HOST PATHOGEN INTERACTIONS: DETERMINING THE ROLE OF THE COXIELLA BURNETII VIRULENCE PROTEIN CINF IN HOST AUTOPHAGY INDUCTION

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ANNA LYNN RODRIGUEZ

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ANNA LYNN RODRIGUEZ

APPROVED:

Dr. Emerson Crabill
Dr. Nicholas Negovetich
Dr. Loren K. Ammerman
Dr. Leslie Kelley

December 2023

APPROVED:

Dr. David Bixler, Dean, College of Graduate Studies and Research
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ABSTRACT

Bioinformatics searches indicated with high degrees of confidence that CinF, a Coxiella burnetii virulence protein, is a fructose-1,6-bisphosphatase (FBP1) enzyme most closely related to the archaeal species, Thermoproteus neutrphilus. However, in this study, I observed a lack of FBP1 enzymatic activity for CinF from C. burnetii in two biochemical assays. I hypothesized that C. burnetii utilizes the bacterial effector protein, CinF, to induce autophagy in the host cell and to recruit and promote fusion with autophagic vesicles to gain the nutrients and components necessary for its proliferation and for expansion of the Coxiella containing vacuole (CCV). Results obtained in this study indicate that despite the lack of metabolic activity expected from bioinformatics, the presence of CinF in HeLa cell culture increased autophagic flux, thus increasing the amount of microtubule-associated protein 1A/1B-light chain 3 (LC3) detected in cells transfected with cinF when compared to mock transfected cells and cells expressing a catalytic mutant.
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INTRODUCTION

Q-fever is a zoonotic disease caused by the bacterial pathogen *Coxiella burnetii* (1,2,4). The symptoms of Q-fever in humans can be mild, resembling those of the flu, but in some cases, it can persist long after the initial infection, causing chronic Q-fever (1,2). This chronic condition may result in a life-threatening illness such as endocarditis, hepatitis, or encephalitis (1). Q-fever is common in ruminants such as sheep and goats, but it also occurs in other livestock (3,4). The death of livestock from spontaneous abortions associated with the disease can cause significant economic losses and is an ongoing problem for pastoralists (3,4). Q-fever is a major concern around the world, with well-documented outbreaks in the Netherlands between 2007 and 2010, and in the northwestern United States in 2011 (3,4). The number of cases reported is thought to be grossly understated because of the lack of surveillance and access to testing in developing regions (5).

Most commonly, Q-fever can be acquired by direct contact with the birthing fluid or placenta of infected livestock (3,4). This poses the greatest threat to farm workers and those who process livestock for meat. Q-fever can also be acquired by inhalation of airborne particles, namely dust from farms. Infected particulate matter carried by the wind can expose close neighbors of a farm and nearby towns or villages, and as little as one bacterium can cause disease (3,4). For this reason, *C. burnetii* is classified by the CDC as a Category B agent for its potential use as an agent of bioterrorism (1,3,4,6).

*Coxiella burnetii* can lie dormant in the environment for long periods of time, making
it a formidable opponent to combat (3,4). This is due in part to the ability of *C. burnetii* to alternate between a less metabolically active, but highly infectious small cell variant, and a larger metabolically active and replicative large cell variant. The small cell variant is able to persist in the environment, and is resistant to drying and UV light, conditions which would normally be detrimental to a bacterium (7). Persistence in the environment increases the probability that the infectious agent can be picked up and distributed via the wind (3,4). While both variants are infectious, the small cell variant appears to be significantly more infectious (2).

*Coxiella burnetii* is an obligate intracellular organism with a unique ability to thrive in the harsh environment of the lysosomal vacuole of a host cell (2). *C. burnetii* infects alveolar macrophages in both humans and livestock. Once occupied by the pathogen, the vacuole is referred to as a *Coxiella*-containing vacuole, or CCV (2). *Coxiella burnetii* enters the cell in an endosome, and passively traverses the normal endosomal pathway, allowing autophagosomal fusion, followed by lysosomal fusion (9). Lysosomes are acidic and proteolytic membrane bound vacuolar compartments that have evolved in eukaryotes as part of the innate immune system and function in clearance of intracellular bacteria (8). The function of the lysosome in clearance of bacteria is most apparent when dealing with nonpathogenic bacterial species, which are easily cleared by the immune response. However, successful pathogens have evolved mechanisms to overcome this system in order to persist.

Autophagy is an evolutionarily conserved process utilized by cells for different purposes. The best characterized way a cell uses this process is as a house keeping
mechanism, whereby old or damaged cellular components are engulfed and degraded (11-14). Their component parts are then utilized for production of new protein or for nutrients to power cellular processes (11-17). Autophagy can be induced by a variety of mechanisms including amino acid starvation, depletion of energy, and by signals from the immune system, among others (11). When a cell is starved, the autophagic pathway can recycle its cellular proteins by breaking them down into their component parts (11,12). Those parts can then be used as a source of nutrients until conditions improve (11).

After invasion of a host cell, intracellular bacteria find themselves in an environment where potential sources of nutrients are tied up in macromolecules, or in fully assembled proteins (11). To be successful, bacteria must develop strategies for accessing those materials in order to utilize them as a source of nutrients (11). Frequently, bacteria evolve elaborate strategies for inhibiting or inducing autophagy for their own benefit (11-13). This subversion of the system is beneficial to the pathogen and is usually harmful to the host.

Two bacteria that employ such strategies are Legionella pneumophila and Chlamydia trachomatis. Legionella pneumophila hijacks the host endoplasmic reticulum (ER) and Golgi secretory pathways by use of type IV secretion system effector proteins. Vesicles transiting these networks emerge containing lipids and proteins L. pneumophila can use as nutrients (14). Chlamydia trachomatis utilizes a type III secretion system for biogenesis of its inclusion body, which is the membrane-bound compartment in which C. trachomatis resides during infection. Chlamydia trachomatis recruits Rab GTPases to the inclusion membrane. Rab GTPases are proteins that are involved in the sorting and delivery of cargo emerging from the Golgi, and C. trachomatis utilizes them to support further expansion of
the inclusion by facilitating the acquisition of iron and other essential nutrients needed for replication (14). Similarly, it is known that *C. burnetii* benefits from host autophagy. In addition to the CCV being decorated with microtubule-associated protein 1A/1B-light chain 3 (LC3) during infection, Gutierrez et al. showed that chemical inhibition of autophagy blocks the normal biogenesis of the CCV, and chemical induction of autophagy increases CCV size and intracellular replication (15,16). LC3 are proteins involved in autophagosomal formation and are members of the Atg8 family of proteins (20).

An additional function of the autophagic pathway in host cells is the clearance of intracellular pathogens, termed xenophagy (Figure 1). In response, intracellular pathogens must evolve strategies for surviving the host’s immune response, and subversion of the autophagic pathway is many times an option (11,13,17). Intracellular bacterial pathogens have evolved elaborate mechanisms to avoid fusion of the endosome with the lysosome. One such bacterium is *Francisella tularensis*. This pathogen enters the cell via an endosome, but immediately escapes it by degrading the membrane. Replication then occurs in the cytosol of the host cell (18). *Legionella pneumophila*, a bacterium closely related to *C. burnetii*, has evolved the ability to disguise itself as an endoplasmic reticulum (ER)-derived vacuolar compartment by fusing with the ER membrane (19). By no longer looking like an endosomal vacuole, *Legionella* avoids lysosomal fusion. Another intracellular bacterium, *Mycobacterium tuberculosis*, arrests phagosomal maturation, thus avoiding acidification of its vacuolar compartment (20). *Coxiella burnetii* is unique in that it passively allows fusion with the lysosome, which acidifies its environment (Figure 2).
**Figure 1:** Graphical representation adapted from Choi et al. of xenophagy depicting the outcome for a non-pathogen (21).

**Figure 2:** Graphical representation adapted from a review by van Schaik et al. of the autophagic pathway comparing a typical *C. burnetii* infection in humans, to an infection by a non-pathogenic bacterium (22).

Fusion with the lysosome and acidification of the vacuole triggers the conversion of *C. burnetii* to its metabolically active large cell variant (10). After acidification and conversion to the large cell variant, *C. burnetii* turns on the genes it needs for production of its virulence factors. Some bacteria utilize specialized secretion systems to translocate...
bacterial proteins from the bacterial cytosol into the host cell cytosol. *Coxiella burnetii* employs a Dot/Icm type IVB secretion system as its main virulence factor and the system is required for pathogenicity (2). It uses this system to inject bacterial effector proteins into the cytosol of the host cell (2). These effector proteins are capable of manipulating the host cell in a way that allows *C. burnetii* to replicate efficiently and avoid detection by the host immune system, while also supporting expansion of the CCV for its rapid replication (2,3). *Coxiella burnetii* is known to translocate more than 130 effector proteins into the host cell, many of which play an important role in virulence (2).

The *C. burnetii* effector protein CvpB is reported by Wang et al. to play a role in manipulating host immune responses, which allows the bacterium to establish its replicative niche. CvpB also plays a vital role in enabling fusion of the CCV with host autophagosomes (23), thereby providing material for expansion of the CCV. An additionally well characterized effector protein employed by *C. burnetii* is CvpF. CvpF interacts with small GTPases involved in organizing cellular trafficking, which leads to the recruitment of LC3 to the CCV membrane and signals an upregulation of the hosts autophagy machinery (23). One additional effector protein encoded by a *C. burnetii* plasmid, CpeB, was found by researchers on the CCV membrane and was shown to interact with LC3 and Rab11a (23). Rab11a is known to be involved in membrane trafficking in the autophagy pathway. Researchers concluded that mutants of each of these effector proteins resulted in reduced replication, multi-vacuolar phenotypes instead of one large CCV, and a decrease in detectable LC3, which indicates their importance in *C. burnetii* virulence (23).
The effector protein employed by *C. burnetii* in this study is the protein, CinF (2). Results from Crabill et al. (2018) indicated that a cinF mutant severely reduced fusion with autophagosomes compared to wild type infections, and that the cinF mutant resulted in small CCVs with significant replication defects (2). Further, the CCVs that were formed by the cinF mutant were missing markers associated with autophagosomal fusion, namely LC3 (2).

Based on these facts, I hypothesized that CinF functions in the autophagic pathway, and operates by promoting the host cell’s normal autophagy mechanisms, resulting in more autophagic vesicles for the CCV to fuse with. This in turn would provide the components necessary for the replication and proliferation of *C. burnetii* within the vacuole. CCVs are highly fusogenic, interacting with many organelles in the endocytic pathway (20), and multiple fusion events provide nutrients for the bacterium to grow and replicate. It also provides the materials necessary for the formation of a spacious environment, within which *C. burnetii* is able to replicate to high numbers (20). This proposed role of CinF would explain why cinF mutants are noticeably smaller in size and have significantly reduced replication (2). During fusion with host autophagic vesicles, CCVs become decorated with LC3 (25), and a lack of fusion events would explain why cinF mutants are reported to lack LC3 markers (2, 25).

Bioinformatic results predicted that CinF is a fructose-1,6-bisphosphatase (FBP1) that is most closely related to a fructose-1,6-bisphosphate aldolase/phosphatase (FBPA) known to exist in the archaenal species *Sulfolobus takodaii* and *Thermoproteus neutrophilus* (25). FBP1 is an enzyme that promotes gluconeogenesis, a process known to contribute to an upregulation of autophagy and is the reverse reaction of glycolysis (26). FBP1 enzymes
remove a phosphate from fructose 1,6-bisphosphate, producing fructose 6-phosphate (Figure 3). This process is identical to the catalytic step in gluconeogenesis (26). Autophagy and gluconeogenesis occur at the same time, and both are regulated by the same transcription factor (25). Further, autophagy and gluconeogenesis both occur during times of starvation, and glycolysis and gluconeogenesis are regulated by the interconversion of fructose1,6-bisphosphate to fructose 6-phosphate (26).

Based on this knowledge, I hypothesized that CinF is a functioning fructose 1,6-bisphosphate aldolase/phosphatase enzyme. I further hypothesized that C. burnetii utilizes CinF as a bacterial effector protein to induce autophagy in the host cell to recruit and promote fusion with autophagic vesicles in order to gain the nutrients and components necessary for its proliferation and for expansion of the CCV. Here I show that while CinF may no longer possess its predicted enzymatic activity, at least in vitro, expression of CinF in cells is sufficient to induce autophagy.
Figure 3: A partial graphical representation of the reversible reaction for glycolysis and gluconeogenesis. Along the glycolysis pathway, the graphic begins with Fructose 6-phosphate and ends at the catalytic step performed by Aldolase, which results in the production of Glyceraldehyde 3-phosphate and Dihydroxyacetone phosphate. This represents the portion of the gluconeogenesis pathway that CinF would be involved with if it had its predicted enzymatic activity, specifically the steps catalyzed by fructose 1,6 bisphosphatase and aldolase.
RESULTS

Results of bioinformatic searches

The C. burnetii genome has been fully annotated and CinF is annotated as a fructose 1,6 bisphosphatase in the National Center for Biotechnology Information (NCBI) database (35). An online platform for analyzing and conducting bioinformatics searches, HHpred, returned query results with a one hundred percent probability (e-value 5.8e-132) that the sequence submitted for analysis was a fructose-1,6 bisphosphate aldolase/phosphatase most closely related to the archaeal species Thermoproteus neutrophilus. It further indicated a one hundred percent probability (e-value 7.2e-132) that the sequence was homologous to ST0318, a protein with a fructose -1,6-bisphosphatase-like fold, modeled from the archaeal species Sulfolobus tokodaii (Figure 4a).

An additional search on the online platform Phyre² indicated with one hundred percent confidence and ninety-three percent coverage of the submitted sequence, that the protein is a fructose-1,6-bisphosphate aldolase/phosphatase modeled from the archaeal species Thermoproteus neutrophilus. Phyre² further indicated with one hundred percent confidence that the protein has a fold consistent with a fructose 1,6-bisphosphatase modeled from the archaeal species Sulfolobus tokodaii (Figure 4b).

I wanted to determine if CinF had the predicted enzymatic activity of an FBP1 enzyme in vitro. To do this, CinF was purified from E. coli using a maltose binding protein (MBP) tag epitope to bind to amylose resin on a gravity column. Samples were eluted from the resin using an elution buffer containing maltose. Four elutions were collected, E1-E4.
Figure 4: Bioinformatics predicts CinF to be a FBP1/Aldolase enzyme. Bioinformatics output for sequence alignments, analysis, and model predictions for cinF sequences obtained from NCBI, and entered into HHPred and Phyre².

Results of CinF biochemical assays.

A commercially available, colorimetric, FBP1 assay kit was utilized to measure the enzymatic activity of the protein I purified. Purified protein from elution samples E1 and E2 consistently tracked with the negative control over the entire time course of the experiment (Figure 5), and indicated no phosphatase activity occurred after the addition of the purified...
FBP1, thus, no conversion of fructose-1,6-bisphosphate to fructose-6-phosphate was detected.

**Figure 5:** *In vitro* assays fail to show FBP1 activity for CinF. The results of the fructose 1,6-bisphosphatase activity assay kit using the change in absorbance detected at 450nm, in a Tecan, SparkCyto plate reader. E1 and E2 represent elutions from the purification of CinF-MBP. Samples E1 and E2 compared to a positive and a negative control provided in the kit.

Similarly, a colorimetric aldolase activity assay kit was utilized to detect the conversion of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone after the addition of purified FBP1. Purified protein from samples E1 and E2 consistently tracked below the negative control in this experiment, while the positive control steadily trended upward (Figure 6). These results indicated the absence of aldolase activity, and thus no conversion was detected of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone.

**Results of expression of wild type cinF in HeLa cell culture.**
Because it was previously determined that cinF mutant infected CCVs did not readily fuse with autophagic vesicles, I next wanted to determine if expression of CinF by transient transfection in HeLa cells was sufficient to induce autophagy. To investigate the role of

![Figure 6](image.png)

**Figure 6:** *In vitro* assays fail to show aldolase activity for CinF. The results of the aldolase activity assay kit using the change in absorbance detected at 450nm, in a Tecan, SparkCyto plate reader. E1 and E2 represent elutions from the purification of CinF-MBP. Samples E1 and E2 are compared to a positive and a negative control provided in the kit.

CinF in inducing autophagy, HeLa cells were transiently transfected with either wild type CinF or with a version of the protein containing an introduced mutation at the catalytic site, Y362A. Bafilomycin was added to block the fusion of autophagosomes with lysosomes, thus, preventing the degradation of accumulated LC3.

By use of immunoblotting, I first determined there was an increase in the presence of LC3, a marker widely accepted as an indicator of autophagosomal formation (14,15), in HeLa cells transfected with wild type cinF (Figure 7). This suggested there was an increase in cellular autophagy in transfected cells when compared to mock transfected cells (Figure 7). However, because autophagy is a degradative process, the increase observed might also
have been caused by a blockage of autophagic degradation. If the amount of LC3 increased with the addition of lysosomal inhibitors, this would confirm that autophagy had been upregulated.

Figure 7: Expression of CinF-Myc increases LC3 levels in HeLa cells. Photographs of Western blots utilizing LC3, c-Myc, and beta-Actin rabbit antibodies as primary antibodies. All photographs include a control in lane 1, which was mock transfected in HeLa cells, and Lane 2 was transfected with wild type cinF. LC3 antibodies show the amount of native LC3 expression, c-Myc antibodies depict expression of CinF-Myc expression for transfected cells, and Actin is used as a loading control to show relative amounts of total cell lysate used. 7b. Bar graph of an Image J analysis of LC3 relative to actin in each lane of a Western blot utilizing LC3 as primary antibody.

The c-Myc Western blot was conducted to determine if the transfection was effective. The transfection was determined to be successful, as the band only appeared in cells transfected with wild type cinF, and not in mock transfected cells (Figure 7 and 8). A beta-
actin Western blot was conducted as a loading control to ensure uniform loading of protein across the gel, and was also considered to be successful (Figure 7 and 8).

**Results of expression after incubation with bafilomycin.**

The conversion of LC3-I to LC3-II by conjugation to phosphatidylethanolamine (PE) is by many considered the standard by which autophagy is monitored (34). The lipophilic properties of the PE group facilitate its insertion into the membrane of autophagosomes. Later in the process of endosomal maturation, LC3-II is degraded when autophagosomes fuse with lysosomes (34). Treatment with bafilomycin blocks the fusion of autophagosomes and lysosomes, thus, preventing the degradation of LC3-II (34). To halt the degradation of LC3-II, cells were incubated with bafilomycin prior to lysis.

Bafilomycin was added to each of three HeLa cell culture plates at approximately twenty-six hours post transfection. Each culture dish was incubated for four hours, and cell lysis was performed at thirty hours post transfection. A western blot indicated a substantial increase in the amount of LC3 detected for transiently transfected cells in the wild type (B+WT) and mutant (B+Mut) plates incubated with bafilomycin when compared to untreated plates (Figure 8). This result suggested that *cinF* expression caused an increase in autophagosomal biogenesis. This was made more evident thanks to the decrease in degradation of LC3 when bafilomycin was added. The control samples for both the bafilomycin treated (B+Ctrl) and untreated (B-Ctrl) plates showed similar levels of LC3 confirming that the increase observed was specific to CinF (Figure 8).
Results of expression of cinF and a Y362A mutant in HeLa cell culture.

To determine if the upregulation of autophagy was the result of CinF and not an unpredicted secondary function, a point mutation was introduced at nucleotide 362, which was previously determined to be the catalytic site of the FBP1 enzyme (26,35). The introduction of this mutation was intended to render the enzyme non-functional by altering its catalytic site. Western blots were performed and indicated that in samples not incubated with bafilomycin (B-Mut), LC3 levels were less in the mutant than both the control (B-Ctrl) and wild type (B-WT) (Figure 8). Cells incubated with bafilomycin showed less expression of the mutant phenotype (B+Mut) when compared to wild type (B+WT) samples, but still more than the control (B+Ctrl) (Figure 8).
Figure 8: Autophagic flux is induced by expression of CinF-Myc in HeLa cells. 8a. Photographs of Western blots utilizing LC3, c-Myc, and beta-actin antibodies as primary antibodies. LC3 antibodies show the amount of native LC3 expression, c-Myc antibodies depict expression of CinF-Myc expression for transfected cells, and Actin is used as a loading control to show relative amounts of total cell lysate uses. B- indicates no bafilomycin, and B+ indicates bafilomycin treatment. Mock are mock transfected, cinF were transfected with wild type cinF, and Y362A were transfected with cinF a catalytic mutant. 8b. Bar graph of an Image J analysis of LC3 relative to actin in each lane of a Western blot utilizing LC3 as primary antibody.
DISCUSSION

The intracellular pathogen *C. burnetii* utilizes host autophagy to its advantage during infection. Here I showed that one of the type IV effector proteins involved in this process is CinF. Based on the increased levels of LC3 detected, CinF is believed to increase host autophagy. These findings are consistent with previous studies that determined a *cinF* mutant had markedly decreased fusogenicity with autophagosomes (2). My data indicated that the expression of CinF is sufficient to induce an increase in autphagic vesicles as measured by increased levels of LC3 detected. In infections with the *cinF* mutant, the CCVs formed experienced fewer fusion events with autophagosomes and therefore, less LC3 decorating the vacuole. This suggests that CinF functions to induce host autophagy leading to an increase in CCV fusion events with autophagosomes, which provides the nutrients and membrane components necessary for the proliferation of *C. burnetii*, and expansion of the CCV.

The results of the bioinformatics searches indicated with high degrees of confidence that CinF was a fructose-1,6-bisphosphatase enzyme. Taken together with the data from the biochemical assays conducted in this study, the results suggested that an ancestrally derived enzyme (FBP1) is no longer a functioning enzyme used in metabolism. Zhang et al. (2021), also reported a lack of enzymatic activity by FBP1 in their study (26). However, the results obtained here and by Zhang et al. (2021) were both recorded *in vitro* (26). The results may be different if taken *in vivo* for a few reasons. Additionally, while Zhang et al. had evidence of phosphatase activity, it was still dependent on the catalytic residue predicted for FBP1 activity (26).
In this study, the expression of CinF was IPTG-induced in *E. coli* cells, and then column purified for use in biochemical assays. It is possible that *E. coli* does not contain a particular factor or enzyme activator needed to produce a fully functional enzyme. It is also possible some type of necessary post translational modification did not occur in *E. coli* or in HeLa cells that would be observed in a wild type *C. burnetii* infection. All of these things would result in a lack of detectable enzymatic activity.

An example of a post translational modification made to a bacterial effector protein by the host is the *Pseudomonas syringae* effector, AvrPto. Upon entry, the host cell phosphorylates two serine residues on the terminal domain, then activating the effector protein contributing to its virulence in the cell (38). One additional example of necessary post translational modifications by the host is the *Chlamydia trachomatis* effector protein, Tarp. Tarp requires tyrosine phosphorylation for activation, and leads to an increased number of interactions with host machinery (38).

Zhang et al. (2021) investigated the role of CinF in the inhibition of NF-κB signaling, rather than its role in autophagy (26). It has been established that bacterial effector proteins can function in multiple pathways simultaneously (23). The Zhang research group’s focus remained on NF-κB signaling, while my focus was on the upregulation of autophagy in the presence of CinF. Further research will be needed to determine how the NF-κB and autophagy phenotypes are related.

To investigate the role of CinF in inducing autophagy, HeLa cells were transiently transfected with either wild type CinF or with a version of the protein containing an
introduced mutation at the catalytic site, Y362A. Bafilomycin was added to block the fusion of autophagosomes with lysosomes, thus, preventing the degradation of accumulated LC3, and the results clearly showed the treatment was effective. The LC3 bands from the CinF transfected sample on the western blot associated with bafilomycin treatment were noticeably darker, and an Image J analysis indicated the presence of substantially more protein when compared to untreated samples (Figure 8). The use of bafilomycin provided some clarity in evaluating the extent of the upregulation in autophagy by CinF.

The results for the Y362A mutant were not as clear. In plates treated with bafilomycin, the amount of protein for samples taken from plates transfected with the catalytic mutant was less than the wild type CinF transfected cells but still noticeably more than mock transfected cells (Figure 4). This indicates the catalytic site may be dispensable, or it may display promiscuous substrate binding, which would enable it to function in the absence of the mutated portion (35). Another explanation for this phenomenon may be the FBP1 enzyme has in fact evolved to function in another pathway and is no longer participating in metabolism. This is supported by the results of the biochemical assays conducted in this study.

The difference in LC3 expression in mock transfected cells and in wild type samples is obvious but somewhat subtle. The results of transient transfections are limited by the efficiency of the transfection. This means not all the cells in a cell culture plate receive the plasmid containing the gene of interest and are therefore equivalent to the mock transfected cells. There are many factors affecting transfection efficiencies, and while I tried to control for as many as possible, it is difficult to tell how many of the cells actually received the gene
encoding CinF. This may explain the muted differences observed between the wild type and control samples when examining LC3 levels. A less than optimal transfection rate may have played a part in the differences observed for the mutant phenotype as well, especially in the bafilomycin untreated cell culture plates. Further replicates of these tests may provide a clarity.

In conducting basic biological studies, like this one, a better understanding of the mechanisms of disease and cellular biology emerge. This research has the potential to uncover new targets for drug and vaccine therapies. It also has the potential to improve the lives of not only the animals who are most affected, but also human lives. Autophagy is ubiquitous across all cell types in multicellular organisms, and dysregulation of the process, whether induced by a pathogen or by cancer, or by any number of other processes, places the organism at a disadvantage. This study reveals another piece of the autophagy puzzle.
Table 1: Primer sequences designed and used in this study.

<table>
<thead>
<tr>
<th>Sequence ID</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>513 pcdna3.1 Kozak</td>
<td>5'-CAC CAA GCT TGC CGC CAC CAT GGA AAT TAC CCT GAG CGC G-3'</td>
</tr>
<tr>
<td>513 PCDNA3.1 R</td>
<td>5'-CAC CGG ATC CGC CGT TTT TGC GCA GTT C-3'</td>
</tr>
<tr>
<td>Y362A F</td>
<td>5'-GAA GCG GAA ATT GCG GCT ACC GGC CTG GTG GAT ACC-3'</td>
</tr>
<tr>
<td>Y362A R</td>
<td>5'-GGT ATC CAC CAG GCC GGT AGC CGC AAT TTC CGC TTC-3'</td>
</tr>
</tbody>
</table>

Table 2: Plasmid vectors.

<table>
<thead>
<tr>
<th>Plasmid Vectors</th>
<th>Use</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA 3.1 Expression Vector</td>
<td>Mammalian Expression Vector</td>
<td>Ampicillin resistance gene, CMV promoter, restriction enzyme cloning</td>
</tr>
<tr>
<td>pMAL-p5X</td>
<td>Bacterial Expression Vector</td>
<td>Ampicillin resistance gene, encodes maltose-binding tag, restriction enzyme cloning</td>
</tr>
</tbody>
</table>
METHODS

Bioinformatics.

Bioinformatics tests were conducted, including protein homology and multiple sequence alignments, using two different platforms, HHpred (30) and Phyre2 (32), for the cinF gene sequence from *C. burnetii* (Accession # WP_012570289.1).

Expression of cinF in eukaryotic cells.

The cinF gene sequence was originally obtained from NCBI. The cinF sequence was submitted to and ordered from Genewiz (Genewiz.com). The cinF gene was cloned into the eukaryotic expression vector pcDNA 3.1 (+)(B) (Thermo Scientific, Waltham, MA) and maintained in DH5α *E. coli* cells. The gene was then expressed in HeLa cells (Mirus, Marietta, GA), which allowed the ability to test for the upregulation of cellular autophagy in eukaryotic cells by Western blot detection of LC3.

Point mutation of the catalytic site of the FBP1 enzyme.

Amino acid 362 was identified by bioinformatics searches (30) to be the FBP1 enzyme catalytic residue. Primers were designed (Table 1) with a point mutation at amino acid 362, which changed a tyrosine to an alanine, rendering the catalytic site non-functional (IDT, Coralville, IA).

Polymerase chain reaction (PCR) was performed on the cinF gene using the Y362A primers (Table 1) according to a protocol developed by Korbie and Mattick (2008) (33), with slight modification to include using Pfu Turbo DNA polymerase (Agilent, Santa Clara, CA) and its associated buffer in the reaction mix. Phase 1 thermocycling parameters were set to perform touchdown PCR (32) with an initial annealing temperature of 81°C. The temperature
is then set to decrease by one degree each cycle, for ten cycles. The phase 2 thermocycling protocol was performed according to the Quick-Change II Site Directed Mutagenesis Kit instructional manual (Agilent, Santa Clara, CA), which introduced and amplified the desired mutation.

The restriction enzyme, DpnI, was utilized to degrade the *E. coli* derived, methylated parental DNA template strand and to select for the newly synthesized strands (NEB, Ipswich, MA). The newly synthesized plasmid DNA was then transformed into DH5α *E. coli* cells (AddGene, Watertown, MA), and grown on lysogeny broth agar plates containing ampicillin (1µl/mL).

**Transfection of HeLa cells, bafilomycin treatment, and sample preparation for immunoassays**

Ten-centimeter tissue plates were used to culture HeLa 229 cells (ATCC, Manassas, VA) from stock, according to the manufacturer’s guidelines. Once the newly seeded plates reached approximately 90% confluency, the Mirus TransIT-LT1 plasmid DNA transfection protocol was completed according to manufacturer’s guidelines (Mirus, Marietta, GA), using either cinF or Y362A miniprepped plasmid DNA.

After transfection, cell culture plates were allowed to grow as per usual for twenty-six hours. After which time, cells were incubated with 1µl of 100nM bafilomycin per ten-centimeter culture plate (35). Cell lysis was performed at thirty hours post transfection using RIPA buffer according to the manufacturer’s protocol (Thermo Scientific, Waltham, MA).

A sodium dodecyl sulfate polyacrylamide gel (SDS-page gel) (Bio-Rad, Hercules, CA) was run according to a Bio-Rad protocol (Biorad.com/Bulletin_6040). Then, a
polyvinylidene difluoride membrane (PVDF) was utilized to electrophoretically transfer protein from the gel to the membrane for blotting (Biorad.com/Bulletin_6040).

**Immunoassay utilization.**

Western blots were performed (Cell Signaling Technologies, Danvers, MA). Primary antibodies used included LC3B rabbit antibodies (Cell Signaling Technologies, Danvers, MA) used to test for the presence of LC3 which signals autophagy, beta-Actin (13E5) rabbit antibodies (Cell Signaling Technologies, Danvers, MA) used as a loading control to ensure uniform application of protein across the membrane, and c-Myc antibodies (Cell Signaling Technologies, Danvers, MA) were used to determine if the transfection worked. Goat anti-rabbit IgG (H+L) antibodies conjugated to horse radish peroxidase (Thermo Scientific, Waltham, MA) were used as secondary antibodies in the protocol. The utilization of Pierce 1-step Ultra TMB blotting solution (Fisher Scientific, Waltham, MA) allowed visualization of the blot.

**Determining the function of CinF utilizing a biochemical assay.**

*Coxiella burnetii* has a genome with a low GC content (37). Having a low GC content can significantly decrease the amount of protein produced when expressed in a different host, and in order to maximize the production of protein when the *cinF* sequence was cloned into and expressed in *E. coli*, the *cinF* gene was first codon optimized. This was accomplished by inputting the *cinF* sequence into Genewiz (genewiz.com), which uses an artificial intelligence algorithm to optimize the sequence for translational efficiency when expressed in *E. coli*. 
The codon optimized cinF gene was cloned into a pMAL-5X vector (AddGene, Watertown, MA) maintained in BL21 E. coli cells (NEB, Ipswich, MA). Production of the protein was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG) (100µl/mL) (Millipore Sigma, Burlington, MA). The protein was purified by use of a maltose binding tag and an amylose-agarose gel column (NEB, Ipswich, MA). In order to confirm the enzymatic activity of the CinF protein, a fructose 1,6-bisphosphatase assay was conducted using the column purified protein (30), and a fructose 1,6-bisphosphatase activity kit (Abcam, Waltham, MA) according to the manufacturer’s guidelines. Additionally, an aldolase assay was completed by use of an aldolase activity assay kit (Abcam, Waltham, MA) according to the manufacturer’s guidelines. Both assays were conducted at 37°C, and were analyzed in real time using a TECAN, Spark Cyto plate reader.
REFERENCES


7. Sandoz KM, Popham DL, Beare PA, Sturdevant DE, Hansen B, Nair V, Heinzen RA, 2016. Transcriptional profiling of Coxiiella burnetii reveals extensive cell wall...


30. Say RF, Fuchs G. 2010. Fructose 1,6-bisphosphate aldolase/phosphatase may be an ancestral gluconeogenic enzyme. Nature 464: 1077-81. doi: 10.1038/nature08884


APPENDIX A

IBC Approval Letters

Institutional Biosafety Committee

9/6/2022

Dr. Emerson Crabill & Ms. Anna Rodriguez
Department of Biology, College of Science & Engineering
Angelo State University
San Angelo, TX 76909

Dear Dr. Crabill & Ms. Rodriguez,

The proposed project submitted by you titled, "Host Pathogen Interactions: Identity and Function of Cbu0513" has been approved by the Institutional Biosafety Committee. The research is considered BSL-2 and will be performed in room 005 in the Cavness building on the ASU campus.

The approval is effective beginning September 2, 2022. Please be aware that the protocol will expire one year from its original approval date. If the study will continue beyond that date, you must submit a request for continuation before the current protocol expires. In addition, any protocol changes must be resubmitted and approved by the IBC.

The approved addendum is for protocol #IBC-ROD-090222. Please include this number in the subject line of all future communications with the IBC regarding the protocol.

Sincerely,

[Signature]

Sam Spooner
Co-Chair, Institutional Biosafety Committee
08/21/2023

Dr. Emerson Crablll & Ms. Anna Rodriguez
Department of Biology, College of Science & Engineering
Angelo State University
San Angelo, TX 76909

Dear Dr. Crabilll & Ms. Rodriguez,

The proposed research project falling under #IBC-ROD-090222 submitted by you titled “Host-Pathogen Interactions Identify and Functional CBU 0513” was approved by the Institutional Biosafety Committee for continuance on August 17, 2023, at the virtual WebEx meeting. The research is considered BSL-2 and will be performed in your laboratory in rooms 001A/005 in the Cavness building on the ASU campus.

The continuance is effective beginning 090223. Please be aware that the research protocols will expire one year from this continuance date. If the research will continue beyond that date, you must submit a request for continuation before the research protocol expires. In addition, any proposed protocol changes must be submitted and approved by the IBC before initiation.

The approved continuance is for protocol #IBC-ROD-090222 which has been renamed IBC-CRA-090222A to reflect the supervision of Dr. Crabilll. Please include the number in the subject line of all future communications with the IBC regarding the protocol.

Sincerely,

Sam Spooner
Co-Chair, Institutional Biosafety Committee
BIOGRAPHY

Anna Lynn Rodriguez began her college career at Sul Ross State University in 2002. She then moved to San Angelo, Texas where she attended Angelo State University. Anna then left college for several years to pursue a professional career. She returned to college in 2018 and attended online at Midland College. She then returned to Angelo State University where she earned her Bachelor of Science in Biology in August of 2021. Anna went straight into the graduate program at Angelo State University in August 2021. She conducted research under Dr. Emerson Crabill, which involved determining the function of a bacterial effector protein employed by the intracellular pathogen, *Coxiella burnetii.*