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Purification of a Bacteriophage Protein Involved in Host Range Specificity

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Honors Project

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Abstract:

The Escherichia coli ferric hydroxamate uptake receptor FhuA serves as the receptor for ferrichrome-Fe(III) complexes, with TonB protein energizing the active transport of the complex. The FhuA receptor is exploited by a variety of bacteriophages as a conduit into the cell. Interestingly, certain of these phages carry a gene called "Cor", the product of which, when cloned and expressed from a plasmid, blocks transport by FhuA. In the present study, components of the cor gene from the bacteriophage ϕ80 were used to construct an IPTG-inducible MalE-Cor-His6 fusion protein, which allowed for affinity purification of the Cor protein. At 61 residues in length, purified Cor protein was not readily demonstrable by standard SDS polyacrylamide gel electrophoresis. The purification of Cor protein via maltose-binding and nickel exchange chromatography as well as visualization of Cor protein by silver staining of samples resolved on Tricine-SDS polyacrylamide gels is described.

Background:

Bacteriophage

Bacteriophages, commonly known as phages, are viruses that parasitize bacterial hosts. These viruses are unique in their structure and are made up of an icosahedral head known as a capsid and a long tail. The head region is where the viral gene are held, and when the tail region is where the virus will attach to a cell and then eject its genes form the head, through the tail and into the newly infected cell.

Infection and entry of virus DNA commonly leads to two possible outcomes. Either the DNA from the virus becomes incorporated into the host DNA and then remains dormant. Or the incorporated DNA send instructions to the host cell, causing the host cell to create more of the virus until the cell eventually bursts. These two stages are called Lysogeny and Lytic respectively. This combination of "lytic" and "lysogenic" activities is prominent in the survival and reproduction of viruses. One such class of bacteriophages are known as T4 Coliphage. These describe a class of virus that specifically infects *Escherichia coli* (*E.coli*). Studies into this coliphage suggest that its survival and reproduction is dependent upon two highly specific interactions with E.coli. First, the virus must attach to the outer membrane surface structures of the cell. Second, subsequent to binding, transport through the outer membrane often requires another cell envelope protein, TonB (Noinaj et al., 2010).

Utilization of *E.coli* Surface Structures by Bacteriophage

Previous studies with coliphage has led to observations in attachment sites and pathways utilized by virus for injection of genetic material into the host cell (Ivanov 2012). This process is commonly referred to as transduction. In order for transduction to occur, the coliphage must be able to inject its DNA into a pathway that will allow the viral genes to travel into the nucleoid region of the bacteria. Previous studies show that one preferred pathway is the FhuA-TonB complex. (Noinaj et al., 2010). FhuA is an outer membrane protein of *E.coli* and TonB is a transmembrane protein pathway acts as a conduit between the cytoplasm and the periplasm.

Together, these two structures form a complex whose primary function is to transport iron into the host cell (Larsen et al., 2003). Many bacteriophage however are able to utilize the FhuA protein as an attachment site and then utilize the TonB pathway to aid in successful injection of viral into the nucleoid region of the host cell.

TonB-dependent transporters (TBDTs) are bacterial outer membrane protein structures that bind to a variety of substances and help to transport these substances into the bacteria (Nioinaj et al 2010). For the bacteria to utilize this transport system, it must supply an appropriate amount of energy. The standard explanation for this dependence has been that the virus uses this energy to power injection of DNA into the host. Surprisingly, we have isolated mutations that are TonB independent but still utilize the same docking sites for host attachment. This indicates that the virus does not need host energy for DNA injection. Thus, TonB dependence may be about something else. In order to figure why TonB is the preferred pathway of bacteriophage, it is crucial that studies into the viral genome be conducted and one such gene that is involved in mediating the attachment and utilization of FhuA-TonB is the Cor gene.

Cor Gene and its importance in Viral Infections

Cor refers to a specific gene in the genome of bacteriophage that is expressed during lysogeny to interact with FhuA and block further infection of host cells by FhuA dependent phage (Ivanov 2012). It has been estimated that there are ~85% of FhuA-dependent phages among heterogeneous lambdoid phages from different immunity groups, and about half of phages tested contained the Cor gene (Hernández-Sánchez et al., 2008). Those observations suggest that interactions between Cor and FhuA can provide a means to further examine the protein-protein interactions that mediate the functions of TBDT proteins. The Cor gene and the protein that is codes for plays a key role in preventing interaction between bacteriophage and FhuA and, if utilized properly, has the potential to prevent this attachment. By extension, this would prevent viral infections by bacteriophages for all FhuA-TonB dependent viruses. Further investigation into the Cor protein was the focus of the research period.

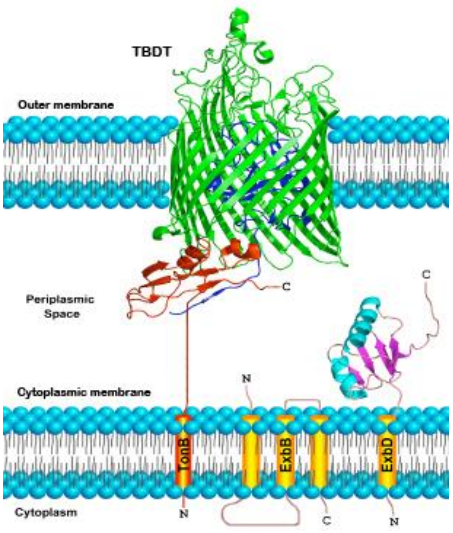


Figure 1. **Components of the TonB system.** Ton-B dependent outer membrane receptor FhuA (green) coupled with the surrounding components of the TonB energy transduction system. The FhuA receptor is shown as a β-barrel plug that can be opened and closed by the N-terminal plug (dark blue). The ribbon structure represents the solved co-crystal structure of FhuA with TonB (Pawelek *et al*., 2006) and the NMR structure of the ExbD periplasmic domain (Garcia-Herrero *et al*., 2007). Adapted from Ivanov, (2012).

Specific Aims.

Aim 1: Expression of Cor protein via use of induction using IPTG

Aim 2: Digest of Cor protein via treatment with Factor Xa

Aim 3: Purification of Cor protein via maltose-binding chromatography.

Aim 4: Further purification of Cor protein via nickel exchange chromatography

DNA Vector Construct:

Prior to the start of induction and purification methods, a DNA plasmid would have to be designed that would allow for desired expression of the Cor protein. pMAL-p2X is an expression vector designed by New England Biolabs which, when treated with the proper restriction endonucleases, allows for both insertion and controlled expression of a desired gene.

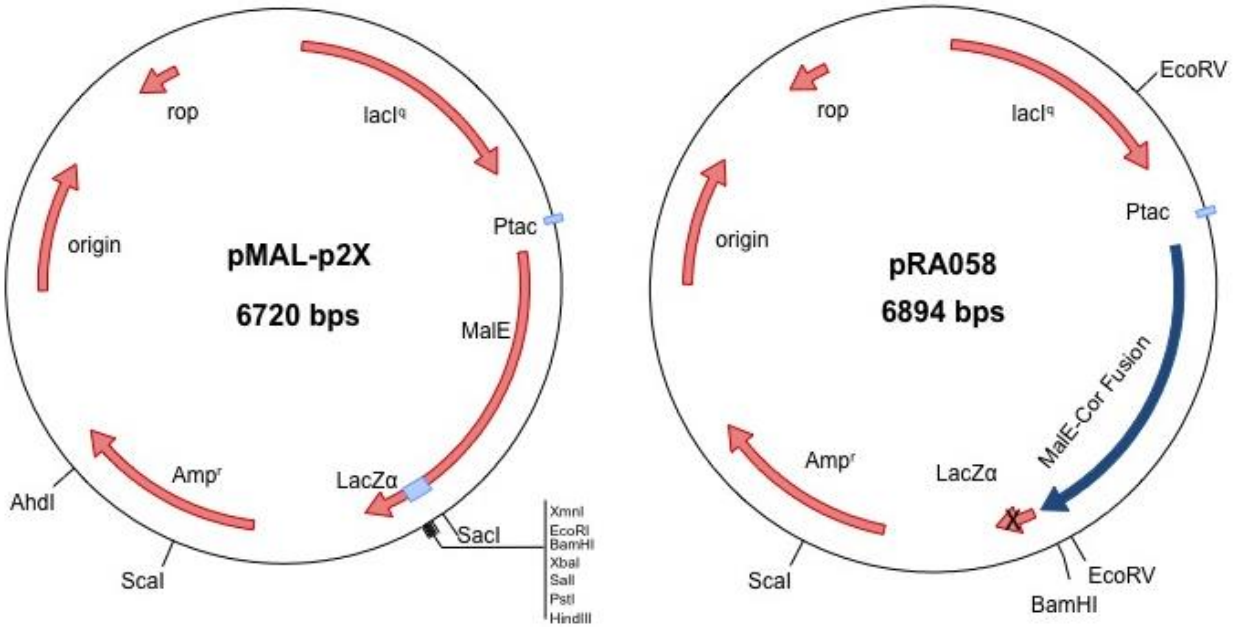


Figure 2: The *malE-cor* fusion gene. The coding sequence excluding the leader sequence of the cor gene had been cloned into the MCS site of the pMAL-p2X vector to produce the *malE-cor* fusion gene. This gene was under the promoter Ptac, which was inducible by IPTG. The product of the fusion gene was the fusion protein MBP-Cor. The fusion protein could be cleaved by a protease, Factor Xa; to isolate Cor. A second plasmid (pRA059) was prepared in the same way to produce a double fusion protein, MBP-Cor-His, which could be cleaved to release Cor-His. Cor-His can be recovered from the solution by Ni-affinity chromatography.

Results:

Aim 1: Induction of genes and expression:

Cor and cor-his genes were induced using 100mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). An empty control vector (pMal-P2x) was also used. As expected, certain proteins were not expressed without IPTG induction. With IPTG induction for 2 hours and beyond, these proteins are clearly seen when run in a 11% tricine gel (photo below).

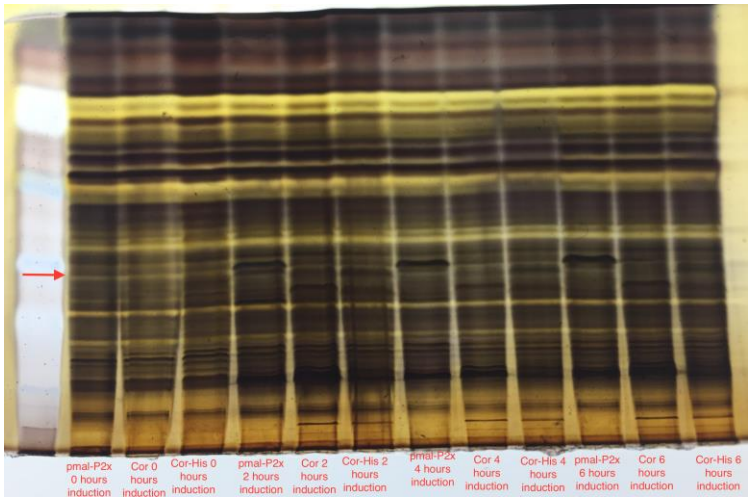
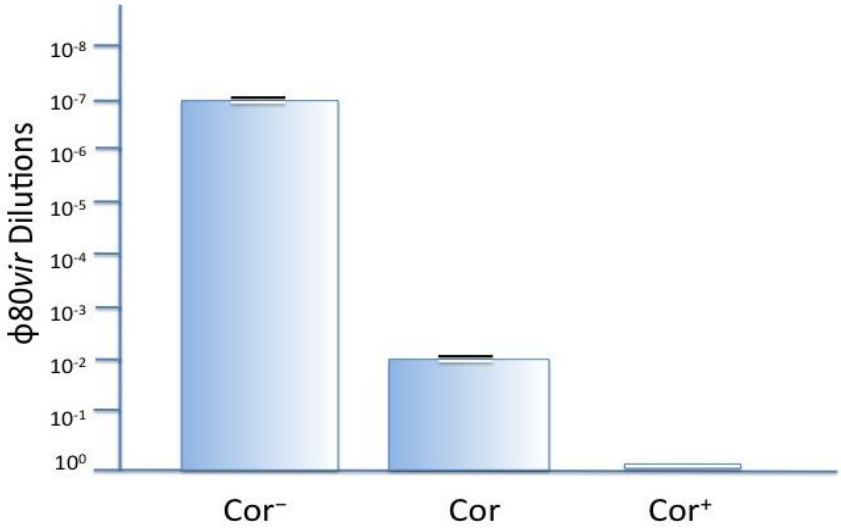


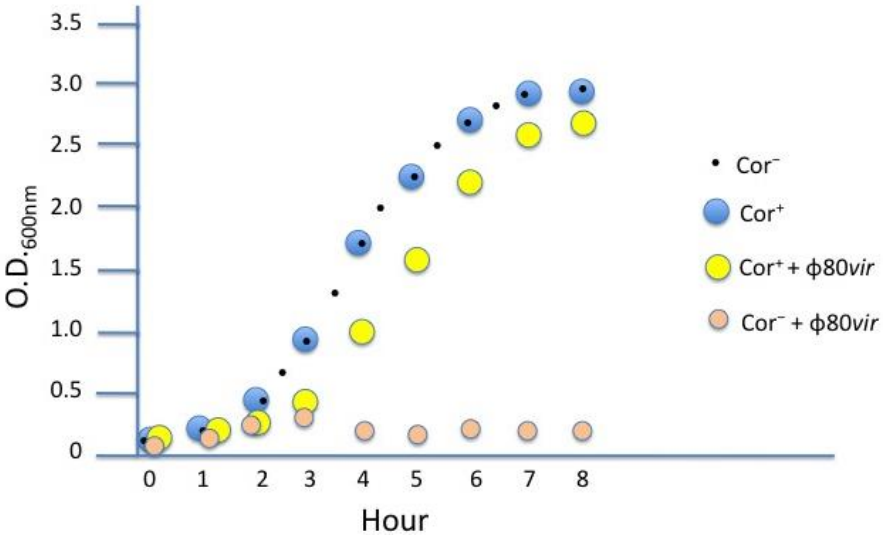
Figure 3: The red arrow represents the Mass where induction is most clearly visible (around 45 kDa). At 2 hours of induction, clearly defined dark bands become visible. These bands represent high concentrations of proteins. Thus, IPTG induction allows for expression of genes that would otherwise be less likely to be expressed.

When inducing W3110 containing the cor and cor-his gene with IPTG, we see that these *E. coli* cells are less susceptible to bacteriophage infection. Using bacteriophage phi-80, viral infection of W3110 is seen in dilute samples of phi-80 with concentrations of virus down to 10^-7. W3110 that have the cor gene incorporated and induced with IPTG do not show any visible signs of viral infection past 10^-2 under the exact same conditions. This experiment was repeated in triplicate, all yielding the same result. This serves as a strong indication that the cor gene can A. be

incorporated into W3110 and B. will aid in protecting W3110 against bacteriophage that utilize the TonB-FhuA complex.



Spot titer assay: The bars represent the greatest dilution of ϕ80*vir* dilutions at which a zone of clearing was evident. W3110 (pBAD24), the negative control with Cor^- phenotype showed plaques up to 10^{-7} dilution. Uninduced W3110 (pRA033) with Cor^+ phenotype showed resistance after 10^{-2} dilution; probably because of leaky expression of *cor* controlled by the pBAD promoter. .01% IPTG induced W3110(pRA033) with Cor^+ phenotype showed complete resistance against ϕ80*vir* infection. All experiments were performed in triplicate.



Growth curve assay: Growth of W3110 (pRA033) at .01% IPTG induced or un-induced state, in presence or absence of ϕ80*vir*; was measured by taking the optical density of the growing culture. There was no significant difference of growth rate in IPTG- induced (Cor^+) and uninduced (Cor^-) state of W3110 (pRA033). The growth curve of induced (Cor^+) W3110 (pRA033) with ϕ80*vir* showed a shift to the right. This suggested most of the cells in the culture were expressing Cor^+ phenotype, protecting the cells from ϕ80*vir* infection; few were not induced in the population and were Cor^- phenotype. The Cor^- phenotype cells were being killed by the virus. Most of the un-induced (Cor^-) W3110(pRA033) cells were being killed by ϕ80*vir*, with few living cells. It was likely that the leaky expression of Cor from the pBAD promoter conferred the Cor^+ phenotype, providing protection against ϕ80*vir* infection. All assays were performed in triplicate.

Preliminary Purification studies:

Maltose-binding Affinity chromatography was the method of choice to purify the cor-his gene. The DNA vector to which the cor and cor-his genes were initially transformed into (pMal-P2x) was selected, in part, due to its ability to produce maltose binding protein.

100mL sample of W3110 containing cor-his was induced with 100microliters of 100mM IPTG for 6 hours. The sample was then centrifuged for 10 minutes and the pellets were later re-suspended in column buffer. Using a Sonic Dimembrator, these cells were lysed so that proteins that these cells contained would be easier to separate when running them through an affinity chromatography column.

This process lead to 13 factions of run through. These samples were then examined on a 11% tricine gel. The results were as follows:

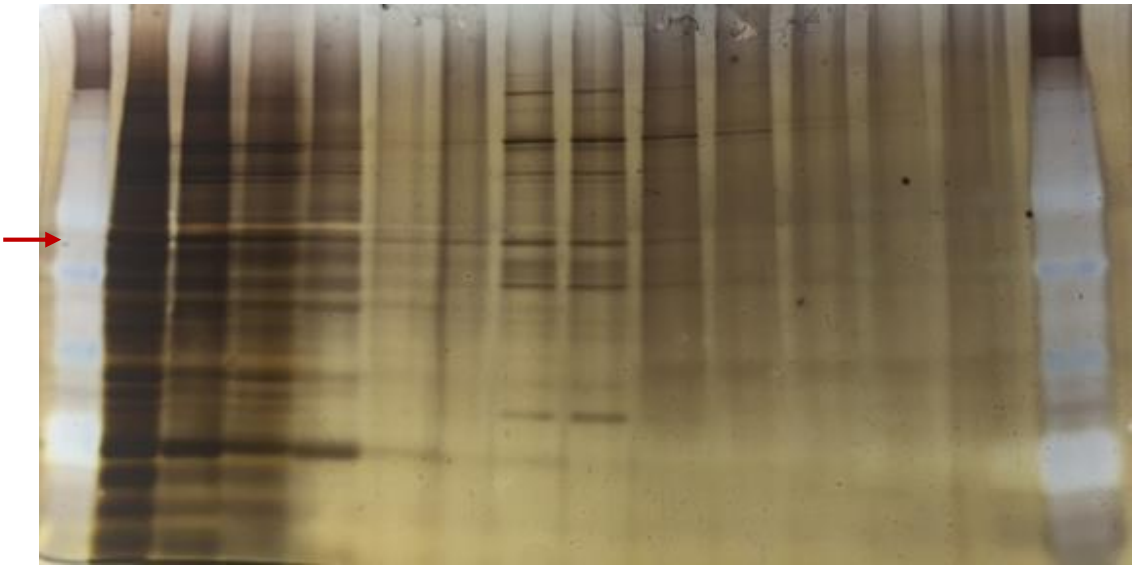


Figure 4: SDS-PAGE gel analysis post sonication. The lanes going from left to right were as follows. Mass marker, original protein sample (supernatant), cell pellet, flow through, wash, elution 1 (E1), E2, E3, E4, E5, E6, E7, E8, E9, mass marker. The red arrow represents 45 kDa. Protein of interest expressed by IPTG is expected to be found in this area of the gel.

Protein bands around 45 kDa are seen in original protein sample, cell pellet, flow through and wash lanes. The bands reappear with good intensity at elution 3-5. When compared to the original protein sample, elution 3-5 contains far less protein bands, thus a more purified solution was achieved.

Aim 2: Digest of Cor protein via treatment with Factor Xa

For further purification of the cor protein, enzyme factor Xa (which is used to cleave cor protein from other associated proteins) was introduced to the elution samples.

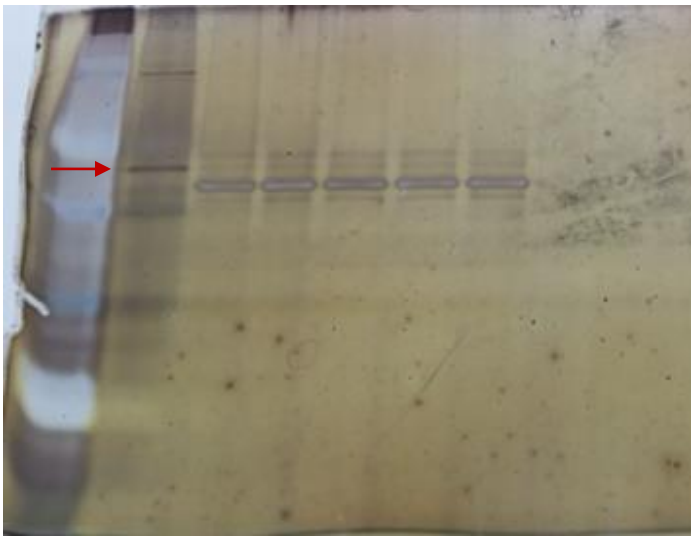


Figure 5: 2 µL of Factor Xa was added to solution of 80 µL of elution 3-5 protein with 318 µL of distilled water. The sample was allowed to incubate at room temperature from 24 hours. The lanes starting from left are mass marker, protein sample with no added Factor Xa (control), Protein sample with Factor Xa incubated for 2 hours, 4 hours, 6 hours, 8 hours and 24 hours. Red arrow represents 45 kDa.

Addition of Factor Xa resulted in formation of white bands. These bands appear as such because the silver used to stain the protein in the gel does not stain these bands. When comparing to the control lane (no added Factor Xa) it is clear that the white bands only appear when Factor Xa is introduced. Furthermore, the intense dark band at 45 kDa that is seen in the control lane is diminished in the lanes with added factor Xa. This may indicate that Factor Xa successfully cleaved the protein, leading to two smaller pieces. One of which is the white band and the other being the Cor protein. Further evidence of this is that the Cor protein is about 4 kDa and the white bands are slightly below 45 kDa. This may imply that the cor protein was successfully cleaved and the white bands are the other cleaved portion. Further analysis will be required to successfully stain the cleaved Cor protein.

The problem with only using this procedure to purify the Cor protein is that cleaved Cor protein is relatively small, which makes it difficult to visualize using common staining procedures. A more reliable pathway towards purification is to purify the non-cleaved Cor protein using a variety of purification methods. Once purification is achieved, then cleavage of the pure Cor protein from the maltose binding protein will result in a pure product with less interfering proteins, which makes cleavage and visualization of the Cor protein a more straightforward process. Methods and results of further purification methods are discussed in the following sections.

Aim 3: Purification via Maltose-binding chromatography:

A Maltose binding chromatography column was used to visualize IPTG induction in a previous step. After induction by IPTG was confirmed, a more selective maltose-binding chromatography procedure was used in order to further purify the protein of interest. For this step of the overall procedure 4 mL of amylose resin was used to pack the column and 3 column volumes of column buffer was run though the column. 800 µl of filtered protein sample was then placed in the column. All fractions were collected in 0.5 mL aliquots. 20 fractions of flow through with elution buffer 1 were collected and stored at 4c. Then 20 fractions of flow through with elution buffer 2 were collected and stored at 4c. Fractions collected were later loaded onto 11% tricine gels and visualized using Commas Blue stain procedure.

Column Buffer (Buffer A):

20mM Tris pH 7.4, 200mM NaCl, 1mM EDTA

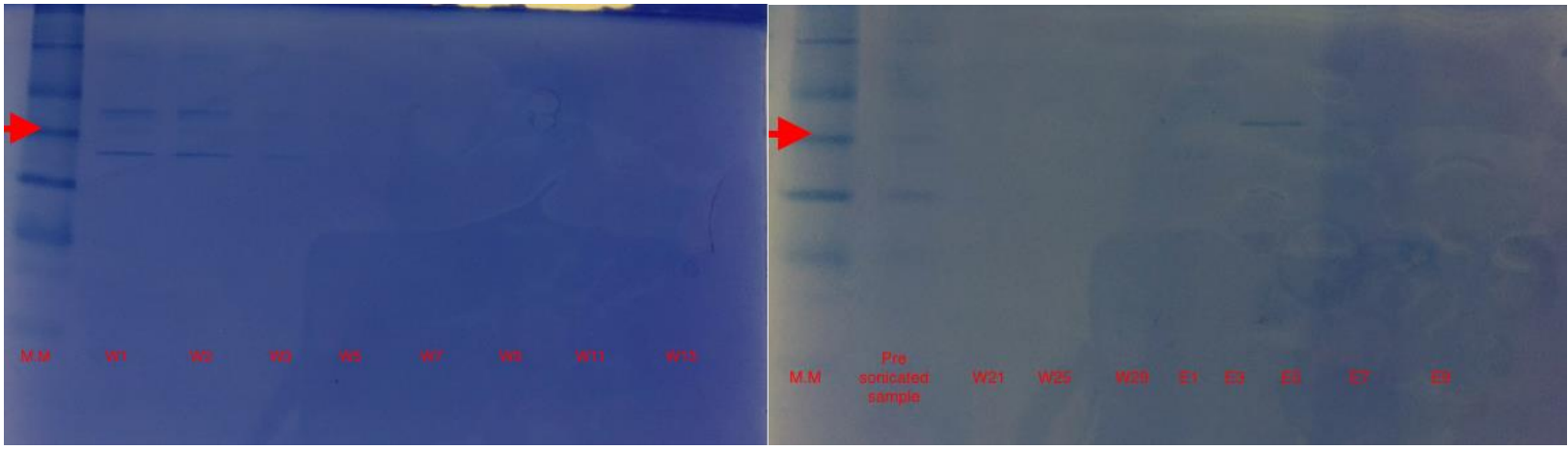
Elution Buffer 1:

Buffer A + 1mM Maltose

Elution Buffer 2:

Buffer A+10mM Maltose

Table 1: List of all buffers used for maltose-binding chromatography. Column buffer contained no maltose, while elution buffers contained increasing levels of maltose. The maltose would competitively bind to the column against the Cor protein. By increasing the amount of maltose between buffers, only proteins with high affinities to bind maltose will remain stuck to the column, removing all other proteins from the column. Eventually, enough maltose is added to remove only the proteins that bind to the column strongly, resulting in collected fractions that are purified.



Figures 6 & 7: SDS-PAGE gel analysis post Maltose-binding chromatography using Coomasie blue stain. The left gel shows protein samples from specific wash fractions. The right gel shows protein samples from later wash fractions and early elution 1 fractions. Red arrows indicate a molecular mass of 46 kDa. Of particular interest is a single, dark band around 46 kDa seen in the E5 lane of the lower gel.

Wash fractions lanes show a decrease in band intensity starting from wash fraction 1 to wash fraction 13. This is result was expected as non-maltose binding proteins in solution would elute out from the column prior to adding in maltose to the column. Lanes with elution fractions show no bands in the E1 or E3 before showing a well defined band in E5 at around 46 kDa. It is important to note that the elution lanes seen above used only elution 1 buffer. All further collected fractions from elution 1 and elution 2 buffers showed no observable bands using Coomasie blue staining. To verify this result, SDS-PAGE gel analysis was replicated. Elution fractions E2-E9 were loaded onto the gel and visualized via silver stain.

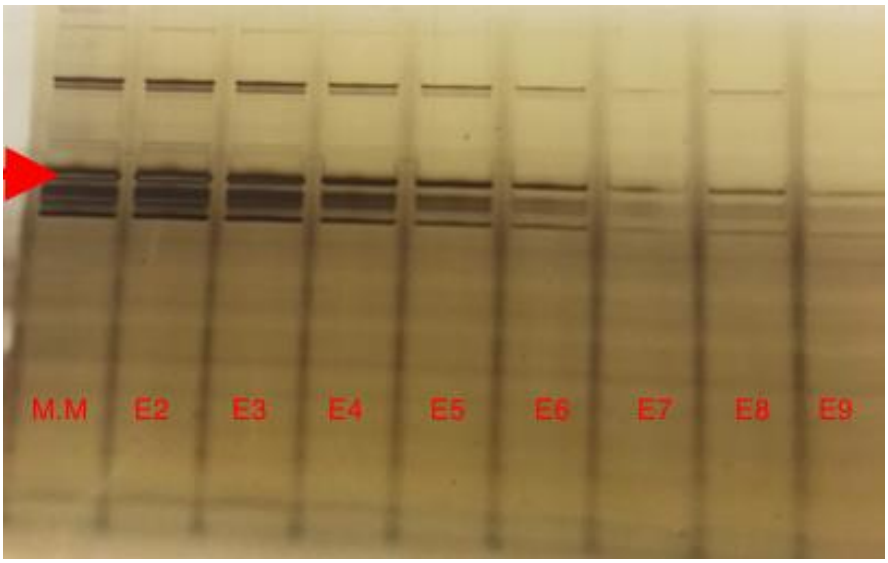


Figure 8: SDS-PAGE gel analysis of elution 1 fraction post maltose-binding chromatography via silver staining. Red arrow represents 46 kDa. Of particular note is elution 5, which shows multiple bands using silver staining procedure but only one visible band using Coomasie blue staining.

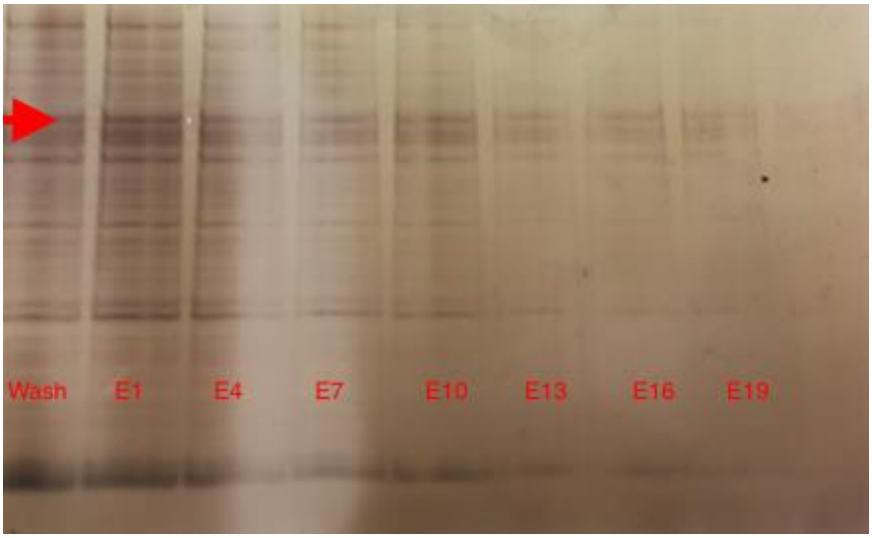
Results of the silver stained gel reveal many protein bands present in the E5 lane as well as in other closely collected fractions. Coomasie blue staining is both a simpler and cheaper staining procedure than silver stain. With many collected fractions, there were many gels to run. For preliminary results, it was believed that Coomasie blue staining would act as a proper way to gauge which collected fractions showed the highest level of purification. Silver stain of E5 shows further purification of the target protein was required.

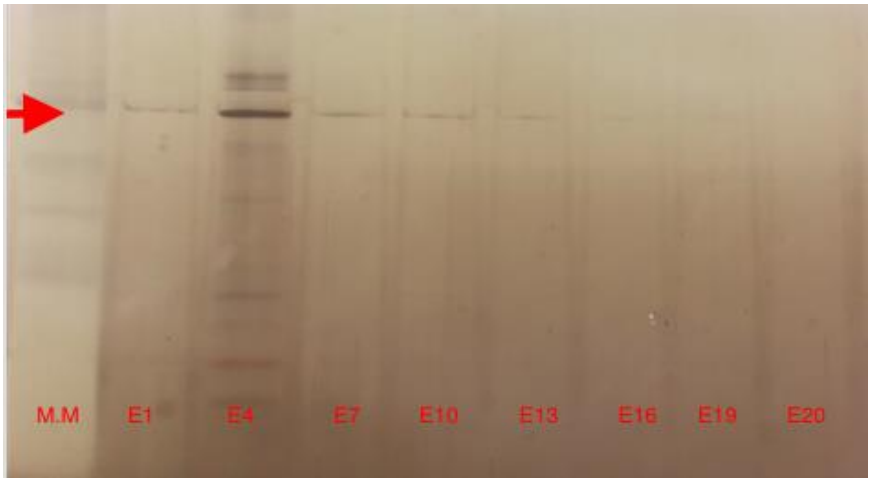
Aim 4: Purification via Nickel-Exchange Chromatography

The studied Cor protein contains a connected chain of 6 histidine amino acids. These histidine molecules (which contain an imidazole side chain) serve a wide array of functions. Of particular interest is their ability to bind Ni^{2+} ions when placed in the appropriate environment. This property allows for the opportunity for further purifications via Nickel-Exchange Chromatography. The same procedure as seen in the maltose-binding chromatography was followed with the following exceptions. First, nickel exchange resin (Specific name of resin) was used instead of amylose resin. Second, buffers found on table 2 were used. 15mL of run through using column buffer was first collected. 20 fractions using elution buffer 1 were collected. Followed by 20 fraction of elution buffer 2, elution buffer 3 and finally elution buffer 4.

Column Buffer (Buffer A)
20mM Tris pH 7.9, 500mM NaCl, 5mM imidazole
Elution Buffer 1
Buffer A + 25mM Maltose
Elution Buffer 2
Buffer A+ 55mM Maltose
Elution Buffer 3
Buffer A + 85 mM imidazole
Elution Buffer 4
Buffer A + 495 mM imidazole

Table 2: List of all buffers used for Nickel-Exchange chromatography. Column buffer contained 5mM imidazole with subsequent elution buffers containing increasing concentrations of imidazole. With a connected 6-histidine chain, to the Cor protein will bind to the resin in the column. A concentration of imidazole higher than what is present in the column buffer is required to elute the protein interest from the column while other proteins are predicted to elute from the column with lower amounts of added imidazole. Using 500mM NaCl and Tris with pH 7.9 aids in ensuring that the majority of the imidazole in solution (both added imidazole and imidazole in the form of a histidine) remain uncharged and thus, capable of binding to nickel.





Figures 9 & 10: SDS-PAGE gel analysis post Nickel-exchange chromatography using silver stain: The top figure shows protein samples form the wash step as well as collected fractions from elution 1. The bottom figure shows protein sample from selected fractions of the elution 2 step. Red arrow indicates 46 kDa. Lane E4 of the bottom figure shows a well defined, dark band around 46 kDa, which is believed to indicate purified Cor protein.

Figure 9 shows a sample of the wash (column buffer fractions) step and subsequent fractions resulting from using elution buffer 1. As more fractions are collected from using elution buffer 1, less and less protein bands are seen. This is the expected result as proteins that have a low affinity to bind Ni^{2+} elute out during this step, and those proteins that have a higher affinity remain on the column. Using Elution buffer 2, E1 shows only faint bands, while E4 shows a few faint bands and one well defined band around 46 kDa. Elution 2 buffer contained 60mM imidazole and this is where the final well defined protein band is seen. Gels of fractions loaded with fraction samples from elution buffers 3 and 4 were run and both showed no protein bands. It is likely that the band seen in lane 4 of Figure 10 is the Cor protein. With this result, it can be reasoned that purification of the Cor protein was achieved during this step.

Conclusion:

Summary:

E. coli cells that have expressed pMAL-p2x-cor-his plasmid are protected from viral infection by bacteriophage virus. The implications of a protein that can protect cells from infection by certain types of viruses calls for further studies of the Cor protein. In order to study the specific structures of the Cor protein that allow for its unique properties, purification of the protein must be achieved. Using IPTG, the Cor protein was expressed in abundance. Using centrifugation, sonication and a 0.45-micron filter, protein samples were processed in a fashion that is conducive to further purification via chromatography columns. Both Maltose-binding chromatography and nickel exchange chromatography utilize physical properties of both the Cor protein and closely associated proteins to purify the Cor protein. This resulted in a final product that was purified and possibly ready for future studies. In addition to these results, an early sample of induced Cor protein was treated with Factor Xa, which showed evidence of cleavage of the Cor protein from its associated protein, leaving only the Cor protein.

Weaknesses of Experiment:

Few if any experiments are fully carried through without a few weak points. In the presented research there are two weaknesses that may cast dispersions on the results. First, the mass marker for silver stain gels were not clearly visible. In person, the protein bands in the marker were more clearly visible than what is represented in the photos. However, the bands were not as distinguishable was would be desired. This is likely due to use of relatively old silver stain marker and possible over loading of protein concentration onto the gel lane.

The second weaknesses is the lack of a pre-sonicated Cor-his protein sample on most of the gel results. This information would allow for a side-by-side comparison of the induced and non-purified Cor to the purified products. Although it is likely that the singular protein band seen in Figure 10 is the Cor protein, a direct comparison to the pre-sonicated protein would help to tell a more complete story.

Future Studies:

Future studies with the Cor protein include treating the purified Cor protein with Factor Xa. Along with more precise staining procedures, it is possible to isolate the 4kDa Cor protein. When this is achieved, protein crystallization can be used to study the structure of the protein via x-ray crystallography and mass spectroscopy methods. This will allow for precise determination of how the protein is structured. And with an understanding of the structure of a protein comes a deeper understanding of the protein's function.

Annotated Bibliography:

Hernandez-Sanchez, J., A. Bautista-Santos, L. Fernandez, R. M. Bermudez-Cruz, A. Uc-Mass, E. Martinez-Penafiel, M. A. Martinez, J. Garcia-Mena, G. Guarneros, and L. Kameyama. 2008. Analysis of some phenotypic traits of feces-borne temperate lambdoid bacteriophages from different immunity groups: a high incidence of cor+, FhuA-dependent phages. Arch. Virol. **153:**1271-1280. doi: 10.1007/s00705-008-0111-0.

One of the more in-depth studies on Cor-FhuA interaction, the work performed in this analysis is a major contributor to the understanding of how Cor functions at the molecular level.

Ivanov, Y. 2012. The role of moron genes in the Escherichia coli enterobacteria phage Phi-80. Ph.D. Dissertation, Bowling Green State University.

In order to study the Cor protein, transformation of Cor genes into a DNA vector and expression of protein via *E.coli* is mandatory. The work described by Yuri Ivanova (a previous graduate student in Dr. Larsen's research lab) is where my specific area of study begins. Yuri describes successful transformation of Cor protein and phenotypes phi-80 characteristics which are important in understanding of cor protein.

Larsen, R.A., Chen, G.J., and K. Postle. 2003. Performance of standard phenotypic assays for TonB activity, as evaluated by varying the relative levels of functional, wild-type TonB. J.Bacteriol. 185:4699-4706.

If not placed under the right environmental conditions, proteins will lose their intended structure, and by extension, their intended function. The work performed in this scientific inquiry aids in the understanding of TonB activity in a biologically active state. The work described is crucial to accurately understanding TonB and the TonB-FhuA complex as a whole.

Noinaj, N., M. Guillier, T. J. Barnard, and S. K. Buchanan. 2010. TonB-dependent transporters: regulation, structure, and function. Annu. Rev. Microbiol. **64:**43-60. doi: 10.1146/annurev.micro.112408.134247.

Seven years after the published findings by Dr. Larsen, Chen and Postle were published, the work of Dr. Noinaj continues the characterization of TonB. This article focuses on the structures and mechanisms of TonB that allow for iron transport across the outer membrane and through the periplasmic space. Findings in this article continue to support a relationship between TonB pathways and bacteriophage activities.

Pawelek, P. D., N. Croteau, C. Ng-Thow-Hing, C. M. Khursigara, N. Moiseeva, M. Allaire, and J. W. Coulton. 2006. Structure of TonB in complex with FhuA, E. coli outer membrane receptor. Science. **312:**1399-1402. doi: 10.1126/science.1128057.

One of the most important research findings pertaining to the understanding of how TonB is associated with FhuA. Work described in this article shows a clear connection between *E.coli* receptors, FhuA and TonB. With an understanding of the pathway, a better understanding of how Cor protein interacts with this pathway and how the Cor protein aids in preventing viral infection can be achieved.

Uc-Mass, A., E. J. Loeza, M. de la Garza, G. Guarneros, J. Hernandez-Sanchez, and L. Kameyama. 2004. An orthologue of the cor gene is involved in the exclusion of temperate lambdoid phages. Evidence that Cor inactivates FhuA receptor functions. Virology. **329:**425-433. doi: 10.1016/j.virol.2004.09.005.

The results of this study phenotypically shows a sharp decrease in FhuA receptor function when exposed to Cor protein. The study utilized Cor mutants to study the effects of wild-type core and the effect of genotype on cor protein activity. In the presence of wild type Cor protein, FhuA was unable to bind adequate amounts of iron and the pathway was shut off. These results help to describe the physical interaction between Cor and FhuA.