RESEARCHREPORT
SWINE HEALTH

Title: Utilization of Neutrophil Extracellular Trap DNA by *Mycoplasma hyopneumoniae*, **NPB 15-147**

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Industry Summary:

Mycoplasmas are bacterial pathogens that colonize and cause disease in a variety of animal hosts. *Mycoplasma hyopneumoniae* (*Mhyo*) is a key participator in the development of the Porcine Respiratory Disease Complex (PRDC) by mitigating host immune responses and promoting secondary infections. The molecular mechanisms by which this is achieved are not understood. Due to their limited genome capacity, mycoplasmas should be considered as true parasites; they acquire all of the requisite macromolecular precursors needed for growth from their host. This includes amino acids for proteins, purines and pyrimidines for nucleic acids, and phospholipids and cholesterol for their membranes. If we can better understand the mechanisms by which mycoplasmas acquire these components, we will have more suitable targets for therapeutic interventions and vaccines.

The overall objective of this project is to determine if *Mhyo* can acquire nucleic acid precursors for DNA replication through the degradation of neutrophilic extracellular traps, a complex of chromosomal DNA and protein extruded from neutrophils that embed or trap bacteria and assist in their destruction. Mycoplasmas have surface nucleases that should be capable of degrading these extracellular nets, releasing abundant levels of precursors for use by the mycoplasma. Our specific objectives were to 1) determine if *Mhyo* can degrade the extracellular nets released by THP-1 cells, a human monocytic cell line that can be induced to form these extracellular traps; and 2) label chromosomal DNA in THP-1 cells with fluorescent nucleotides, induce extracellular nets and follow the fluorescent label into mycoplasma DNA following infection. This will demonstrate *Mhyo*'s ability to degrade chromosomal DNA in extracellular traps, and take up and incorporate the precursors into its own DNA.

These studies established that *Mhyo* can utilize extracellular trap DNA produced by white blood cells for its own use by employing its cell surface nuclease and nucleotide transport processes. The objectives for the study were met by utilizing a bioorthogonal nucleotide detection method known as Click-iT® Labeling, or "click" chemistry, where the modified nucleotide EdU was incorporated into a cell's DNA and detected by reacting the cellular DNA with a fluorescently labeled azide group. The resulting fluorescence was viewed via confocal microscopy. We used the human monocytic cell line THP-1 as an *in vitro* model and labeled the cell's DNA with EdU by incorporating the label in growth media followed by extensive washing to eliminate any residual EdU in the culture supernatants. This was tested by incubating *Mhyo* in the final wash supernatant. The suspended THP-1 cells were then induced to produce macrophage extracellular traps (METs) composed of chromosomal DNA and proteins. Once the traps were produced, *Mhyo* was incubated with the cells to look for the transfer of the EdU label from the METs to the DNA of *Mhyo.* Since the only source of EdU in the samples was these extracellular traps, we confirmed that *Mhyo* was indeed able to obtain nucleotides from METs by fluorescent microscopy. This potentially helps explain how mycoplasmas circumvent the host immune response while also identifying a source of DNA precursors *in vivo* that does not require the direct destruction of host epithelial cells. This study also established the importance of surface nucleases in the metabolism and growth of *Mhyo.* Consequently, new therapeutic strategies could be developed targeting the nuclease function. By combining nuclease inhibitors with vaccines (which do not prevent colonization of *Mhyo*), it may be possible to completely eliminate *Mhyo* infections from swine herds Since acquisition of DNA precursors is essential to mycoplasma viability, inhibiting the membrane DNase activity either through pharmacological intervention or vaccine targeting could potentially protect the host against infection. The reported studies are the first steps in

demonstrating an *in vivo* source for nucleic acid precursors with further studies focused on identifying the membrane DNase target and developing intervention strategies.

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Scientific Abstract:

Mycoplasma hyopneumoniae infection of swine is a major disease problem for producers world-wide. Vaccines have had some success in reducing disease, but colonization of swine herds continues unabated with the potential for severe disease in naïve animals and exacerbation of disease associated other pathogens. Few antibiotics are effective against this organism because of structural and molecular differences from other bacteria. New intervention targets could be associated with the process of macromolecular precursor acquisition, which is necessary for growth. One such target is the membrane DNase that is thought to participate in the acquisition of DNA precursors. Our hypothesis is that neutrophils are recruited to areas of infection in the lung, where they release extracellular chromatin called NETs (neutrophil extracellular traps) that serve to entrap bacteria while additional host immune responses can be mounted against the pathogen. According to our hypothesis, these NETs would be degraded by mycoplasma surface nucleases and the released purines and pyrimidines taken up through membrane transporters for use in DNA replication. By demonstrating the flow of nucleotides from NETs to the mycoplasma, we can begin to consider these nucleases as viable intervention targets either through pharmacological approaches or vaccination.

The overall approach was to take THP-1 cells labeled with a modified nucleic acid base, induce extracellular traps (METs), add mycoplasmas and then monitor the destruction of the METs with concomitant uptake of the labeled nucleotide and its incorporation into the mycoplasma DNA. Our initial attempt was to incorporate Cy3-labeled nucleotides onto DNA and monitor its uptake into mycoplasmas by fluorescence microscopy. We could produce Cy3-labeled PCR products and demonstrate their degradation by *M. hyopneumoniae* whole cells. We could also show uptake of Cy3-labeled nucleotides into mycoplasma DNA. We also optimized the conditions needed for induction of the THP-1 extracellular traps. However, we were unable to label THP-1 DNA with Cy3-labeled nucleotides despite repeated attempts. Thus, we modified our approach by substituting BrdU for Cy3-labeled nucleotides. BrdU is a smaller modification and can be monitor by antibody binding. Detection of BrdU requires fixation of cells, permeabilization to allow for antibody penetration, DNase I treatment to expose the incorporated BrdU to the antibody, and then fluorescence microscopy. Despite success in incorporating BrdU into THP-1 DNA, we failed to visualize the BrdU in the METs due to the need for DNase treatment to expose the label, which degraded the METs prior to adding *Mhyo*. Finally, we tested the thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU). EdU can be detected by treating labeled DNA with a fluorescent azide through a Cu(I)-catalyzed cycloaddition reaction also referenced as "click" chemistry. We first demonstrated *Mhyo* can incorporate EdU into its DNA by incubating *Mhyo* with EdU, reacting the cells with an Alexa555-labeled azide, and detecting fluorescence via confocal microscopy. In addition, we validated *Mhyo*'s membrane nuclease function and its ability to degrade extracellular DNA. Next, the THP-1 cells were incubated with EdU and induced with phorbol 12-myristate 13-acetate (PMA) to produce EdU-labeled METs. *Mhyo* was then incubated with the induced THP-1 cells for a period of time to determine whether the bacterium was able to degrade the METs and integrate EdU into its own genomic DNA. Visualizing the resulting *Mhyo* via fluorescent confocal microscopy exhibited Alexa555 fluorescence indicating that EdU was incorporated into *Mhyo* nucleic acids. These findings showed *Mhyo* could procure free nucleotide bases from NET-like structures in this monocytic cell line by nuclease action and incorporate them into its genome, supporting our hypothesis that *Mhyo* can utilize extracellular traps as a source of nucleic acid precursors.

Introduction:

Despite efforts by the swine health industry, *Mycoplasma hyopneumoniae* (*Mhyo*) continues to have a significant negative economic impact on swine producers. Our lack of knowledge concerning pathogenic mechanisms continues to hinder progress towards resolving this disease in pigs and others like it in various mammalian species including humans. Mycoplasmas have a small genome and are limited in their biosynthetic potential. They lack the biosynthetic pathways for amino acids and purines and pyrimidines. Thus, they must acquire these basic macromolecular precursors from their environment. Since mycoplasmas are exclusively a

host-associated pathogen (probably due to this reason), they must obtain these precursors from the host. This laboratory was the first to identify membrane-associated nucleases in mycoplasmas, presumably to serve as an activity associated with the uptake of nucleic acid precursors. The source of that external DNA in the host has not been defined, however.

The recent observation that neutrophils produce an extracellular trap composed of chromatin (chromosomal DNA and proteins) to control bacterial diseases suggests that this might be a good source of nucleic acid precursors for mycoplasma growth since they have potent nucleases on their surfaces. To support this idea, mycoplasma disease is characterized by a large influx of neutrophils into the area of colonization. This recruitment is thought to be induced by the induction of cytokines in colonized epithelial cells along the upper respiratory tract. Previous studies have shown that cytokine production in the lung accompanies *Mhyo* infection.

These studies will establish whether *Mhyo* can utilize neutrophil extracellular trap DNA for its own use and thereby render neutrophil extracellular NET function non-functional. That can help explain how mycoplasmas can circumvent the host immune response while identifying a source of DNA precursors *in vivo* that doesn't require the direct destruction of host epithelial cells. This will also establish the importance of surface nucleases in the metabolism and growth of *Mhyo.* Consequently, new therapeutic strategies can be developed targeting the nuclease function. By combining nuclease inhibitors with vaccines (which do not prevent colonization of *Mhyo*), it may be possible to eliminate *Mhyo* infections from swine herds. If these surface nucleases are essential to the survival of *Mhyo*, they should also be essential to other mycoplasmas including the other swine pathogens. Perhaps treatment with mycoplasma nuclease inhibitors can eradicate or eliminate all mycoplasma infections in swine, improving the health status and profitability of pig production.

Objectives: To test the hypothesis that mycoplasmas can utilize the extracellular trap DNA as a source of nucleotides, we will complete the following objectives:

First, we will establish a precursor uptake assay using fluorescently labeled nucleotides to establish that such molecules can be taken up and incorporated into DNA by *M. hyopneumoniae*, and subsequently be detected by its fluorescence. This will require growth of *Mhyo* in the presence of various concentrations of all four nucleotides and the monitoring of the uptake by confocal microscopy.

Second, we will produce PCR fragments with fluorescently labeled nucleotides and show that these fragments can be digested by external mycoplasma nucleases and the precursors taken up and incorporated into mycoplasma DNA. The PCR fragments will be random sequences or sequences produced from targets of the neutrophils themselves. This objective is a reasonable facsimile of the NET DNA and will confirm that our overall hypothesis is reasonable.

Third, we will grow the human monocyte cell line THP-1 with labeled nucleotides and show they can be incorporated into the cell's DNA. Confocal microscopy will be used to monitor uptake into the chromosomal DNA. Extracellular traps will then be induced in THP-1 cells, and they will be interacted with *Mhyo* to show by scanning electron microscopy and confocal microscopy that the mycoplasmas can disrupt/destroy the extracellular traps. This will establish the optimal infection ratio needed for NET disruption as well. Then, we will interact *Mhyo* with fluorescently labeled THP-1 extracellular traps and monitor uptake of the nucleotides into the mycoplasma DNA by confocal microscopy and by use of ImageStream multi-spectral imaging flow cytometry (MIFC), which can detect and quantify fluorescent mycoplasmas. Of course, each step will require optimization of the conditions for uptake and transfer of the nucleotides into *Mhyo*.

Materials & Methods:

Mhyo degradation of extracellular DNA via membrane-bound nucleases. This experiment was performed to confirm that *Mhyo* contained surface nucleases capable of digesting linear DNA fragments. *Mhyo* culture was grown to mid-log phase and pelleted. Mycoplasmas were resuspended at a concentration of 100 μg protein per mL in PBS or culture media containing 1% dextrose and 5 mM $MgCl_2$. Two hundred fifty ng of a 4 kb PCR product was added to the mycoplasmas. Cells and DNA were incubated at 37°C for 30 min. Eight μl were loaded on a 0.7% agarose gel and ran at 100 V for 45 min. The gel was stained in EtBr for 15 min and imaged.

Induction of THP-1 cells and the production of macrophage extracellular traps (METs) using PMA. The induction of extracellular nets in THP-1 cells was optimized by varying concentrations of nm phorbol myristate acetate (PMA) and fetal bovine serum (FBS) and varying the length of incubation. The optimized conditions

were as follows. THP-1 cells were grown in suspension, pelleted and the cells resuspended in RPMI plus 1% FBS. The cells were seeded onto collagen-coated, glass cover slips in a 24-well plate at $5x10^5$ cells/well. Two hundred nm phorbol myristate acetate (PMA) was added to each well and incubated at 37°C with 5% CO_2 for 48 hours. By 4 hours the cells had begun to differentiate, lay down on coverslips and develop extracellular nets. The cells were carefully washed with PBS, stained with Cell Tracker Green, fixed with 4% paraformaldehyde and mounted onto slides with ProLong Gold Antifade Reagent with DAPI stain. Slides were viewed via confocal microscopy.

Labeling THP-1 cells and Mhyo DNA with labeled dNTPs. THP-1 cells grown in suspension were incubated with one to three different dNTP Cy3 labeled analogs. The ratios of labeled to unlabeled *analogs* were varied and incubation was carried out for 24-48 hours. *Mhyo* cultures were treated in similar fashion. Both THP-1 cells and mycoplasmas were then visualized by fluorescence confocal microscopy.

To label THP-1 *cells* and *Mhyo* with BrdU, a modified eBioscience protocol was used. THP-1 cells are incubated with 10 µM BrdU for 24 hours. Cells were then fixed and permeabilized with per manufacturer's instructions. DNase I solution was added to provide the anti-BrdU antibody with access to BrdU in the DNase nicked DNA. $Alexa_{555}$ conjugated anti-BrdU antibody was incubated with the cells (1:1000). The cells were gently washed and the cell were viewed via confocal microscopy. *Mhyo* was treated in a similar way except that mycoplasmas were pelleted and washed by centrifugation.

EdU-Labeling and Visualization of THP-1 Cells. THP-1 monocytes were incubated with 10 µM EdU for 24 h at 37°C with 5% CO_2. After incubation, the labeled cells were spun down and the EdU-containing supernatant was aspirated for incubation with *Mhyo*. The labeled THP-1 cells were washed three times in 5 mL of RPMI-1640 at 1000 x g for 5 min. The supernatant of the final wash was set aside for incubation with *Mhyo. Mhyo* was incubated with the pre-wash and post-wash supernatants to demonstrate EdU was in the media used to label the THP-1 cells and that *Mhyo* can uptake and incorporate the nucleotide. The post-wash *Mhyo* incubation was used as a control to confirm there was no residual EdU in the supernatant prior to differentiation of the EdU-labeled THP-1 cells. Trypan blue staining of the EdU-labeled THP-1 cells was preformed to affirm cell viability prior to differentiation. To visualize the EdU label within the THP-1 cells, cells are fixed with 4% paraformaldehyde (PFA), permeabilized with 0.5% Triton® X-100 in PBS, and reacted with a Alexa555-labeled azide according to the protocol provided by Life Technologies. The cells are washed with PBS, stained with 1X Hoeschst® 33342 solution, and mounted on coverslips for confocal microscopy.

THP-1 Differentiation and MET Visualization. THP-1 cells were suspended in RPMI-1640 containing 1% FBS and seeded onto acid-washed coverslips at $5x10^5$ cells/well. Phorbol 12-myristate 13-acetate (PMA) was added *to* each well at a final concentration of 200 nM. The cells were incubated at 37°C with 5% CO_2 for 36 h to allow for the production and expulsion of the METs from the THP-1 cells. After 36 h, a final concentration of 4% PFA was added to each well and incubated at room temperature for 20 min. Fixed cells were washed with 1 mL of PBS twice for one minute each. The Alexa555-labeled azide was reacted with the fixed cells and METs according to the protocol listed in the Click-iT Plus EdU imaging Kit (Life Technologies) and incubated at room temperature, protected from light. After a 30-min incubation, the reaction cocktail was removed from the wells and the cells were washed with 1 mL of PBS followed by staining with 1X Hoechst® 33342 solution for 30 min at room temperature. Each well was washed with PBS twice and the round coverslips were mounted onto glass coverslips for confocal microscopy using DAPI and TRITC filters.

MET Degradation by Mhyo and Visualization Labeled and non-labeled THP-1 cells were differentiated as described above for the production of METs. After 36 h of differentiation with PMA, an equal volume of *Mhyo*, grown to mid-log phase, was added to each well. $MgCl_2$ was added to a final concentration of 5 mM to activate *Mhyo* nuclease function. The differentiated THP-1 cells and *Mhyo* were incubated together for 6-8 h at which time the cellular media containing *Mhyo* was aspirated and centrifuged at 10,000 x *g* for 1 min to pellet the cells. *Mhyo* was washed with PBS followed by fixation with 4% PFA for 20 minutes at room temperature. After fixation, the cells were pelleted at 800 x *g* for 5 minutes to carefully pellet the cells and washed with PBS. The bacterial cells were permeabilized with 0.5% Triton® X-100 in PBS at room temperature for 20 minutes followed by two washes with PBS using the same centrifuge settings as used prior in fixation. The fixed and permeabilized cells were reacted with the Alexa555-labeled azide according to the manufacturer's protocol (Life Technologies). Staining with a 1X Hoechst® 33342 solution for 30 min at room temperature proceeded the prior reaction following washing with PBS. The processed *Mhyo* cells were mounted on a coverslip and visualized using confocal microscopy using DAPI and TRITC filters.

Results:

Objective 1: Can Mhyo take up fluorescently labeled nucleotides and incorporate them into DNA? In order to *determine* whether *Mhyo* has the capability of taking up the EdU and incorporating the thymidine analog into its genetic material, *Mhyo* was incubated with a 10μM solution of EdU in Friis broth for *Mhyo* for 18-24 h. Incorporation of the modified nucleotide was determined using an Alexa555-labeled azide group that binds to EdU within the cell followed by confocal microscopy to look for fluorescence. The images in Figure 1 provide evidence that *Mhyo* is fully capable of incorporating EdU into its genetic material.

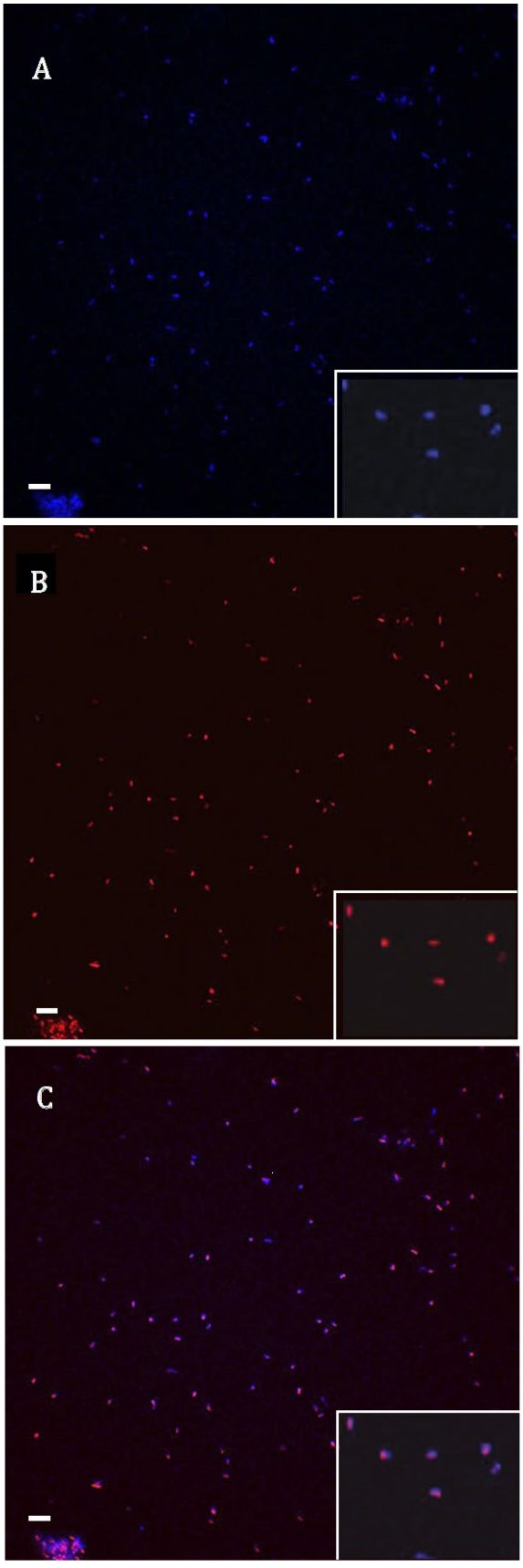


Figure 1. EdU *incorporation* into *Mhyo* DNA. Scale bar represents 10 µm. (A) DAPI (blue) stain of *Mhyo.* (B) *Mhyo* viewed through TRITC filter (red) to observe the presence of EdU. (C) Composite image of (A) and (B) showing the presence of EdU within *Mhyo* DNA. The color purple indicates where the two overlap. The inset is a 300X magnification of a region of the field.

Objective 2: Because of the nature of the EdU label and the utilization of "click" chemistry for visualization, we were not able to confirm its incorporation into PCR products. The degradation of extracellular DNA by *Mhyo*'s membrane nuclease was reconfirmed demonstrating that *Mhyo* can readily dismantle external DNA when stimulated with 5mM $MgCl_2$ (Figure 2). As demonstrated in objective 1, it is already known *Mhyo* can utilize EdU for the building of nucleic acids.

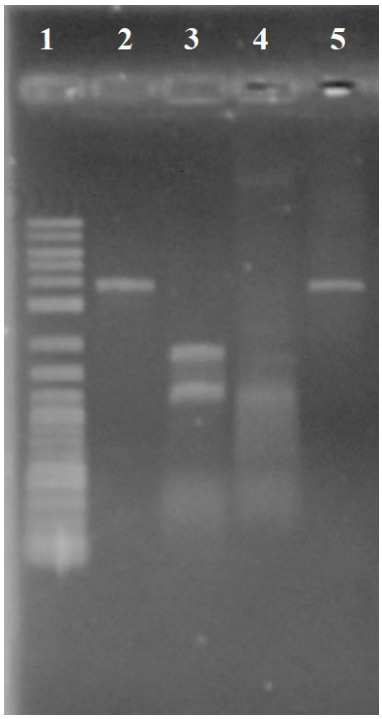


Figure 2. Analysis of surface nuclease activity in *Mhyo* by agarose gel. Lanes: 1. NEB 2-Log *DNA* Ladder; 2. 4 kb PCR product (-) control; 3. DNA and *Mhyo* in PBS; 4. DNA and *Mhyo* in culture media; 5. DNA in culture media (a control for any nucleases in the media). Lanes 3-5 contain 5 mM Mg^{2+} as a co-factor for the nuclease.

Objective 3.

Optimizing the conditions for inducing extracellular traps in THP-1 cells.

Phorbol myristate acetate had been used to induce differentiation of THP-1 cells. During induction, THP-1 cells begin to adhere to collagen-coated surfaces and start releasing its DNA to form METs. Prior to induction, THP-1 cells are grown in suspension culture. Optimal conditions for inducing METs in THP-1 cells was determined by varying serum concentrations, PMA concentrations and incubation times. METs were visualized by staining with Cell Tracker Green and DAPI

(Figure 2). The optimized incubation time and concentration of PMA allowed us to establish the flow for incubation with mycoplasmas.

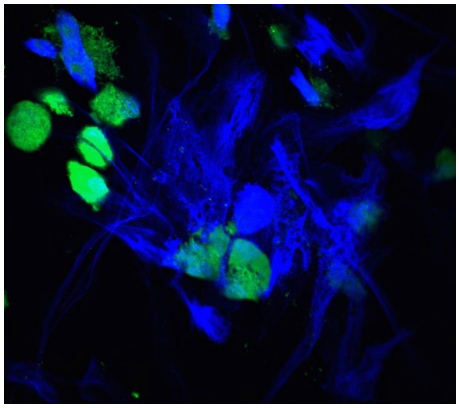
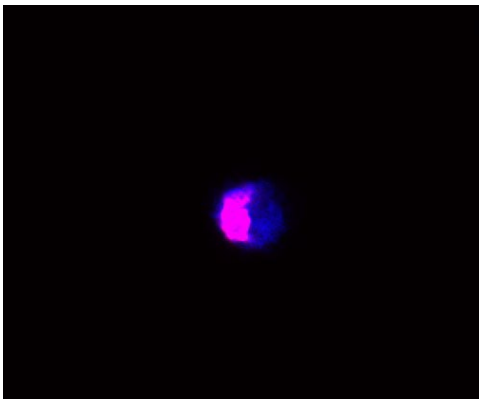


Figure 3. THP-1 cells producing extracellular traps. Cell Tracker (green) was used to identify the THP-1 cells. DAPI (blue) was used to stain the nucleic acids that have been released upon PMA treatment to form METs. The dark blue represents that released chromosomal DNA (METs) and the green is the cellular membrane.

Utilization of nucleotides in NETS by Mhyo. To investigate whether *Mhyo* can utilize extracellular traps produced by human monocytic cells, the cell line THP-1 was used as a model for the expression of the extracellular traps. EdU-labeled THP-1 cells were induced for the release of METs using PMA followed by a 6-8 h incubation with stimulated *Mhyo.* Figure 4 presents the successful incorporation of EdU into the nucleus of THP-1 cells followed by visualization of the label in the METs upon induction (Figure 5). To control for residual EdU remaining in cell culture supernatants following the labeling and washing of THP-1 cells, *Mhyo* was incubated with labeling media prior to washing the THP-1 cells and with the final wash supernatant (Figure 6). There was no indication that EdU remained in the media after three washes prior to the MET induction. Many EdU-labeled METs were formed upon induction. When *Mhyo* was added to the labeled METs, MET structures disappeared after 6-8 h of incubation suggested *Mhyo* was able to degrade the extracellular DNA (Figure 7). In addition to the destruction of the extracellular traps, the EdU label was detected in *Mhyo* genetic material after incubation with the METs (Figure 8).

Figure 4. THP-1 cell with an EdU-labeled nucleus. Cells were visualized with DAPI (blue) and Alexa555 (red). Purple indicates the DAPI-Alexa555 overlap.



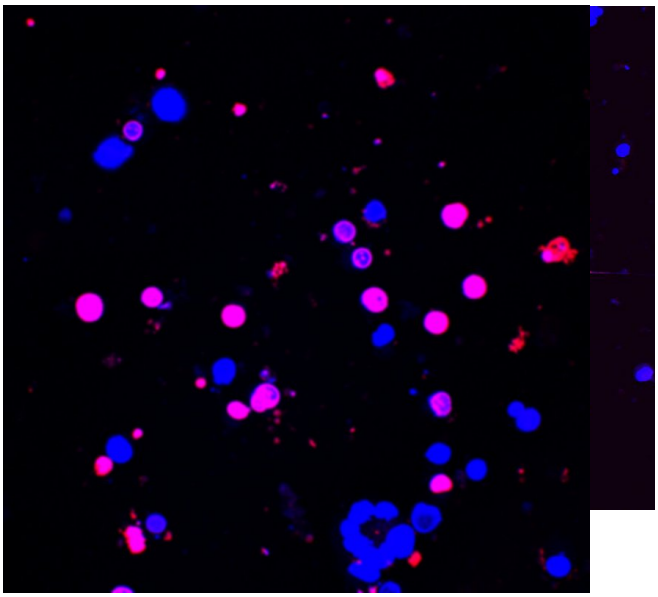


Figure **5.** EdU-labeled THP-1 cells induced for the production of METs. DNA is stained with DAPI(blue) and EdU is labeled with Alexa555 (red). Arrows indicate location of MET structures containing the EdU label.

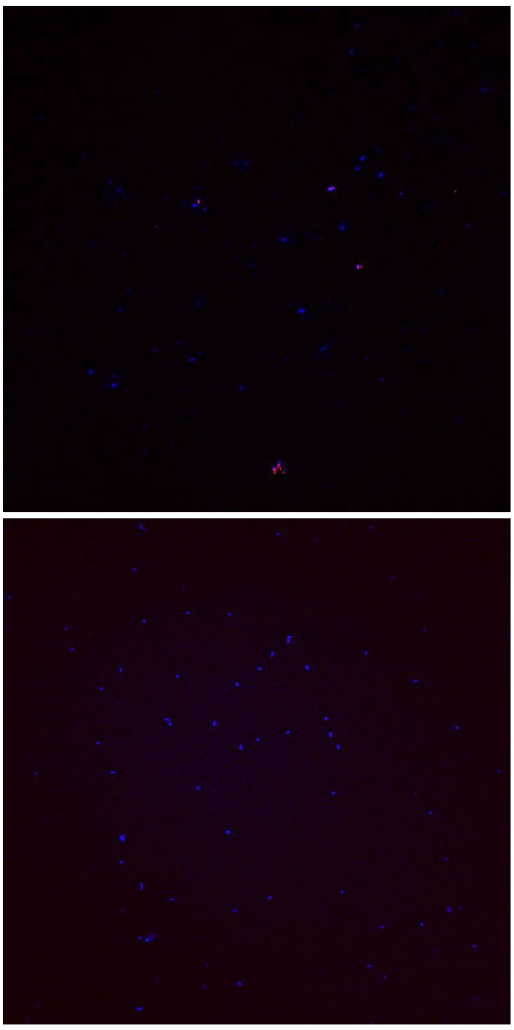
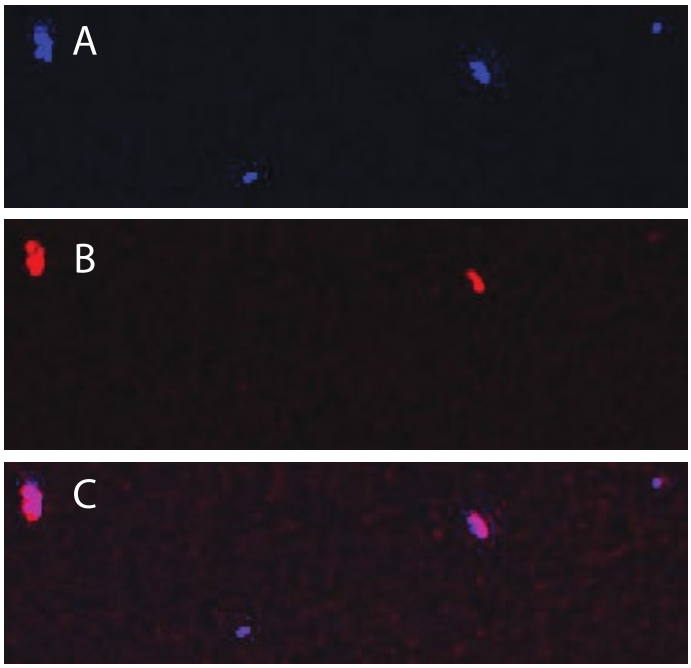


Figure 6. *Mhyo* pre- and postwash to control for residual EdU prior to MET induction. Cells are stained with DAPI (Blue) and labeled with Alexa555 (red) that recognizes EdU (upper) Prewash showing there was EdU in the media used to label the THP-1 cells and that *Mhyo* can utilize it for synthesizing DNA. (lower) Postwash showing there is no residual EdU in the media prior to inducing the THP-1 cells to release METs.

Figure 7. Induced EdU-labeled THP-1 cells that have been incubated with stimulated *Mhyo* resulting in the destruction of extracellular trap structures.

Figure 8. EdU label visualized in *Mhyo* post incubation with EdU-labeled METs. (A) DAPI (blue) stain of *Mhyo.* (B) Alexa555 dye (red) labeling the location of EdU. (C) Composite of (A) and (B) indicating the overlap of the *Mhyo* genetic material and the location of the EdU label. The images represent a 300-fold magnification.



Discussion

Mhyo is a key participator in the development of Porcine Respiratory Disease Complex (PRDC) by mitigating the host immune response and encouraging secondary infections. *Mhyo* has a minimal genome lacking major biosynthetic pathways, making it reliant on its host to obtain nucleic acid precursors. It is possible *Mhyo* obtains these precursors by using membrane nucleases to degrade the DNA found in neutrophil extracellular traps (NETs), chromatin structures ejected from neutrophils as a anti-bacterial response at sites of infection.

The *innate* immune system plays a crucial role as the first line of defense against invading pathogens, such as *Mhyo.* Neutrophils, macrophage, and other phagocytic white blood cells prevent early infections by phagocytizing the bacteria or, in particularly difficult cases, release of extracellular traps. These extracellular traps have intrinsic antimicrobial properties and are able to clear infections successfully unless hindered by mechanisms employed by the pathogen. Bacteria have been found to have mechanisms to resist being killed by extracellular traps, with one of the most common mechanisms being the expression of an extracellular nuclease to degrade the eDNA released by innate immune cells. We confirmed previous observations that *Mhyo* has a membrane-bound extracellular nuclease that can degrade eDNA, providing support for our hypothesis that *Mhyo* can procure nucleotides from host extracellular traps. Further, the appearance of EdU fluorescence in Mhyo following exposure to EdU-labeled METs indicates that *Mhyo* was able to degrade the extracellular DNA, transport the released nucleotides and incorporate them into their own DNA.

The destruction of neutrophil NETs by *Mhyo* can explain the difficulty in clearing the organism from infected pigs. A common feature of lung lesions during mycoplasma infection is an influx of neutrophils and their subsequent activation. Although not directly detected, it is expected that part of this neutrophil response would be the production of NETs by some of the neutrophils. These serve to immobilize the bacteria while the host can mount additional anti-bacterial responses. The degradation of these NETs by *Mhyo* as we have demonstrated, they could circumvent that aspect of the immune response while being provided a pool of nucleic acid precursors needed for growth. In summary, our findings show that *Mhyo* can degrade extracellular labeled DNA from activated monocytes, transport labeled free nucleotides and integrate them into its genome.