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Structural formation and functionality of the SpoIIE protein in Clostridium difficile

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Structural formation and functionality of the SpoIIE protein in *Clostridium difficile*

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the requirements for Honors Studies in Biology

By

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Introduction

Clostridioides difficile formerly known as *Clostridium difficile* is an opportunistic pathogen that is scientifically and medically relevant due to its ability to cause infectious disease such as infectious diarrhea or colitis [1,2]. Globally, *C. difficile* is among the leading cause of hospital-acquired infectious diarrhea and is associated with significant morbidity and mortality with an estimated half a million infections per year [21, 22]. It is most often found within the long-term healthcare setting due to the abundance of susceptible hosts [3]. A susceptible host is a patient that has a weakened immune system or has decreased or eliminated their normal intestinal flora in response to antibiotic therapy [4]. Due to antibiotic therapy, a patient's normal flora within the intestines is suppressed or removed, producing an unintended optimal growth environment for *C. difficile* [5].

This gram-positive, endospore-forming, obligate anaerobe bacterium has a variety of virulence factors such as resilience to disinfectants, antibiotics, and stomach pH, which allows it to thrive in a healthcare setting [6,7]. As a result of these characteristics in combination with a compromised gut biota in a susceptible host, *C. difficile* can then produce transmissible and dormant endospores that are able to withstand harsh chemical and physical environments as well as survive for long periods of time [8]. These spores can travel the gastrointestinal tract, where the optimal environmental factors will trigger germination and permit the growth of vegetative cells [7]. For transmission, the spores are excreted from the body and into the feces. Once there, these spores are unknowingly transmitted to other surfaces until they are swallowed by another person, which can cause an infection in a new host [8].

This model organism is also able to produce two toxins, enterotoxin A and cytotoxin B, which are responsible for the disease symptoms with the timing of their release from infecting cells paralleling sporulation [9,10]. These two toxins are important for *C. difficile* as they play an important role in the sporulation and infection process. There are methods to treat this infection of the gastrointestinal tract such as antibiotics or a fecal transplantation. The patient would need to stop the antibiotic treatment they were on previously and start an entirely new one, but this method is not always effective. In the case of the fecal transplantation, a donor's fecal matter is used to reestablish the normal intestinal microbiome in the patient. Neither treatment is completely effective, however. Treatment failures and the recurrence of *C. difficile* infections have pushed the scientific community to search for potential treatment options [21].

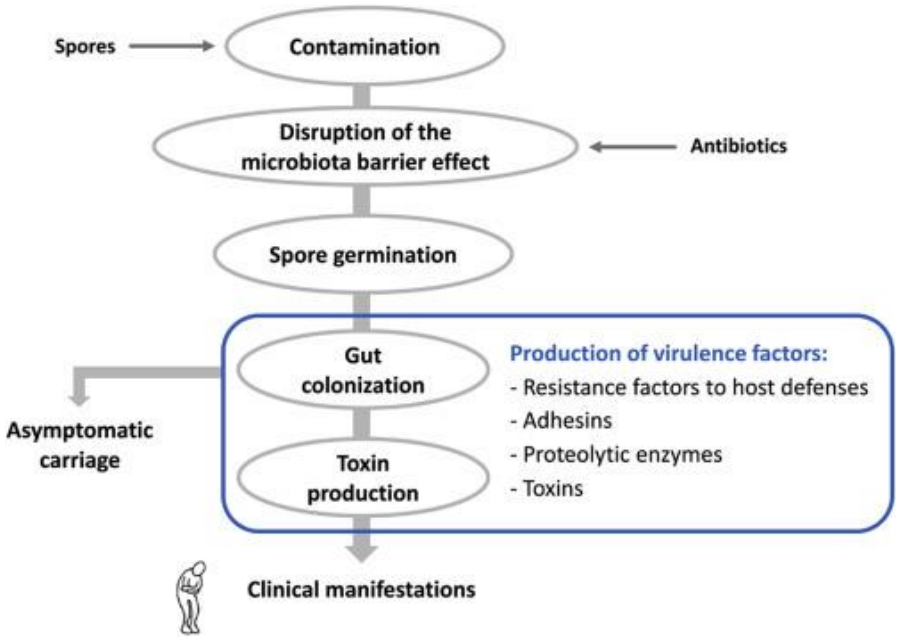


Figure 1. *C. difficile* infection pathway [23]

My research efforts were directed toward the structure and functionality of a protein, designated SpoIIE [11], which previously has been shown to regulate *C. difficile* peptide utilization in addition to sporulation and toxin release. The SpoIIE protein is found in all spore-

forming gram-positive bacteria and is a crucial component in activation of transcription factors and septum formation thus affecting the regulation and timing of spore development [11,12]. A truncated form of the SpoIIE protein named SpoIIEΔTM was used in an effort to determine the structure of an important but uncharacterized linker domain of the protein. If the structure and specific function of this region were to be discovered, it would provide valuable information regarding the complex reaction cycle of SpoIIE and its relevant virulence factors.

To understand more about the structure and function of the protein, there were two components of my research. To begin, The SpoIIEΔTM protein segment was modelled using ab initio modeling systems computationally. The benefit of using this type of modeling is it can predict the structure of a protein without using any previously known structures [13]. This is useful for proteins that are newly discovered and do not have many structures in the Protein Data Bank (PDB) such as the currently unmodeled linker region of this protein [13]. Multiple programs were used to provide a variety of structures of SpoIIEΔTM to compare. Based on the models provided, ideal structures would show an exposed region that of SpoIIE that might define the protein-protein interaction domain of SpoIIE. This first component was completed providing multiple viable structures created from modeling programs such as Robetta [14] and I-Tasser [15]. The structure(s) are preeminent in the second component of research. Further discussion about modeling programs will be completed in Results and Discussion section.

In contrast to the purely electronically acquired data, the second component was in vitro and provided physical data. The protein SpoIIE is known to regulate peptide transport within *C. difficile* which is essential for cell function and plays a vital role in the sporulation process. To understand this relationship and determine which specific area of the SpoIIE protein is responsible for regulating peptide transport, site-directed mutants in SpoIIE were analyzed. If the

peptide transport process becomes inactive after mutagenesis, it can be concluded which segment is responsible for regulating the transport process. Specifically for in vitro experimentation methods, two strains of the SpoIIE gene named pUA626 and pUA629 were used throughout all reactions. Their sequencing and annotated plasmid map can be show in figure 2 and 3. In addition, models completed previously and ab initio data from completed previous threading results from the Ivey lab will be compared to collect substantial evidence supporting or denying the various segments tested.

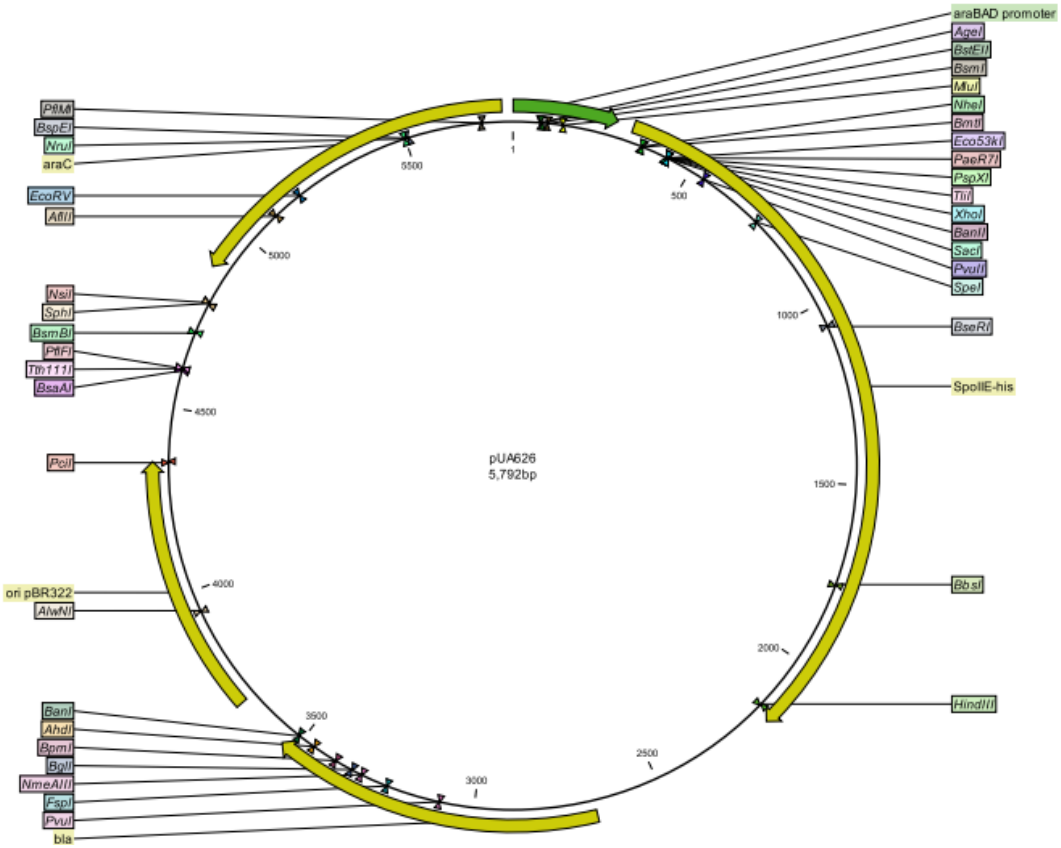


Figure 2. pUA626 annotated plasmid map generated by the Ivey Lab

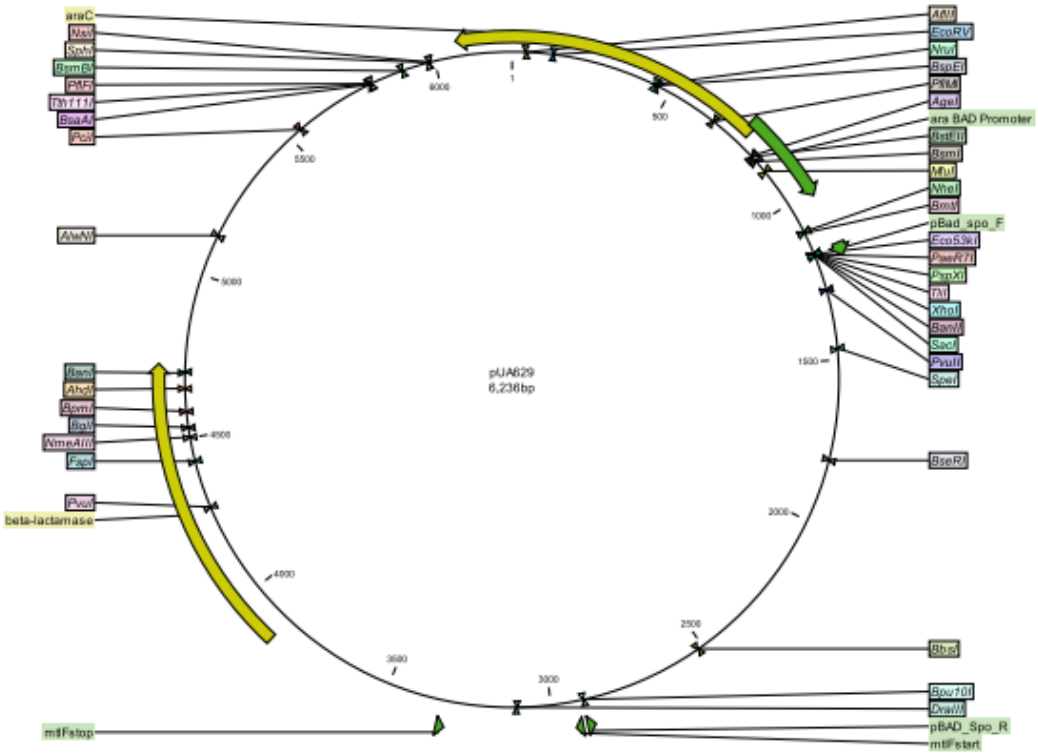


Figure 3. pUA629 annotated plasmid map generated by the Ivey lab

This research project used preliminary data gathered by previous students within the Ivey lab to guide this thesis project. This data provides multiple structures created using threading through a multitude of programs as well as experimental methods that were not able to be completed due to the COVID-19 pandemic. This project was a continuation and extension of this research idea started in the Ivey lab. It was anticipated some of the experimental methods and/or procedures would need to be altered to comply with the regulations or problems that occurred as this research took place. Described below were experimental designs and methods in compliance with the respective COVID-19 regulations at the respective time.

Materials and Methods

Computational Modeling

For this component of my research, primarily two ab initio protein modelling software programs, I-Tasser and Robetta, were accessed and used through free online servers to develop models of the *C. difficile* SpoIIE. The first 100 amino acids of hydrophobic residues were deleted from the SpoIIE fasta file to create a final sequence of 471 amino acids. I-Tasser did require the user to make an account therefore that was completed. The sequence which can be observed in figure 4 was then uploaded into each respective program in fasta format. Once the models were produced, comparisons between the various models for each program were made to determine which protein model was thought to be most accurate in terms of the unknown transmembrane region.

```
>Cdiff_SpoIIE_Final_from_UA001/101-571
RLFNKIEKLIKSNVASNEIVYDYIMRSKNLTNSRLNSIYKTYDDLADTFDKIREKDKVLDQRDIANVIDMIH
NDECKSCSMRRMCWESRFNHTYTMVYNILEKIEEKGELSLNDIPKNFRKECMKPESIVKISNHYYKMFVLDY
DWSVKFSESRKLIANQIRSISKSIKSLSQDLEGDIMLDIEKEKNIYEQLERYDITVDKVSYLTKSNSEFEIS
IEKKTCHDGCMCEDKIVNIISDLVGENMSVRKIGCHCLGGKCKATFVKSQKYKAVTEVSAMSRDGHILCGDN
YTYMEINDGKYMMAISDGMGKGKKAYEESSATIDILEKMIDAKIKDEIVIDTINNMLLLKSSEEMFSTLDLG
ILDLKRGCLETIKMGACSTYIKREDGEVDLISSSSLPVGILSDVKIDRKNVKVKEGDYVIMVSDGIVDAGRN
NNLGDNWLIYFLKNIETTNPKEISNLILDRALELQALQI
```

Figure 4. SpoIIE Sequence with the first 100 Amino Acids removed as shown in a fasta format.

Polymerase Chain Reaction (PCR)

Multiple polymerase chain reaction (PCR) cycles were completed to amplify targeted genomic DNA sequences, such as pUA626 and pUA629, for subsequent recombinant techniques. Primers were used as a complementary single-stranded DNA to our targeted sequence, SpoIIE, to initiate replication with DNA polymerase. Table 1 shows the primers used

in the PCR cycles, their sequences, and the source. The master mix used was GoTaq Long PCR Master Mix by Promega Corporation. This contains the higher performance GoTaq Hot Start Polymerase for longer DNA amplification. Each PCR reaction for initial DNA amplification was performed in 49.5 µL, comprised of 25 µL GoTaq Long PCR 2x Master Mix, 22.5 µL of deionized or nuclease free water, 1 µL of pUA626 and pUA629 respectively, 0.5 µL Eurofin 1/2 primer, and 0.5 µL Eurofin 2/2 primer to create a 49.5-50 µL sample in a 0.2 mL PCR tube. The Thermal cycler (MWG-Biotech) was used to complete PCR amplification, and the reaction conditions were 95°C for 2 min, 30 cycles of 92°C for 30s, 45°C for 30s, 65°C for 6 min, and extension at 72°C for 10 min. These conditions were determined from previous experiments. Verification of PCR amplification was completed through gel electrophoresis.

Table 1. Primers used for PCR

Primers	**Sequence (5'→ 3')**	**Source**
Spo2e TMF 1/2	CCAGAGCGATTATTTAATAAG	Eurofins
Spo2e TMR 2/2	CTCGAGCTCGGATCCCCATCG	Eurofins

Gel Electrophoresis

Many gels were completed to examine and distinguish different samples of DNA based on their size and length traveled through the gel. Quantity of DNA was observed and used as confirmation of success for completed procedures prior to moving to the next step by comparing the brightness of each band as a brighter band suggests increased amounts of DNA. Two different types of gels were used throughout my research processes, but the protocol was the same fundamentally for each. To make the first gel, 160 mg agarose was added to 20 mL of TAE

buffer from a lab stock in a flask and then heated until bubbles formed. After letting cool, 2 µL of EZ-Vision Bluelight DNA Dye by VWR Chemicals LLC was added to the flask. The mixture was then poured into gel mold with variable comb size needed and allowed to cool for 15 minutes. Once DNA molecular weight marker and samples were loaded into the gel, it was allowed to run at 100V for 20 min using the Embitech Run One Electrophoresis Cell. The second type of gel had similar protocol, however, the mixture contained 40 mL of TAE, 280 mg of agarose, and 4 µL of EZ-Vision Bluelight DNA Dye. The gel was allowed to run at 60V for 40 min using the EC Apparatus Corporation Gel Electrophoresis. The DNA marker mix used consisted of 4 µL 1 Kb DNA marker, 6 µL deionized water, and 1 µL 2x concentration dye for all gels completed. The amount of products loaded into the gel varied throughout my research with specific measurements provided for each respective process, however, the concentration of 2x dye were always added to the products. Ideally, the wells loaded had 10 µL, however, procedures completed did not produce a full 10 µL therefore less was loaded into the gel well and allowed to run.

Site-Directed Mutagenesis

Site-directed mutagenesis was completed in efforts to remove the transmembrane anchor region from the SpoIIE gene of the strains pUA626 and pUA629. To complete these procedures, manufacturer's instructions were followed of the Q5 Site-Directed Mutagenesis Protocol by New England Biolabs (NEB) located on the website protocols.io for the kinase, ligase, and DpnI (KLD) Treatment. Additionally, the manufacturer's instructions were followed for the transformation process as well but with an adjustment to the amount of PCR product and incubation time used in the process. This protocol was not followed for the PCR reaction as previous protocol yielded positive results; therefore, the exact same protocol was followed when

repeated. PCR reaction was completed as stated in Polymerase Chain Reaction (PCR) section and the products produced from it were used in the subsequent site-directed mutagenesis procedures.

DpnI Digestion

The purpose of the DpnI digestion following a PCR of plasmids was to destroy all parental template plasmid DNA due to DpnI only digesting the methylated portions of DNA. Because my PCR product because is unmethylated, DpnI will select for the mutated plasmids by only removing methylated plasmid. Protocol for this procedure was the addition of 1 µL of DpnI to PCR products and subsequent incubation at 37°C for 1 hour.

Purification

Following DpnI digestion, purification was immediately followed. Manufacturer's instructions of the MicroElute DNA Cleanup kit (Omega Bio-tek). Steps 1, 3, and 14 from the manufacturer's instructions were range values therefore specification used in my procedure will be provided. For step 1, about 40 µL was measured for each tube; for step 3, 120 µL was added to the PCR tubes; lastly, for step 14, 10 µL was added. All other steps were followed accordingly to manufacturer's instructions

Kinase and Ligase Reaction

The purpose of the Kinase and Ligase reaction was to allow efficient phosphorylation, intramolecular ligation and circularization, and template removal. This procedure uses a KLD Enzyme mix which is a blend of Kinase, Ligase, and DpnI enzymes and an enzyme buffer. Due to this mixture, this step is the most crucial and sensitive step within the site-directed mutagenesis process. To begin, 5 µL of 2x KLD Reaction Buffer, 1 µL KLD Enzyme mix, and 4

µL of PCR product that was prepared in earlier section were added into a PCR tube. To mix properly and gently, the reagents were pipetted up and down. The mixed solution was then incubated for 5 minutes at room temperature and then the transformation procedure according to manufacturer's instructions with slight variation was completed. An additional identical cycle of kinase and ligase reaction was performed with the only exception being the sample was incubated at 15℃ overnight in the Biotech Thermal cycler. Two cycles were completed due to time constraints and to increase the likelihood of colony formation within the plating step which will be discussed.

Transformation

As stated earlier, the transformation process used the manufacturer's protocol of the Q5 Site-Directed Mutagenesis by New England Biolabs (NEB) located on the protocols.io website. NEB C2987 chemically competent *E. coli* cells were transformed so that my targeted plasmid sequence of each pUA626 and pUA629 with the transmembrane region presumably deleted was inserted into the cells. The only difference noted in procedure was that 4 µL of KLD product was used instead of the 5 µL that is called for in the protocol. Each cycle of KLD products underwent transformation at separate times but was completed as stated.

Plating

For the plating process, the plates used were Luria-Bertani (LB) medium with antibiotic ampicillin added (LB-amp) that were created according to the Biotechnology Explorer Microbial Culturing Kit Preparation of LB-Ampicillin Agar protocol. The plates had a concentration of 10 mg/ml ampicillin to LB medium as outlined in the protocol and were stored at 4℃ until used. Transformed *E. coli* cells were then pipetted onto the plate surface and then streaked with a

sterile loop by an open flame. The set of plates were then incubated overnight at 37°C. Table 2 shows the cycle, plasmid, as well as amount pipetted onto the plates. Cycle two used a control of pUC19 Control DNA by New England Biolabs (NEB) to ensure the plates were viable and could allow for colonization of the *E. coli*. 1 µL of pUC19 was added to the tube of competent *E. coli* cells following protocol and later the transformed cells were plated in the process identical to my samples.

Table 2. Plating Protocol

Cycle	Plasmid	Amount (µL)
1	pUA626	10
	pUA626	100
	pUA629	10
	pUA629	100
2	Control	100
	pUA626	10
	pUA626	100
	pUA629	10
	pUA629	100

Colony Selection and Broth Cultures

After incubation of transformed *E. coli* cells on LB-ampicillin plates and colony formation, colonies were picked and inoculated into liquid broth for further growth. Plates resulted in two viable colonies for pUA626 and four viable colonies for pUA629. Sterile wooden applicator sticks were used to pick a single colony by pressing gently to it and then swishing the stick into the liquid broth tube. The broth is made of 33 µL of carbenicillin and 33 mL of

laboratory LB stock broth. About 4 mL of this broth mixture was deposited in a total of 6 tubes and each held their respective colony picking. These inoculated tubes were then shaken overnight at 37°C. Carbenicillin was used over ampicillin due to the more difficulty to build up a resistance to carbenicillin.

Viogene Plasmid DNA Extraction

Following incubation, about 1.6 mL of broth culture and was processed using manufacturer's protocol within the mini plus plasmid DNA extraction system (Viogene). Products created from this procedure were then separated by agarose gel electrophoresis to confirm the viability of the mutant plasmids. The new mutant plasmids should be smaller than the original plasmids due to the deleted region allowing the DNA to run faster in the gel and thus travel farther.

Restriction Endonuclease Digest

Two different digests were completed to observe the weight and brightness of mutants on a gel. The first digest was using the restriction enzyme EcoRV. The locations of this restriction enzyme for each mutant can be observed on the original strain plasmid map shown in figures 2 and 3. The original pUA626 and pUA629 were included in this digest and gel because we wanted to observe the difference between my mutant plasmids and the original strains. Ideally, the mutants should be smaller in size due to the portion deleted, therefore they should run faster on the gel showcasing this smaller size. Comparison between the original strains and mutants offers some idea if procedures were a success without sequencing being completed yet to give full confirmation. For the EcoRV digest, 5 µL of plasmids, both mutant and original, were added to 3 µL of deionized water, 1 µL of EcoRV, and 1 µL of NEB 3 Buffer into a PCR tube. The

PCR tubes were then incubated at 37°C for 30 minutes. A gel was then performed using the second style of gel. All products from incubation were used when loading the gel therefore 1 µL of 2X dye was added to about 10 µL of product. Marker was prepared and all products were loaded into gel and ran at 60V for 40 minutes. The second digest performed included the restriction enzyme PvuII with the same protocol as for EcoRV.

Sequencing

Sequencing is to be completed by Plasmidsaurus.com which is said to provide complete, accurate, and full-length plasmid sequencing. Information from the "Prep&Ship" tab located on their website was used to determine what concentration our mutant samples needed to be at. Based on our mutant plasmid DNA length, the concentration of our samples needs to be at least 10 µL normalized to 30ng/µL. Concentration of samples were measured with Qubit quantification, reagents, and buffer. First product concentration was not high enough, therefore it was decided to grow a new culture overnight to achieve more sample product. It was decided that due to limited time, mutation samples 1 and 2 of pUA 626 would be grown first as they were the most promising out of all mutated samples. To grow new cultures, 50 µL of ampicillin and 100 µL of previously grown cultures were added to liquid broth and incubated with shaking overnight at 37°C. Next, the Viogene plasmid DNA extraction was completed for 5 samples of each mutant in hopes of having plenty of product to achieve a high concentration. To help achieve a high concentration, centrifugal filter Centricon-10 was used in conjunction with a Heraeus centrifuge. Concentrations of this product were then calculated using the Qubit manufacturer protocol and device. Due to issues with achieving the correct concentration of product, we were unable to go further past this step in this process for sequencing at the current moment. We are hopeful to send off results and achieve sequencing maps prior to the end of the

semester in order for accurate conclusions to be reached. Determinations for what is next in the Ivey lab can then be concluded.

Results and Discussion

The goal of this thesis project was to model the unknown transmembrane linker region of SpoIIE and compare this model to data gathered from in vitro experiments to determine if the model is accurate. The transmembrane linker region has not been crystalized meaning the structure is still unknown in current scientific literature. If positive results were yielded from this project, possible protein models would be identified for the SpoIIE gene of *C. difficile*. Furthermore, if sequencing results show that our mutant strain effectively deleted the transmembrane region, research efforts in the Ivey lab can be geared toward crystallization.

Ab Initio Modeling

As stated previously, two ab initio protein modeling software systems were used. Each produced variable models, however, the primary goal of using two different systems was to compare the models against one another to look for key similarities or differences. Figures 5 and 6 show two models produced by Robetta that we believe are most accurate due to the higher order of its folding as they have more tertiary structures, specifically alpha helices, than other models produced.

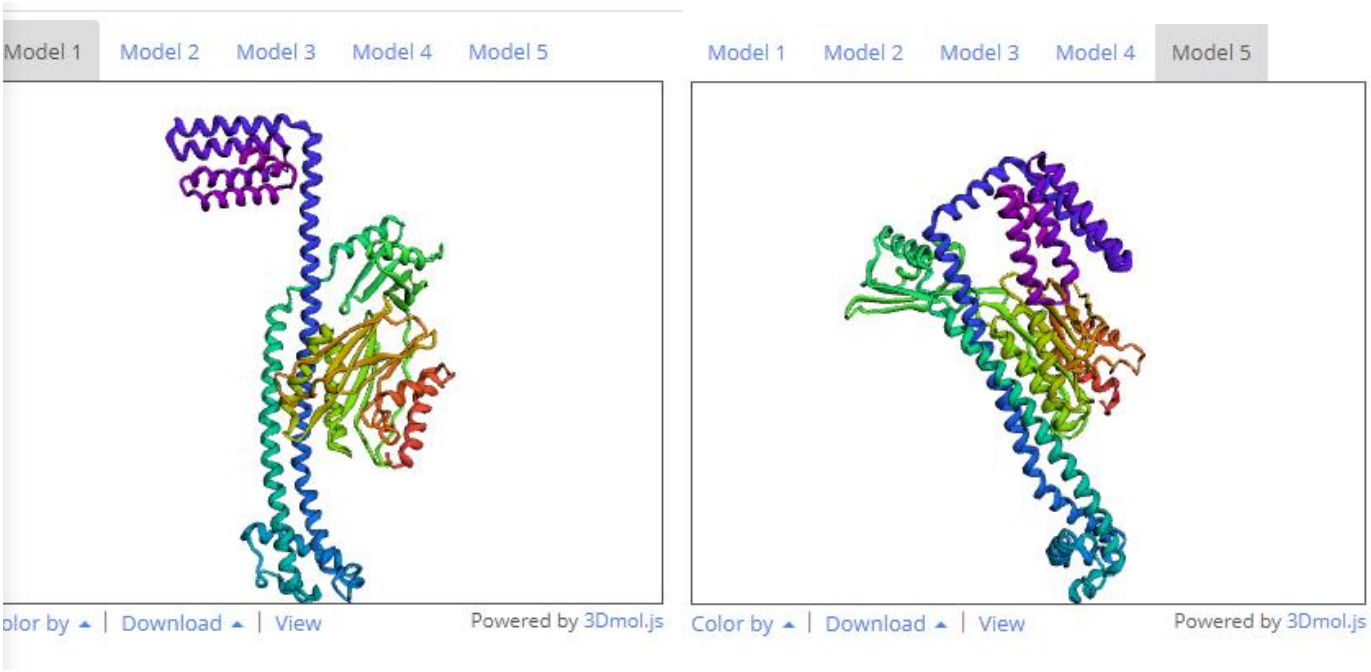
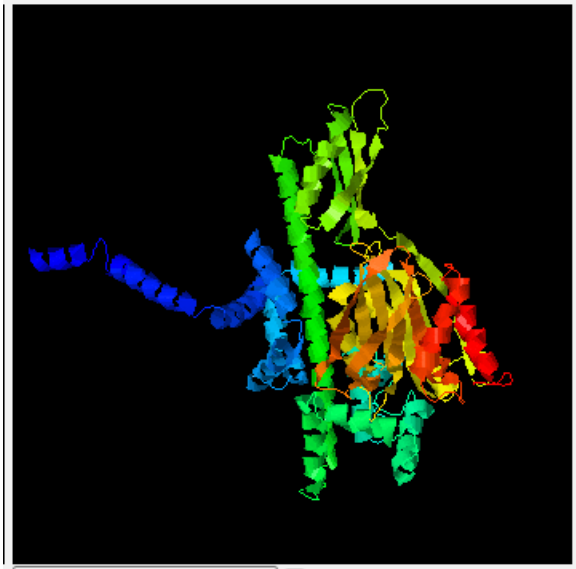


Figure 5 (left) and Figure 6 (right). Robetta models of *C. difficile* SpoIIE fasta format.

The key areas of interest of these models are the dark blue and purple regions which we believe represent the transmembrane region of SpoIIE. The linker region needs to be accessible as it has contact with dimers; The purple alpha helices of these two models show a conformation synonymous with its known needs and functions. The other colored structures are the regulatory regions of SpoIIE and are well known and studied throughout scientific literature. Our focus is on the transmembrane linker region which we believe to be the purple and dark blue alpha helices and is located away from the regulatory regions. Using in vitro lab experiments outlined previously, we believe we removed the transmembrane region from SpoIIE.

Secondly, the program I-Tasser was used to produce protein models and compare against models created by Robetta. Figures 7 and 8 show the models we believed are most likely accurate. It is important to note that comparison of the two program models were done with both

showing the believed transmembrane region located away from the regulatory regions. While the folding of the dark blue region in model 5 (Figure 7) is different compared to models 1 and 5 (figures 5 and 6), it is important to observe the protein structure conformation of alpha helices is similar. Model 2 of I-Tasser (figure 8) is a great example to show the distance between the regulatory regions of the SpoIIE protein and the believed transmembrane linker region. Comparison of models between the two provides evidence of similar structures by means of conformation and level of folding.



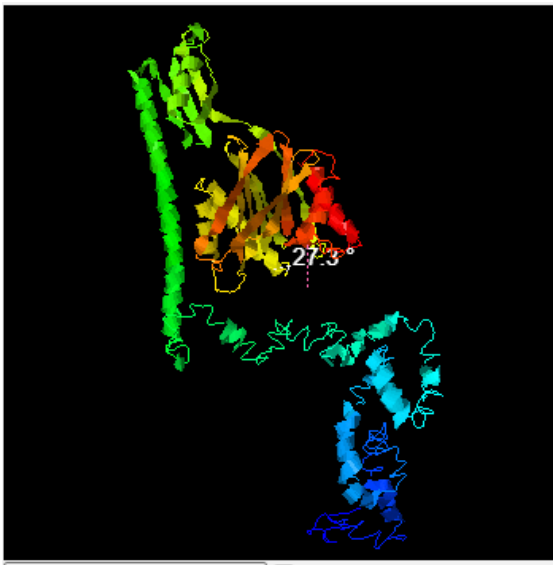


Figure 7 (left) and Figure 8 (right). I-Tasser model of *C. difficile* SpoIIE fasta format with the first 100 amino acids deleted.

In Vitro Experiements

Essentially, three cycles of the procedures were completed until viable results were formed. However, two specific procedures were not used in the final successful procedures

because the results from their specific protocols were deemed unsuccessful. It is important to note that efforts were made to complete these procedures to ensure that the results we received did exhaust all options. Within the first cycle, DpnI digestion was completed but it did not provide positive results therefore it was no longer used in subsequent cycles. Additionally, the purification procedure was completed in the first and second cycle attempts with no successful results and thus it was not used in the third cycle attempt.

To begin the in vitro portion of this project, difficulties occurred with the initial PCR attempts as they proved to be unsuccessful when attempting to confirm DNA amplification through gel electrophoresis. Various master mixes and primers were tested until a positive result was yielded (Figure 9). It was determined that a new master mix and new enzymes were needed as the previous ones were not successful at amplifying the original strains of DNA of pUA626 and pUA629. By having new master mixes and new enzymes, we were able to confirm through gel electrophoresis that we started the project with viable DNA; Thus, if products were shown to be unyielding of results, we would know it would be the subsequent process as the culprit instead of the original DNA strains. Once all reagents of PCR were confirmed to be in good working condition, we were able to continue with the procedures. Prior to beginning the third cycle of procedures, PCR was

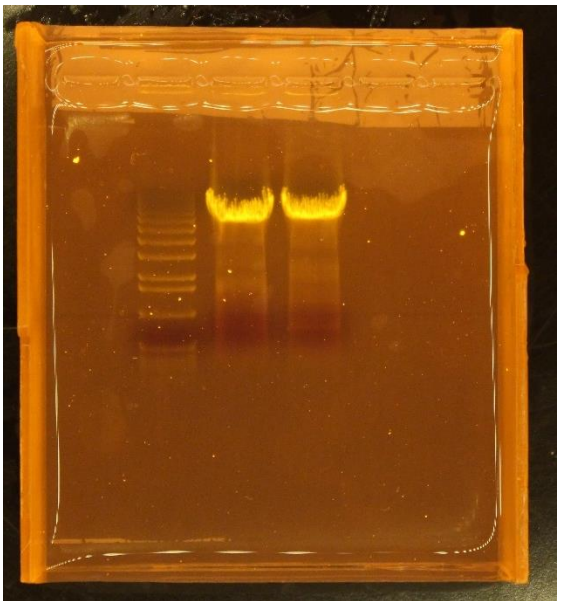


Figure 9. Gel electrophoresis confirming amplification of DNA using new master mix and new primers. From left to right samples are as follows: marker, original pUA626, and original pUA 629.

completed again a gel of the PCR results was completed to ensure viability of DNA and proper amplification had occurred due to negative results of the second cycle of procedures (figure 10). By the third cycle of procedures, we were able to receive viable results.

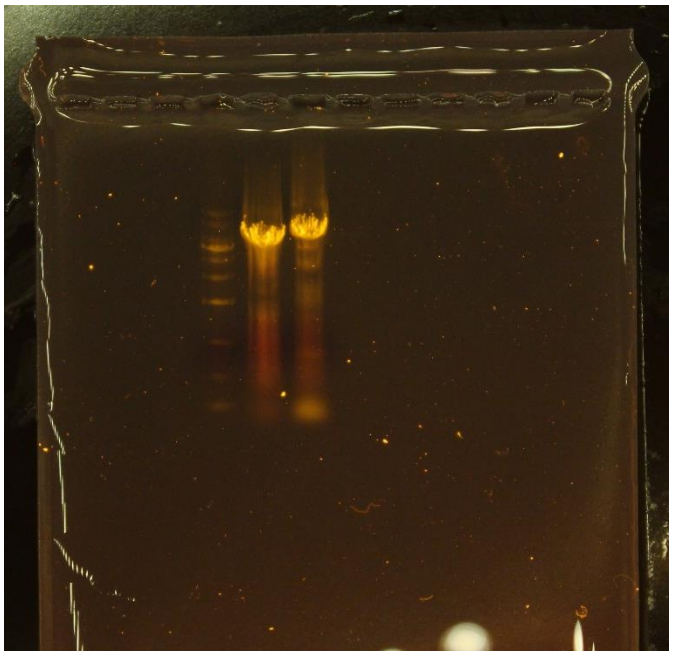


Figure 10. Gel result of PCR prior to final cycle of procedures. Amplification of plasmids can be observed. From left to right samples are as follows: marker, original pUA626, and original pUA 629.

Plating and Culture

Using protocol stated for plating and broth cultures, mutant strains of pUA626 and pUA629 were created through the removal of the transmembrane linker region. Competent *E. coli* cells with our mutant plasmid inserted into it were spread across LB-ampicillin plates with the most successful being the spread of 100 µL. Mutant 626 had growth of a total of two colonies that were picked (figure 11). It is important to note, two Kinase and Ligase reactions were completed to increase the likelihood of possible colony growths, however, the two colonies of 626 strain were picked from the second kinase and ligase reaction. Mutant 629 had growth of four colonies that were picked (figure 12). Lastly, a control was completed to ensure the plates were optimal for cell growth (figure 13).

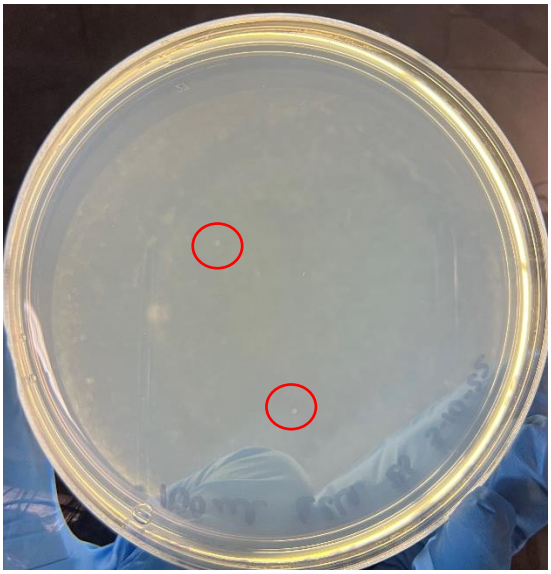


Figure 11. Result of mutant 626 colony growth on LB-ampicillin plate.

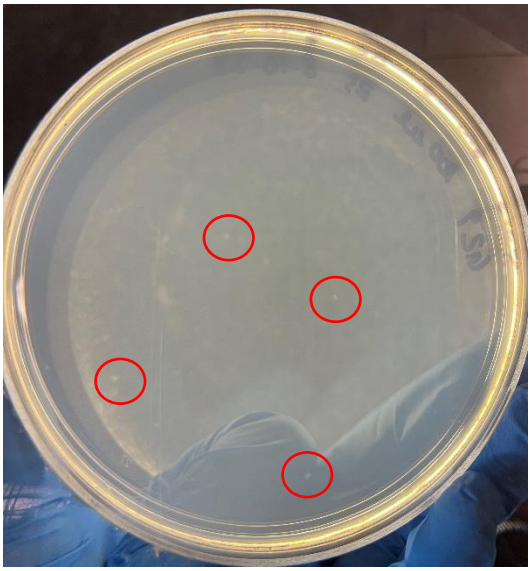


Figure 12. Result of mutant 629 colony growth on LB-ampicillin plate.

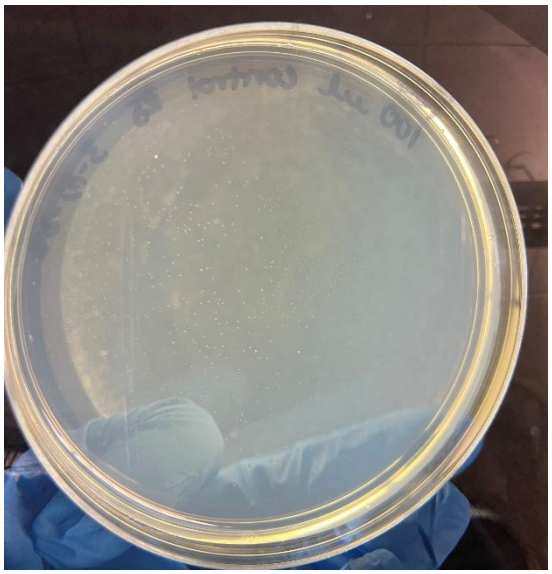


Figure 13. Positive result of control pUC19 colony growth on LB-ampicillin plate.

Once colonies were picked from each mutant plate, they were inoculated into liquid broth (figure 14). As stated previously, mutant 626 had 2 samples and mutant 629 had 4 samples. Cloudiness of liquid broth samples show growth of cells and indicate viability of cells.

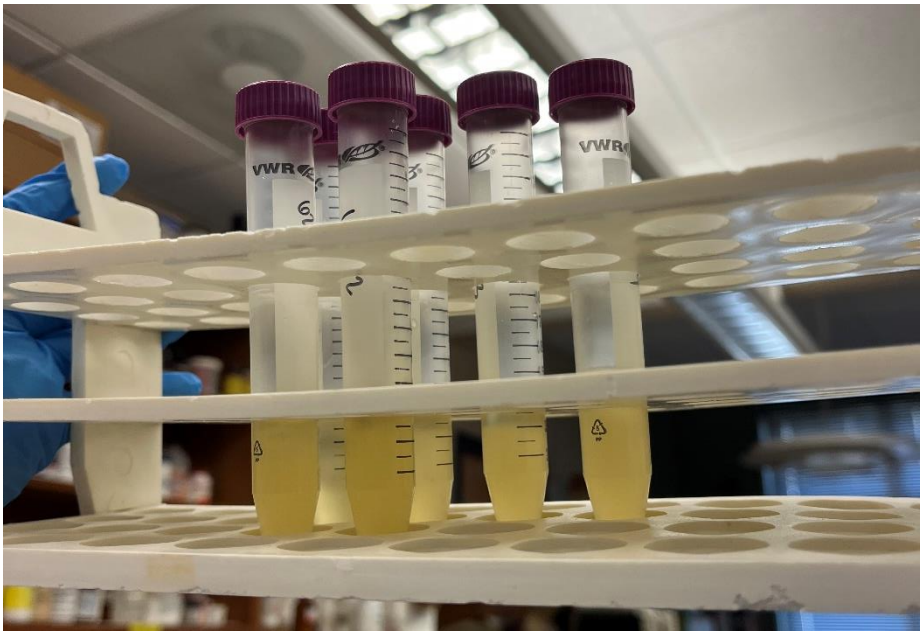


Figure 14. Results of colony inoculation into liquid broth and carbenicillin solution.

Restriction Enzyme Digests

We believe we have successfully removed the transmembrane region of pua626 and 629 which can be shown on the models if proven correct. However, PCR can introduce unplanned changes within the plasmid; therefore, restriction enzyme digestions and subsequent gels as well as sequencing can be completed to verify our mutants are missing the transmembrane linker region. Gel imaging of the restriction enzyme digest can broadly confirm that our mutants have the same pattern we'd expect, however, sequencing would need to be done to have complete confirmation that the correct region was deleted (Figure 15 and Figure 16). Additionally, it needs to be noted that the EcoRV gel's marker malfunctioned therefore it is not visible so we cannot

determine the exact size of the samples; however, generally we can confirm that our mutant samples are smaller than the original plasmids which is what we would want to se. Logically, it makes sense for our mutants to be smaller as they are missing an entire region of DNA. The restriction enzyme PvuII cuts the plasmid DNA in two separate places creating two separate DNA fragments. This causes there to be two bands within the gel for each sample indicating positive results for each mutant. Based on size and brightness of mutants as compared to the original plasmids, the EcoRV gel and PvuII gel shows broad confirmation that a deletion did occur.

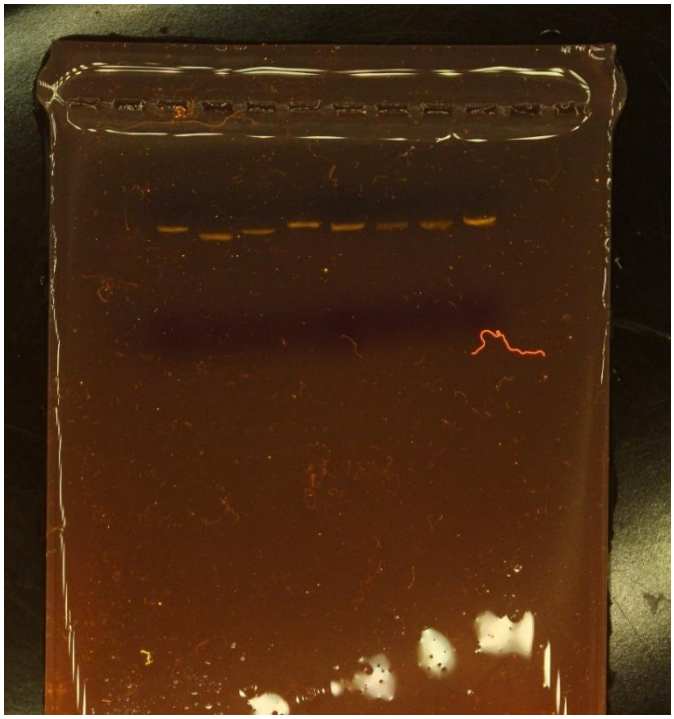


Figure 15. Gel results of EcoRV digest of mutant and original plasmids with mutants being smaller than originals indicating positive result. From left to right samples are original pUA626, 626-1, 626-2, original pUA 692, 629-1, 629-2, 629-3, and 629-4.

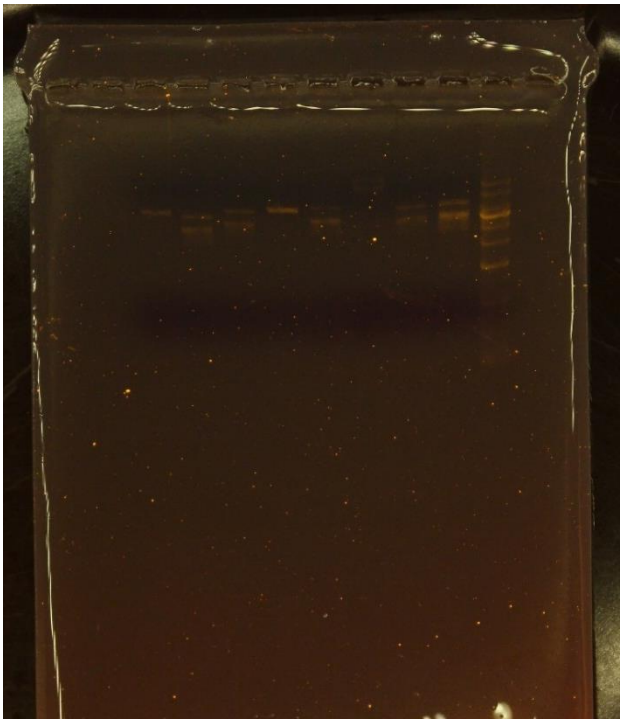


Figure 16. Gel results of PvuII digest of mutant and original plasmids showing two separate bands for mutant strains indicating positive result. From left to right samples are original pUA626, 626-1, 626-2, original pUA 692, 629-1, 629-2, 629-3, 629-4, and marker.

Sequencing

The digests indicated the mutants were smaller than original plasmids which confirmed the need for sequencing to determine with confidence which region was deleted. Problems arose with the sequencing preparation unfortunately so at this time, we currently do not have the mutation sequencing nor the plasmid maps which would be similar to figures 1 and 2. For the plasmid preparations, samples need to be at a certain concentration for Plasmidsaurus to sequence them. Procedures completed to achieve the concentration were unsuccessful even after further concentration efforts. Additionally, the plasmids only ship out once a week and we were unable to meet the deadline in order to receive the sequencing results in a sufficient amount of time. We do plan to sequence our mutants to reach conclusions based on our efforts this far.

The results of the sequencing will either confirm that we have made a mutant of SpoIIE without the transmembrane region, or that all of our research efforts for this project were unable to confirm the deletion of the transmembrane region and thus unable to confirm any models. If sequencing confirms our mutants, the Ivey lab will have a new strain to use in future experiments and endeavors.

Future Directions

Further directions for this research project are subjective to what I am able to accomplish with my remaining time within the lab. It is expected that I will complete sequencing as well as possibly do fluorescence assays, however, results of these procedures may not be presented for this thesis due to time constraints. Possible future directions include but are not limited to fluorescence assays, if I am unable to complete them, as well as immunoprecipitation and western blotting for these specific mutants if they are confirmed to have correctly deleted the

linker region. Fluorescence assays can be completed to assess the protein functionality and determine the peptide transport gene expression level. Thus, the protein functionality could be discovered to correspond to a specific region and intramolecular contact sites may be determined. Immunoprecipitation can be completed to observe the intermolecular interactions of wild-type and the mutated SpoIIEΔTM. Western blotting will follow IP to verify the identities of binding partners used in the IP process and provide further information regarding our three-dimensional protein models. It is hopeful that the mutants created for this thesis will yield positive results and will then become a new strain within the Ivey lab for future endeavors to be completed with. Ultimately, the work and data presented in this thesis may contribute to future research projects within the Ivey lab that can be done by incoming undergraduate students.

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