop reactive arthritis in various cohort studies (Ajene et.al, 2013).

The development of acute disease and persistent post-infectious disorders is associated with the induction of the host inflammatory response. Our group has shown that *C. jejuni* infection results in inflammation of the intestinal epithelium and recruitment of neutrophils, the most prevalent white blood cell in the human body. In order to kill invasive bacteria, neutrophils employ several mechanisms. First, they can engulf and phagocytize the pathogen and kill them using reactive oxygen species (ROS) (Callahan et.al, 2021). Second, upon interacting with the pathogen, they can release granules contents, including cytolytic enzymes such as myeloperoxidase (MPO) and neutrophil elastase (Ela2) (Callahan et.al, 2021). Importantly, the release of these cytolytic enzymes and the ROS generated during pathogen killing can lead to additional inflammation and tissue damage (Mutua and Gershwin, 2021). Third, infection can also result in the production of neutrophil extracellular traps (NETs). NETs are webs made of sticky extracellular DNA, chromatin, and antimicrobial proteins that are typically extruded to kill invasive bacteria. Because the antimicrobial proteins released from NETs have non-specific activities, NETosis can also lead to increased inflammation and damage to host tissues (Mutua and Gershwin, 2021). Our group has previously shown that *C. jejuni* induces NET extrusion during infection (Callahan et.al, 2020).

To better understand the activation of neutrophils by *C. jejuni* and the production of NETs, our group previously conducted a transposon mutant screen to identify genetic determinants of the pathogen that are involved in these processes. Preliminary results indicate that *C. jejuni* possesses a sirtuin-like deacetylase, which we call SliP, that is active during *C.*

jejuni infection of human neutrophils and that the activity of this deacetylase is associated with the ability of *C. jejuni* to activate human neutrophils, induces NET formation, and promote the intestinal immunopathology that is characteristic of human campylobacteriosis. Because SliP is involved in these important processes, this paper will describe our preliminary data examining SliP's role during *C. jejuni* infection of neutrophils and the construction of a reporter system to examine whether SliP is secreted into neutrophils as a possible mechanism for how this effector impacts neutrophil behaviors.

Preliminary Studies

Introducing SliP and Sirtuins

It has been previously identified that neutrophil activation and the induction of NETosis is an important part of *C. jejuni* infection. We identified an insertion interrupting the locus CJJ81176_0779 during a transposon mutant screen that resulted in a mutant that was unable to efficiently activate neutrophils. Bioinformatic analysis of the protein encoded by CJJ81176_0779 revealed that the protein does not share primary sequence homology to other proteins, but does share secondary structure homology with sirtuins, which are NAD⁺ dependent lysine deacetylases. Appropriately, the protein encoded by CJJ81176_0779 was named SliP for sirtuin-like protein.

Sirtuins are NAD⁺ dependent histone deacetylases. Histone modification is an extremely important aspect of cellular function, gene expression, and cell signaling since histones are tightly bound to DNA and condense chromatin. This binding and condensing activity means that histones have the ability to grant or deny access of transcription machinery to the target DNA, which can be impacted by altering the charge of the histone and affecting its electrostatic

interactions with negatively-charged DNA. Unmodified histones are positively-charged due to their high abundance of lysine residues. Lysine can be modified in several ways (acetylation, methylation, citrullination, etc.) that can affect the way in which histones interact with DNA. Acetylation is one of the most common histone modifications and it changes the charge of lysine from positive to neutral, therefore reducing the electrostatic interaction between the histone and DNA. Acetylase enzymes add acetyl groups to lysine residues, while deacetylases remove acetyl groups. Because of its functionality as a sirtuin, it is believed that SliP acts as a lysine deacetylase, serving to tighten the grip of histones to the DNA of the neutrophil in the nucleus.

SliP Interactions with Histone H3

SliP is specifically predicted to act on histone H3, which is involved in a variety of neutrophil functions, and is a key player in the release of NETs from neutrophils (Thålin et. al, 2018). The connection between histone modification and NET induction is well documented, which is also dependent on the citrullination of histone H3. Citrullination is the conversion of an arginine residue of the histone to a citrulline and is governed by the activity of peptidylarginine deiminase 4 (PAD4), which requires deacetylation of H3 prior to citrullination (Poli et.al, 2021). Because of this cascade, deacetylation of H3 by SliP could promote citrullination of H3 by PAD4 and lead to NET production and promote inflammation and intestinal pathology.

SliP in the Intracellular Environment

First, to understand the impact of SliP on the neutrophil, it must be determined whether SliP is translocated into the neutrophil. The function of SliP as previously proposed is dependent on SliP being part of the neutrophil's intracellular environment so that it can gain access to the nucleus and histones. Evidence that SliP enters the intracellular environment was first obtained by examining the impact of SliP on the deacetylase activity present in infected neutrophils. In

this study, synthetic acetyl-lysine peptides were permeabilized into human neutrophils infected with either wild-type *C. jejuni*, the $\Delta sliP$ mutant, $\Delta sliP$ complemented with wild-type *sliP* ($\Delta sliP$ -*psliP*), or $\Delta sliP$ complemented with a *sliP* variant that cannot bind the NAD cofactor ($\Delta sliP$ -*psliP*_{G26A}). This experiment demonstrated that wild-type *C. jejuni* and the complemented strain exhibited deacetylation of the synthetic acetyl-lysine peptides. In contrast, the $\Delta sliP$ mutant and the $\Delta sliP$ -*psliP*_{G26A} substitution displayed a significant decrease in deacetylase activity. This data suggests that the presence of SliP results in less protein acetylation during neutrophils infection with *C. jejuni*, which further suggests that the presence of SliP likely results in differential acetylation of host proteins during infection. This data set is consistent with the hypothesis that SliP acts as a histone deacetylase, as well as strongly indicates that SliP acts within the intracellular environment of the neutrophil.

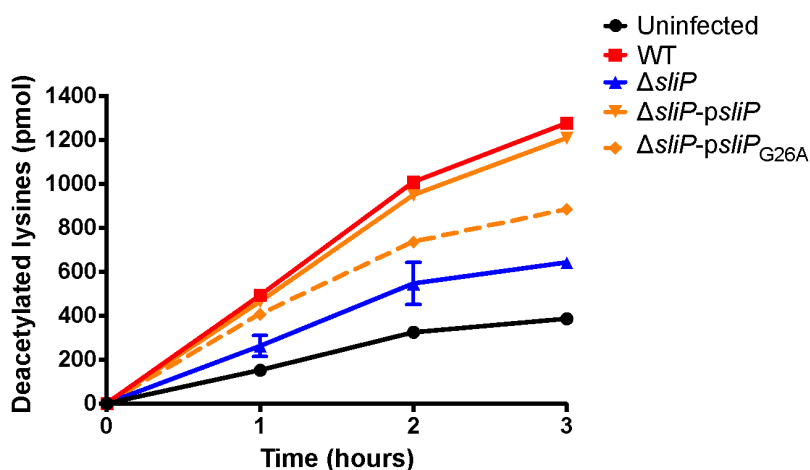


Figure 1: Lysine deacetylation activity of SliP upon introduction of *C. jejuni* to neutrophils.

SliP as an Intracellularly Secreted Protein

After establishing that SliP acts once internalized by the neutrophil, the question remains as to how exactly SliP is delivered to the intracellular environment of the neutrophil. In order to do this, it is predicted that SliP utilizes a secretory system of *C. jejuni* in order to be mobilized into the cell. Gram-negative bacterial species such as *C. jejuni* have evolved secretion systems in

order to translocate effector proteins across the cell membrane and into the host cell. It has been previously shown that *C. jejuni* utilizes a noncanonical type III secretion system (T3SS) for the delivery of virulence proteins to the host cell. Type III secretion systems are characterized by the use of a flagellar apparatus for protein export into the host cell. Traditional or canonical type III secretion systems employ a flagellar-derived apparatus called the injectosome. However, some bacterial species, like *C. jejuni*, use the flagellar apparatus as their type III secretion systems (Christensen, 2009). Traditional flagellar type III secretion systems recognize flagellar proteins for secretion to construct flagella. Interestingly, *C. jejuni* uses its flagellar T3SS to secrete proteins that are not involved in flagellar motility (Burnham et.al, 2018). A significant example of this is the secretion of the family of effector proteins called the Cia proteins. The Cia (*Campylobacter* invasion antigen) family are proteins that are critical factors in *C. jejuni* cell invasion and subsequent establishment of disease. An important experiment characterizing the intracellular secretion of Cia proteins provides details on the key flagellar components in play in the secretion system of *C. jejuni*. In a study in 2004, Michael Konkel and his team identified five genes in *C. jejuni* that constructed the flagellar apparatus for Cia protein delivery. By creating knockout mutations, they summarized the construction of the apparatus as encoding for a basal body (FlgB and FlgC), a hook component (FlgE), and filament (FlaA and FlaB) (Konkel et.al, 2004). This experiment indicated that a knockout mutation of any five of the genes identified resulted in disruption of CiaB secretion. Therefore, the deletion of these genes can be used as an experimental measure to confirm the secretion of an effector protein in *C. jejuni*.

To determine if SliP is secreted, wild-type *C. jejuni* cells were grown in broth culture, introduced to deoxycholate to mimic the gastrointestinal tract and to induce secretion (Malik-Kale et.al, 2008), and then centrifuged to separate cells from the growth medium (supernatant),

which would include any proteins that were secreted. A western blot using polyclonal antibodies that were raised against SliP was used to examine for the protein in these supernatants.

Importantly, these western blots revealed that SliP is present in cell-free supernatants, which supports the conclusion that SliP is a secreted protein.

To determine whether SliP is secreted via the *C. jejuni* flagellar T3SS, a $\Delta flgE$ mutant was used, which encodes the hook of the flagella and is required for assembly. If SliP requires the flagellar T3SS for translocation, we expect that SliP would be absent from the supernatants of the $\Delta flgE$ mutant. As expected, SliP was absent in the supernatants of the $\Delta flgE$ mutant, but was present in wild-type supernatants. The result of this experiment further supports the conclusion that SliP is secreted by *C. jejuni* and that its translocation requires the flagellar T3SS.

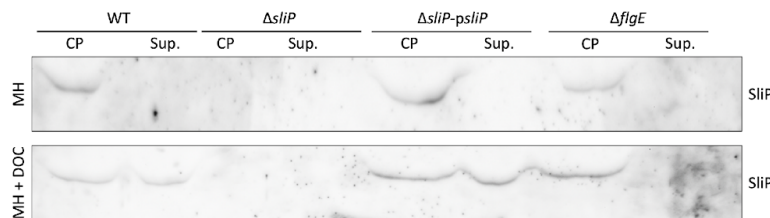


Figure 2: Western Blot analysis detecting the presence of SliP protein in the supernatant of *Campylobacter jejuni* broth cultures supplemented with deoxycholate to induce protein secretion.

Goal of the Project

cAMP ELISA

Considering the strong indicators that SliP is secreted through the *C. jejuni* flagellar apparatus, a reporter system was constructed in order for SliP secretion into the neutrophil to be quantified. The reporter used in this system was the protein CyaA, which is naturally found in *Bordatella pertussis*. *B. pertussis* CyaA contains multiple domains, including an N-terminal catalytic domain that encodes an adenylate cyclase (Voegelé et.al, 2018) and has been shown to

be transported across the membrane of eukaryotic cells through a dedicated type I secretion system (Voegelé et.al, 2018). Because of this, it is a commonly used reporter to examine and quantify protein translocation from bacterial cells. Once in the host cell, the adenylate cyclase domain of CyaA is activated by its interaction with host calmodulin, a calcium-binding protein that is present in only eukaryotic cells. The activation of CyaA by host calmodulin leads to the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) through ATP hydrolysis. Therefore, the presence of CyaA inside the cell leads to the increased production and accumulation of cAMP.

In a translational fusion, the protein of interest (in this case SliP) is fused to a reporter protein by expressing each from the same translation initiation region (TIR) in the same reading frame. By producing this chimeric SliP-CyaA protein, SliP can be translocated into the host cell along with CyaA and the resulting cAMP production from the CyaA protein can be used to quantify the secretion of SliP. In translational fusions, the reporter can be placed at either the N-terminus or C-terminus of the chimera. For the purposes of this study, the CyaA reporter was placed at the C-terminus because SliP is induced during neutrophil infection and driving production of the chimera from its promoter and TIR ensures the fusion will be produced. In addition, having this design, the translocation of the fusion protein will be dependent on any signal sequence SliP requires for translocation from *C. jejuni*. Because the C-terminal domain of the *B. pertussis* toxin is naturally required for the translocation of the adenylate cyclase domain of CyaA (Chenal et.al, 2018) it was also logical that the chimeric gene only encode the N-terminal domain. The cAMP resulting from the secretion of the SliP-CyaA fusion in the neutrophil can then be assayed using an ELISA (Enzyme-Linked Immunosorbent Assay). The

commercially-available cAMP ELISA relies on binding of an antibody against cAMP to provide an output of cAMP levels in the sample.

In addition to the SliP-CyaA fusion, this experiment also included the cloning of fusions for both positive and negative controls. As a positive control, a CiaB-CyaA fusion was used because CiaB belongs to the *Campylobacter* invasion antigen family of effectors and has been documented as using the flagellar T3SS of *C. jejuni* to translocate into host epithelial cells. Therefore, this was an appropriate protein to positively control for translocation into host cells. As a negative control, a HeuR-CyaA fusion was used, since HeuR is a non-secreted cytoplasmic bacterial regulator that controls iron acquisition from host heme (Johnson et. al, 2016). The use of this HeuR-CyaA fusion also allows us to determine whether SliP released into neutrophils might be due to *C. jejuni* autolysis or killing by the neutrophil and not due to active secretion through the flagellar T3SS. The constructs were also introduced into the Δ *flgE* mutant to determine whether the flagellar T3SS is required for SliP secretion into the cell.

Construction of CyaA Translational Fusions

To begin the construction of the translational fusions, the primer sets were designed beginning with four main primers, two for each component of the chimeric gene. The first primer in the construct is the *sliP*, *heuR*, or *ciaB* complement forward primer. This primer encompasses roughly the first 20 base pairs of the gene and a 5' BamHI site, which is a restriction enzyme important for the excision of the chimeric gene from the vector plasmid. The second primer, the *sliP-cyaA*, *heuR-cyaA*, or *ciaB-cyaA* SOE reverse, encodes for the overlap between the effector and *cyaA* sequences, which creates the actual fusion of the genes. The overlap includes a linker region of two alanines and base pairs at the beginning of the *cyaA* sequence as well. The linker region serves as a buffer to ensure that each protein does not disrupt the function of the other and

that both proteins fold properly. Using these primers, *sliP*, *heuR*, and *ciaB* can be amplified from *C. jejuni* genomic DNA (Blue, Figure 1). The third primer used is the *sliP-cyaA*, *heuR-cyaA*, or *ciaB* SOE forward, which begins the sequence of *cyaA* and encompasses the end of the effector sequence. The fourth primer is the *cyaA* complement reverse, which anneals the end of *cyaA* and introduces a 3'BamHI cut site. Using these primer combinations, the *cyaA* product can be amplified from a plasmid containing CyaA conjugated with AnkB from *Legionella pneumophila*, ordered from Dr. Kwaik at the University of Louisville (Orange, Figure 4).

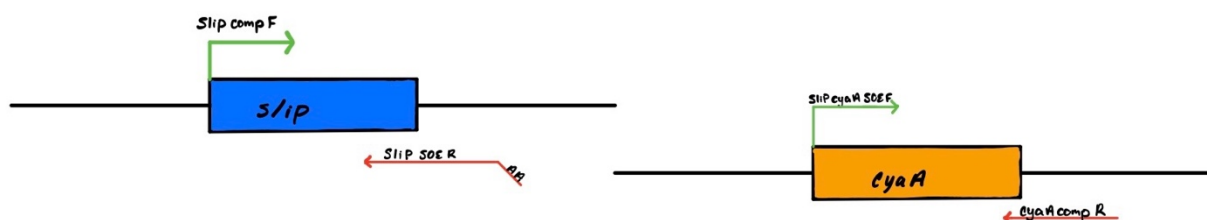


Figure 4: Construction of primer sets for translational fusion of SliP and CyaA.

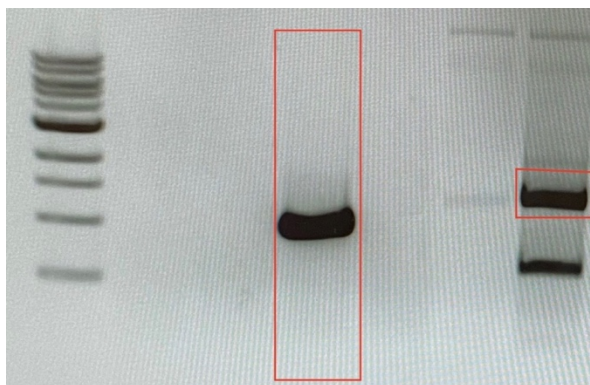


Figure 5: Successful creation of the SliP F and R fragments of appropriate size confirmed by gel imaging as indicated by the red boxes. The standard size of each band on the ladder is as follows from top to bottom: 10kb, 8kb, 6kb, 5kb, 4kb, 3kb (darkened band), 2kb, 1.5kb, 1kb, and 0.5kb.

The *sliP*, *heuR*, *ciaB*, and *cyaA* fragments were produced using standard PCR conditions with Q5 polymerase, a high-fidelity polymerase with low error-rate (Ricardo, 2019). PCR products were run on a 1% agarose gel and confirmed as the bands were of the expected size. Each different fusion had forward fragments of varying sizes. The size of the forward fragments

for *sliP*, *heuR*, and *ciaB* were 987 bp, 654 bp, and 1.8 kb, respectively. Because the reverse fragment was the same for each fusion, it was consistently 1.12 kilobases for the reverse fragment. The successful products were gel-extracted (Figure 5). After extraction, the fragments were fused together using touchdown (TD) PCR. In a TD PCR, the initial annealing temperature begins as higher than the optimal melting temperature of the primers. This creates a temperature gradient to accommodate wider difference in optimal melting temperature of primer sets and to join homologous regions between the forward and reverse gene products. With the TD PCR, the two separate gene products now become fused into a single DNA product of 2.1 kilobases for the *sliP* fusion, 1.8 kilobases for the *heuR* fusion, and 3 kilobases for the *ciaB* fusion. After the TD PCR, the product was run on a 1% agarose gel and the single DNA product was gel-extracted (Figure 6).

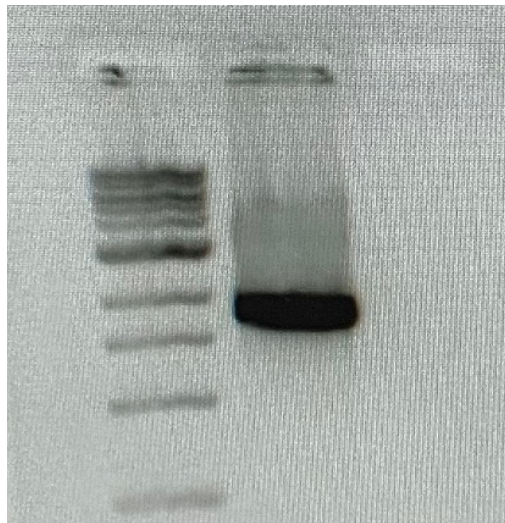


Figure 6: Successful fusion of fragments using TD PCR of *sliP-cyaA* confirmed by gel image.

After the two genes were fused together, the DNA was ligated in the pJET1.2 plasmid vector. Because the pJET plasmid is a high copy number plasmid, it allows for ample proliferation and sequencing of the gene. The pJET plasmid also confers resistance against

ampicillin. Once ligated into pJET, the plasmid was transformed in maximum efficiency DH5a *E. coli*. To transform the plasmid into these cells, the competent *E. coli* are transferred between a heat block and ice to permeabilize and then restabilize the membrane. Once the cells recovered and were outgrown in a 37°C incubator for one hour, and plated on Luria-Bertani (LB) agar, containing ampicillin. This media selects for *E. coli* cells that are resistant to ampicillin because they now harbor the new plasmid. These colonies were then screened for the newly recombinant plasmid using the *slpP*, *heuR*, or *ciaB* comp forward primer and *cyaA* comp reverse primers to flank the entire fused gene. In this screen, GoTaq polymerase was used in the PCR reaction because the purpose of these screen was to detect the sequences present, so it was not necessary to use high efficiency Q5 polymerase. Once the PCR was complete, the reaction for each colony was run on an agarose gel. The presence of the desired sequence was indicated by the presence of a band. If the sequence was not present, no band was visible. Despite the fact that the fragments were stitched, the forward and reverse fragments were screened separately to test for their ligation to pJET, as the optimal melting temperatures of each primer were over 10°C different from each other, the colony PCRs would be more successful if the annealing temperatures were more optimal for each primer. The screenings for both the forward and reverse fragments in pJET were then run on an agarose gel and imaged. The expected size of the reverse fragment was 1.12 kb and the expected size of the forward fragment was 987 bp (Figure 7). Successful colonies were stocked and sent off for Sanger sequencing, which would identify any single nucleotide polymorphisms (SNPs).

At this point in the cloning process, there had been several failed attempts to transform the *ciaB-cyaA* translational fusion into the DH5a *E. coli*. Because of this, a recombinant plasmid

was ordered with the gene already ligated into a the pUC57-mini cloning vector (GenScript). This purchased construct was used for the rest of the cloning process for *ciaB-cyaA*.

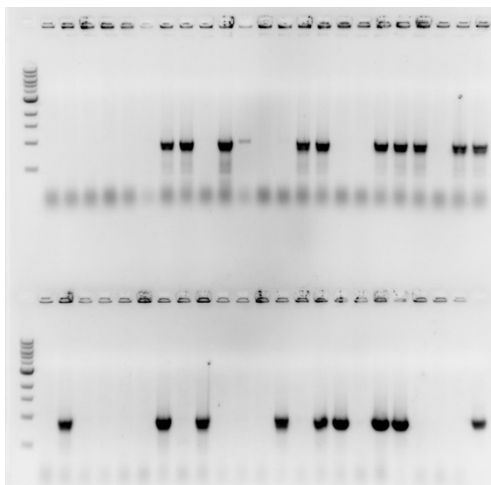


Figure 7: Screening of PCR reactions for successful colonies harboring the forward fragments translational fusions in the pJET plasmid.

Using plasmid DNA containing inserts that were confirmed to not contain SNPs, the fused gene was excised using the restriction enzyme BamH1. The BamH1 cut site is part of the fused DNA and was contained in the forward primers and the *cyaA* reverse primer. In this experiment, Invitrogen BamH1 was used, as previous attempts to use New England Biolabs BamH1 had failed. In order to troubleshoot this, both New England Biolabs BamH1 and Invitrogen BamH1 were used in either the water bath or the heat block to incubate at 37°C for one hour. This resulted in four different digestions with different combinations of vendors for BamH1 and incubation conditions. The only successful combination was found to be the Invitrogen BamH1 incubated in the water bath (Figure 8, Lane 7).

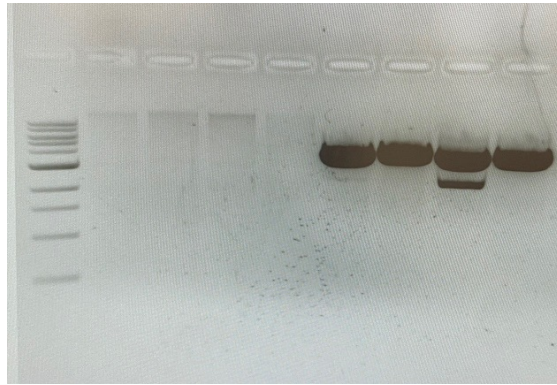


Figure 8: Troubleshooting of BamH1 digestions for both HeuR and SliP. The conditions from left to right are NEB BamH1 in water bath, New England Bam H1 in heat block, Invitrogen BamH1 in water bath, and Invitrogen BamH1 in heat block.

Following the BamH1 digestion, the fusion was gel-extracted and ligated into pECO102. To prepare the pECO102, purified plasmid was digested with BamH1 to open the plasmid for ligation of the fusion DNA with the compatible BamH1 cut ends. After BamH1 digestion, the pECO102 plasmid was cleaned, concentrated, and incubated with Antarctic phosphatase (AP) to prevent plasmid re-ligation (NEB, 2022). The AP-treated pECO102 plasmid was then incubated at 65°C to heat-inactivate the AP enzyme. Afterward, the pECO102 and fusion products were ligated overnight at 16°C with T4 ligase. This new plasmid was then transformed into competent *E. coli* using the same transformation protocol as previously described. Transformations were plated on LB agar with chloramphenicol as pECO102 plasmid encodes a chloramphenicol marker. Chloramphenicol resistant colonies were screened using GoTaq polymerase, the JJ13 primer, and the *cyaA* SOE reverse primer. The use of these primers not only indicates the presence of the desired fusion, but the directionality of the insertion into the pECO102 vector. The PCR reactions were run on a gel to confirm the presence and directionality of the sequence. Again, because of the wide differences in the optimal temperatures of the primers for the forward and reverse fragments, these were screened separately. (Figure 9).

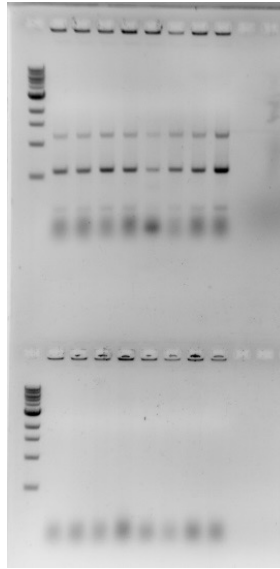


Figure 9: In order, heuR-cyaA (654 bp forward fragment) and sliP-cyaA (987 bp forward fragment) screening PCRs for forward fragments using JJ13 to indicate directionality.

The new plasmids containing the confirmed *cyaA* gene fusions in pECO102 were electroporated into *E. coli* harboring the helper plasmid pRK212.1, which will allow us to deliver the pECO102 fusions into *C. jejuni* by conjugation. The pRK212.1 pECO102 transformations were screened with JJ13 and SOE reverse primers to confirm the plasmid was successfully introduced (Figure 10).

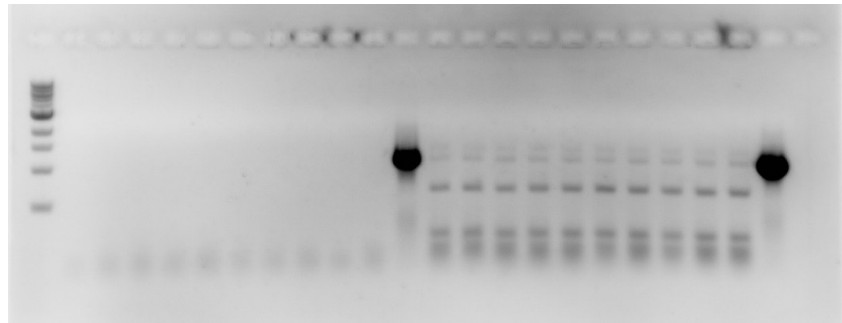


Figure 10: Screening of successful transformation of pRK212.1 with pECO102 (HeuR lanes 1-10, SliP lanes 11-20)

To accomplish this conjugation, the pRK212.1 *E. coli* with pECO102_{cat}*sliP-cyaA*, pECO102_{cat}*heuR-cyaA*, and pECO102_{cat}*ciaB-cyaA* were co-incubated with either wild-type *C. jejuni* DRH212 and or the Δ *flgE* mutant at 37°C overnight under microaerobic conditions.

Following the incubation, the cells were plated on Mueller-Hinton agar containing trimethoprim, streptomycin, and chloramphenicol. Colonies of *C. jejuni* pECO102P_{cat}*sliP-cyaA*, pECO102P_{cat}*heuR-cyaA*, and pECO102P_{cat}*ciaB-cyaA* were identified using the chloramphenicol resistance encoded by the pECO102 plasmid. Colonies were then screened using the forward and reverse primers to confirm the presence of the translational fusion (Figures 11,12). These cells were stocked and stored for the cAMP ELISA.

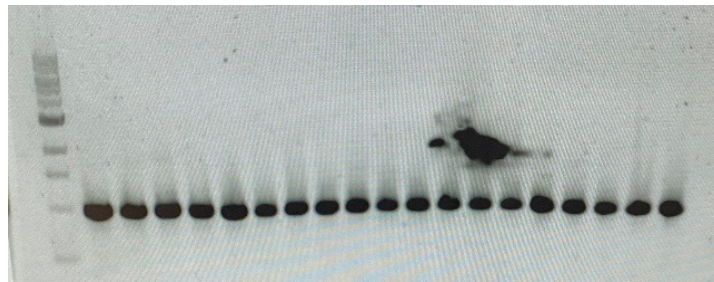


Figure 11: Colony PCR of *ciaB-cyaA* reverse fragment present in colonies of *C. jejuni*.

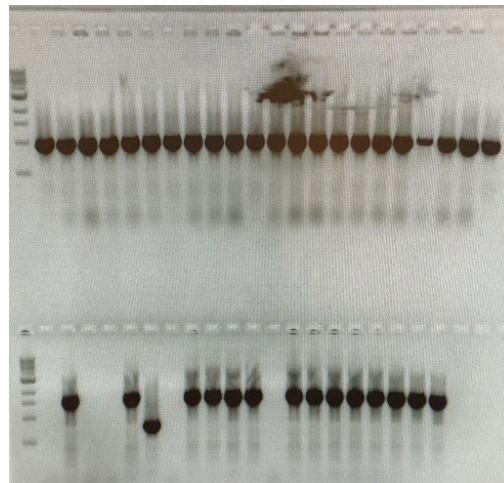


Figure 12: Colony PCRs confirming the presence of *sliP-cyaA* forward fragment (top) and *heuR-cyaA* (bottom) in colonies of *C. jejuni*.

The results of the ELISA using the plasmid we constructed supported our hypothesis that SliP is secreted by the flagellar T3SS in *C. jejuni*. For example, the introduction of wild-type *C. jejuni* expressing the SliP-CyaA fusion into neutrophils resulted in increased cAMP (7.26-fold) comparable to that of the CiaB-CyaA positive control for secretion, which yielded an 8.4 fold increase in cAMP (Figure 3). cAMP levels were also considerably higher in the SliP fusion compared to the HeuR fusion as well. Furthermore, the levels of cAMP were decreased in the $\Delta flgE$ mutant, which supports our prediction that SliP is secreted through the flagellar T3SS in *C. jejuni*. To determine whether endocytosis of *C. jejuni* could also lead to increased levels of SliP inside the neutrophils, phagocytosis was blocked by the introduction of cytochalasin D. This resulted in a decrease in cAMP concentrations despite the presence of an intact flagellar T3SS, which suggests that endocytosis of neutrophils by *C. jejuni* is required for SliP internalization (Figure 13).

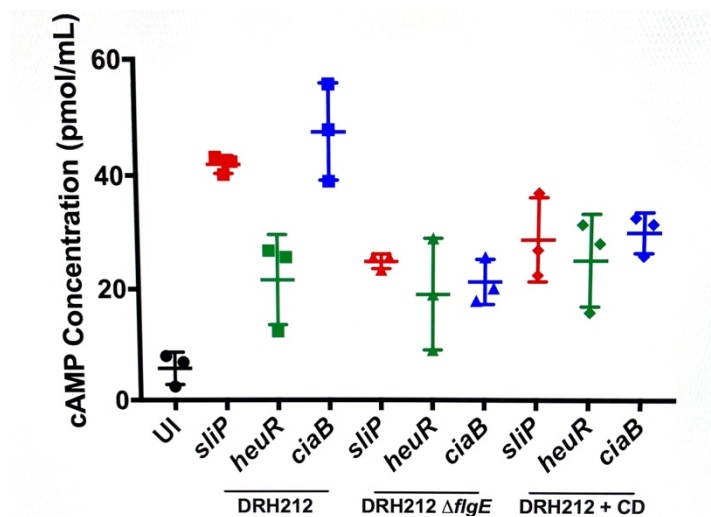


Figure 13: Results of cAMP ELISA with effector-CyaA translational fusions introduced to neutrophils.

Conclusion

Campylobacteriosis has been demonstrated to result in inflammation that is damaging to the host. A source of such inflammation is the activation of neutrophils. Neutrophils employ several mechanisms to kill invasive pathogens. These mechanisms include the release of cytotoxic compounds via degranulation and NETosis. Although these cytotoxic elements serve to kill invasive pathogens, their unspecific activity results in the inadvertent inflammation of host tissue as well. *C. jejuni* has been shown to induce neutrophil response and NETosis, and is believed to deliberately trigger this response to promote disease within the host. These studies identify an effector protein, SliP, that induces neutrophil activation and subsequent NETosis. SliP is predicted to act as a canonical sirtuin to deacetylate histone H3 of neutrophils. This deacetylation would allow space for the citrullination of H3 by PAD4, which causes the release of NETs. In order to act on H3, SliP would need to be delivered to the intracellular environment of the neutrophil. The presence of SliP in neutrophils incubated with *C. jejuni* along with deacetylation of histones in the presence of *C. jejuni* suggest that SliP acts intracellularly on these histones. The mechanism by which SliP is delivered to the intracellular environment of the neutrophil was of particular interest in this project. To determine whether SliP is a secreted protein, fusions with the reporter CyaA were cloned. These translational fusions were designed so that the secretion of SliP into the neutrophil would lead to translocation of the adenylate cyclase, CyaA, and result in increased production of cAMP. Thus, the secretion of SliP could be measured using the cAMP produced by CyaA. The production of cAMP was quantified using an ELISA and these results concluded that cAMP increased upon fusion of SliP to CyaA, which indicates SliP is secreted into the neutrophil during infection. The decrease of cAMP in the flagellar mutant of *C. jejuni* carrying the SliP-CyaA fusion further suggested that the T3SS is used to deliver SliP into the

neutrophil. Characterizing the delivery of effector proteins such as SliP holds immense clinical relevance. The inflammation caused by NET induction mediated by SliP may be responsible for long-term morbidity and increased disease. Ultimately, it is our expectation that studying this system will allow us to identify new therapeutic targets that can reduce the severity of human campylobacteriosis.

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