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Directed Genome Evolution to Identify Genes for Macrophage Survival by Staphylococcus agnetis

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Directed Genome Evolution to Identify Genes
for Macrophage Survival by *Staphylococcus agnetis*

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by

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Abstract

Bacterial Chondronecrosis with Osteomyelitis (BCO) is a debilitating infection that negatively impacts animal welfare and costs the broiler industry billions of dollars annually. We have previously isolated *Staphylococcus agnetis* 908 from BCO samples obtained from broilers at the University of Arkansas research farm. This isolate can induce BCO lameness at greater than 50% in broilers exposed to the pathogen in drinking water. We found that *S. agnetis* 908 is capable of surviving and escaping macrophages compared to a closely related cattle isolate, 1379. Through Directed Genome Evolution (DGE) we identified that this difference is at least partially associated with an alanine to glutamate substitution for residue 164 of the enzyme deoxyribose phosphate aldolase (a.k.a. deoC, DERA). This study further explores whether A164E in deoC is responsible for enhanced survival and escape of *S. agnetis* 908 from macrophages. *S. agnetis* 1379 was transformed with the PCR products of the 908 deoC1 and deoC2 paralogs. The resulting transformants were cocultured with chicken macrophage-like cells in standard phagocytosis assays for DGE. The survivors were characterized for sequence changes in deoC through PCR sequencing. In addition, we investigated the effect of the A164E on host-cell damage by utilizing gentamicin protection assays in tandem with crystal violet staining. We evaluate the drawbacks of the gentamicin protection assay and evaluated an alternative enzyme protection assay. The crystal violet staining revealed that 908 and the transformant of 1379 inflicted more cellular damage. The enzyme protection assay was superior to gentamicin protection and indicated that there were no significant differences in the damage to HTCs caused by the different bacterial strains.

Identification of the bacterial virulence factors important to the infection process, and how these interact with the host immune responses are important in devising management plans for mitigation of BCO which would help the broiler industry control this important and costly disease.

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Dedication

Dedicated to ammi (mom) and appachchi (dad). Thank you for all the sacrifices you have made to help me reach my dreams.

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Chapter 1

Literature review

1.1. Bacterial Chondronecrosis with Osteomyelitis

The poultry industry which consists of broilers, eggs, and domesticated birds such as turkeys, ducks, quail and pheasants is a billion-dollar industry in the United States alone (USDA, National Agricultural Statistics Service, 2021). The broiler industry depends on selective breeding of chickens (*Gallus gallus domesticus*) for meat production. When the broiler industry first started out in the 1920s it took 16 weeks to obtain an adult chicken of 1.1 kg (Aho, 2002, National Chicken Council, 2021). The expansion of the broiler industry was driven by production of high yielding birds through selective breeding (Bradshaw et al., 2002). At present, a typical broiler chicken weighs approximately 4.5 kg as result of selective breeding for early rapid growth rate. This improvement is a nearly 300% increase from gaining 25 g per day to gaining 100 g per day (Knowles et al., 2008).

With the domestication of chickens, the genetic diversity of the species has considerably decreased through inbreeding, creating a genetic drift through selection (Elferink et al., 2012). Selection for an advantageous gene may also have negative consequences. Selection for a beneficial trait in one gene can result in a selective sweep of “neutral” genes in flanking regions in a process called ‘hitch-hiking’. Others have hypothesized that hitch hiking has resulted in producing high-yielding birds with several negative traits such as decreased resistance to infectious disease (Zekarias et al., 2002), increased skeletal deformities (Julian, 1998), osteoporosis (Whitehead and Fleming, 2000) and pulmonary hypertension syndrome (Baghbanzadeh and Decuypere, 2008, Balog, 2003, Wideman et al., 2013b).

The rapid growth rate observed in broilers compared to the layers (for egg production) has resulted in significantly higher incidences of lameness reported (Wise, 1970, Williams et al.,

2000, Wideman, 2016). With increasing muscle mass, the chicken skeletal system must also have concomitant increases (Thorp et al., 1993, Riddell and Springer, 1985, Williams et al., 2000, Wideman, 2016, Rhoads and Wideman, 2020). The femora and tibia undergo a four-fold increase in length along with a three-to-five-fold increase in mid-shaft diameter over the course of the first six weeks (Applegate and Lilburn, 2002). However, it is evident that skeletal development does not sufficiently compensate for the increase in muscle mass as the incidence of lameness is reduced when the growth rate of chicken was reduced.

Lack of sufficient skeletal development is not the sole cause of lameness. Bone defects such as tibial dyschondroplasia (TD) (Leach and Nesheim, 1965), angular limb deformities (Julian, 1984), arthritis and osteomyelitis (Riddell & Springer, 1985) further aggravate the problem. However, currently Bacterial Chondronecrosis and Osteomyelitis (BCO) has been identified as the most common cause of lameness among commercial broiler chicken (McNamee et al., 1998, Thorp and Waddington, 1997, Mitchell, 2014, Wideman, 2016). BCO has been identified using various terminologies including: osteomyelitis, femoral head necrosis, long bone necrosis, proximal femoral degeneration, bacterial chondritis with osteomyelitis (McNamee and Smyth, 2000). However, BCO is most fitting since it considers the infection in the growth plate cartilage and the bacterial aetiology of the disease.

BCO was first reported as a cause of lameness in commercial broiler chickens in Australia (Nairn and Watson, 1972). Subsequently, countries such as US, Canada and Europe have also reported this condition in broilers (Riddell & Springer, 1985; Thorp et al., 1993; Thorp & Waddington, 1997; McNamee et al., 1998). Thorp et al. (1993) investigated birds in the UK suspected to have lesions in the hip but showed a high incidence of BCO. BCO was also a common diagnosis in lame birds examined by Thorp & Waddington (1997). BCO was identified to be the most common cause of lameness in commercial birds through systematic large-scale studies of lame broilers from seven commercial flocks (McNamee et al., 1998, 1999a). It

continues to remain the most frequent form of lameness in the US broiler industry (Wideman, 2016, Mitchell, 2014).

1.1.1. Factors contributing to BCO.

In addition to the increased growth rate and skeletal deformities, a multitude of other factors contribute to BCO. One such factor is immunosuppression (Mutalib et al., 1983, Andreasen et al., 1993, McNamee et al., 1998, Wideman et al., 2013a, Butterworth, 1999). As described by McNamee and Smyth (2000) by infecting a bird with chicken anemia virus and infectious bursal disease led to an increased incidence of BCO while reducing the feed intake decreased its incidence. Additionally, environmental stresses such as competition induced by social pressure can lead to compromised immunity (Wideman et al 2013) and chronic stress caused by the lack of accessibility to floor-litter has been shown to induce immunosuppression with increased blood corticosterone levels (El-Lethey et al., 2003). Additionally, male broilers are more likely to develop BCO due to the fast growth rate compared to slower growing female counterparts (Wideman et al., 2013).

Bacterial infections are a major risk factor for the development of BCO. Bacteria in the breeding farm and the hatchery are potential sources for bacterial infections (McNamee ad Smyth 2000). Pulse field gel electrophoresis had confirmed this by tracing naturally *Staphylococcus aureus* infection in chicken to bacteria isolated from fluff debris from hatcheries. Furthermore, birds hatched from floor eggs were identified as being more susceptible to BCO which could be due to the transmission of poultry strains of *S. aureus* from humans to chickens (McCullagh et al., 1998).

1.1.2. Pathogenesis of BCO

Bacteria in the environment can enter the chicken blood stream through the respiratory system in the form of aerosolized bacteria or through the digestive system (Figure 1). Once in

blood circulation, bacteria that can survive are able to distribute hematogenously and enter the bone marrow vasculature (Wideman, 2016). BCO is most likely to affect the proximal growth plates of the femora and tibiae of broilers. This is due to the combination of possessing abnormally long, unevenly aligned chondrocytes paired with thicker proximal growth plates of birds which lead to higher growth plate turnover during longitudinal growth. Thus, the proximal ends of the leg bones grow faster and are thicker than other species (Church and Johnson, 1964, Leach and Nesheim, 1965, Thorp et al., 1993, Kember et al., 1990, Kirkwood et al., 1989). Torque and physical stress exerted on these bones results in microfractures called osteochondrotic clefts. These microfractures may result in damaged blood vessels resulting in ischaemia leading to regions of necrosis. Bacteria in the bone marrow vasculature colonize these osteochondrotic clefts resulting in necrotic regions, i.e., osteomyelitis. Bacteria residing in these locations are inaccessible to immune responses and antibiotics thus harder to eliminate(Thorp and Waddington, 1997, McNamee et al., 1998, McNamee and Smyth, 2000).

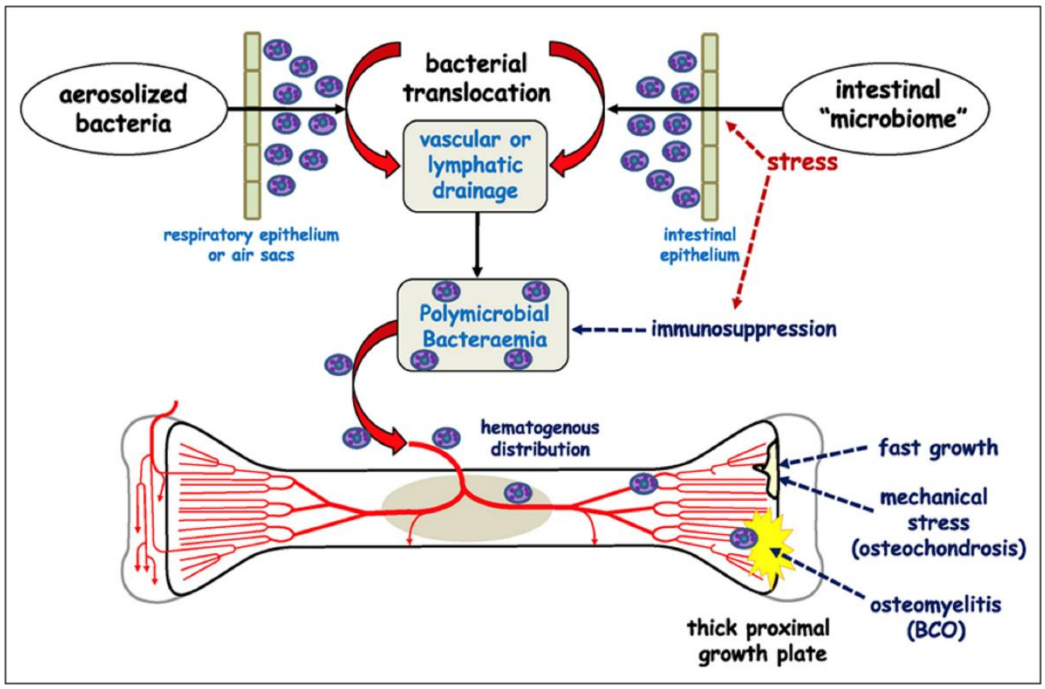

Figure 1. Pathogenesis of BCO describing the possible portals of entry of bacteria and mechanisms in which they enter the bone marrow. (Image reproduced from Wideman, 2016)

1.1.3. BCO as an animal welfare and economic issue

Lameness compromises mobility of the bird which decreases its quality of life (reduced animal well-being). Immobile birds are unable to perform their routine activities such as dustbathing foraging, preening or walking to access food and water (Vestergaard and Sanotra, 1999, Weeks et al., 2000, Butterworth, 1999). Multiple studies concluded that birds with lameness feel pain as shown by McGeown et al. (1999) the birds preferring food with analgesics and performing better after a period of time while others state that there is no relationship between lameness and pain (Caplen et al., 2013, Danbury et al., 2000).

Based on data recorded daily and from weekly necropsy examination of birds from 51 broiler flocks in Western Canada, Riddell & Springer (1985) found the incidence of birds culled due to lameness ranged from 0.46 to 4.08%. In this study, the average incidence of chickens with skeletal deformities was 1.72%, which included 1.1% chickens culled in the field and 0.62% chickens condemned at processing (McNamee and Smyth, 2000). In 1993, a national survey in the US estimated that leg problems cost the broiler industry between 80 and 120 million dollars annually (Morris, 1993). As of present, BCO costs the broiler industry over a billion dollars annually (Rojas-Nunez et al., 2020).

1.1.4. Clinical signs of BCO

Previous studies have shown that an increase in lameness is usually accompanied by increased mortality rates (Nairn and Watson, 1972, Griffiths et al., 1977). Living birds with BCO would display a limping gait, hip flexion and would indicate pain by vocalizing loudly when pressure is exerted to the affected regions (Thorp et al., 1993, Mutalib et al., 1983). Other clinical signs include ruffled feathers, reluctance to walk, having their heads down, closed eyes, sitting on their hocks, and weak response to external stimuli (McNamee and Smyth, 2000) However, in their experiment where broilers were challenged with bacteria in drinking water, Al-

Rubaye et al. (2015) reported that birds with lameness were not overtly detectable until 19 days post-inoculation with bacteria. Reticence to move was a common sign observed toward the latter stages of the disease (Nairn & Watson, 1972) As a result of this, weight loss is yet another identifiable marker of both naturally and experimentally induced lame birds (McNamee and Smyth, 2000).

1.1.5. BCO and bacteria

Bacteria are inevitably present in the environment, from drinking water to the air inside the facility. DNA for a diverse bacterial population was isolated from macroscopically healthy proximal femoral and tibial heads of presumably healthy chicken (Jiang et al., 2015). Thus, it is evident that bacteria can enter chicken blood circulation and distribute hematogenously even among healthy birds. This study identified Proteobacter as the most common species isolated from both femoral and tibial samples of healthy birds, followed by Firmicutes and actinobacter. Similar findings were reported in a study by (Mandal et al., 2016). Yet, a study by (Danzeisen et al., 2011) had identified Firmicutes to be the most abundant organisms isolated from cecum samples which indicates Firmicutes would have been more prevalent. However, it should be noted that Proteobacter has a higher potential to survive and spread hematogenously, crossing the gastrointestinal epithelium which could explain the prevalence of Proteobacter. Jiang et al. (2015), further reported that there were distinctive differences in the microbial population between healthy femoral and tibial samples and samples obtained from BCO infected chicken. Additionally, the femoral and tibial samples did not yield the same bacterial populations and bacteria isolated from the different lesions including normal, Femoral Head Separation, Femoral Head Necrosis, Tibial Head Necrosis, and Tibial Head Necrosis severe or caseous were significantly different from each other.

Several studies on BCO have identified *Staphylococcus aureus*, *Escherichia coli*, coagulase-negative Staphylococci, *Enterococcus* spp., *Enterobacter,* and *Serratia* as common

causative agents of BCO (McNamee and Smyth, 2000, Wideman and Prisby, 2012, Jiang et al., 2015). However, a study by Al-Rubaye et al. (2015) identified the majority of BCO samples from the University of Arkansas Research Farm were *Staphylococcus agnetis* 908. The samples were isolated through broth enrichment from BCO lesions and direct plating on differential media. The isolated colonies were processed for PCR and sequencing of the 16S rRNA gene to identify to species. In contrast to the study by Jiang et al. (2015) which stated that the bacterial samples obtained from the femoral and tibial bones were different, this study detected *S. agnetis* isolates from all lesions regardless of the lesion type or location. Previously, this species had only been associated with cattle mastitis samples (Taponen et al., 2012, Shwani et al., 2020). It was speculated that *S. agnetis* could be a contaminant present in commercial hatcheries possibly passed on by hatchery workers or equipment. Furthermore, administering a single dose of *S. agnetis* 908 in drinking water was able to induce BCO lameness in 50% of the chicken population, recognizing 908 as a highly virulent strain (Al-Rubaye et al., 2015).

S. agnetis is a coagulase variable, Gram-positive bacteria that has been identified to induce lameness in broilers (Al-Rubaye et al., 2015). Phylogenomic analyses based on the 16S region identified that *S. agnetis* 908 is closest to isolates from cattle (Taponen et al., 2012) and microbiome samples from sheep mites (Hogg and Lehane, 1999). *Staphylococcus hyicus* and *Staphylococcus chromogenes* were the two closest species to *S. agnetis* based on neighbour joining tree results of 908 16s rRNA genes (Al-Rubaye et al., 2015).

To further understand the relationship between the *S. agnetis* 908 chicken isolate and the cattle isolates, (Shwani et al., 2020) used whole genomes for elucidating the phylogenetic relationships of known *S. agnetis* isolates. Although the cattle and chicken isolates were very similar the study did not conclusively identify acquisition or loss of any gene or gene cluster that coincided with the switch of hosts from cattle to chicken. Thus, it is hypothesized that 908 may possess alterations such as missense mutations in particular virulence factors that would contribute to its pathogenesis in chickens. Additionally, we have identified that 908 is capable of

surviving and escaping macrophages when compared to the closely related isolate from cattle, *S. agnetis* 1379 (Zaki, 2021). This had led to further investigations to identify and locate the virulence factors by employing Directed Genome Evolution (DGE).

1.2. Deoxyribose phosphate aldolase

To identify the virulence factors that allow 908 to survive and escape macrophages transformants of 1379 were created. *S. agnetis* 1379 was electroporated with whole genome or extrachromosomal 'plasmid' 908 DNA (Zaki, 2021; Shwani, 2021). The transformation product was passaged through chicken macrophage-like cells and after several rounds of selection the survivors were very similar to the 908 phenotype for survival and escape from macrophages. Multiple DGE products were produced, and their genomes sequenced, assembled, and compared to 908 and 1379. These analyses identified two alternative single nucleotide polymorphisms (SNPs) where either SNP could result in converting 1379 into a 908 phenotype for survival and escape from macrophage. The two alternative SNPs were each in one of two paralogs for deoxyribose aldolase phosphate (deoC or DERA). DERA which is a 220 amino acid polypeptide is coded by the paralogs deoC1 and deoC2 for both 1379 and 908. However, the major difference between 1379 from cattle, 1379 and 908 from chicken, was that in 1379 residue 164 for both paralogs is alanine while in 908 both copies encode glutamate.

DERA is an aldehyde-lyase that reversibly converts 2-deoxy-D-ribose 5-phosphate to D-glyceraldehyde 3-phosphate and acetaldehyde. Acetaldehyde can then be converted to acetyl CoA and transferred to the glycolytic and Kreb's cycle to generate energy (Lomax and Greenberg, 1968) . Salleron et al., (2014) revealed that an over expression of DERA in human cells allowed cells to survive by utilizing exogenous deoxyinosines to produce ATP in the absence of glucose and presence of an oxidative phosphorylation disrupter. Structurally, DERA consists of monomers that are TIM α/β barrels (Figure 2) and are categorized as Class I

aldolases which are generally involved in forming Schiff bases at Lys^{167} in the active site (Heine et al., 2004).

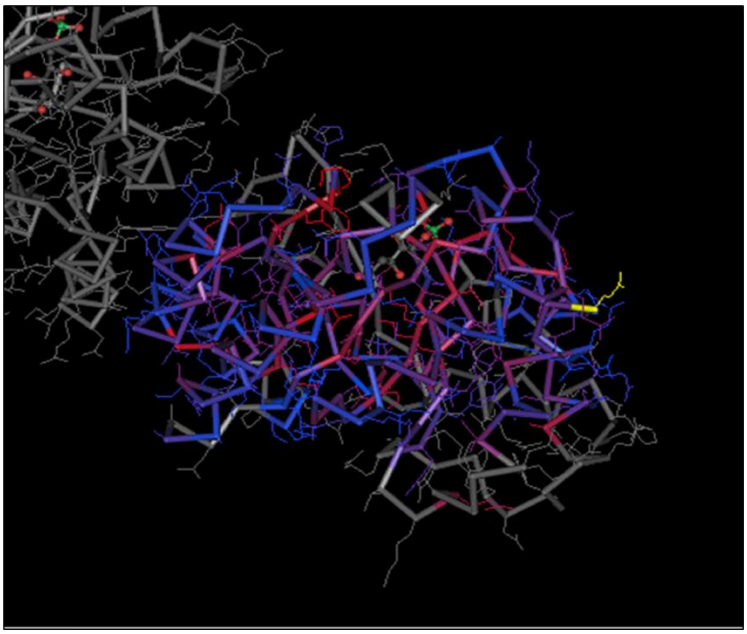

Figure 2. Three-dimensional structure of 908 DERA generated using Cn3D software, NCBI. The A164E substitution is indicated in yellow located on the outer alpha helix of the homotetramer.

1.3. Immune responses

1.3.1. Macrophages

Macrophages are the dominant effector cells of innate immunity but are also important in adaptive immunity found in organs and connective tissue (Abbas, Lichtman and Pillai, 2012). In blood circulation, they are identified as monocytes and once they transverse blood capillaries and enter tissues, termed as macrophages (Steinman and Cohn, 1974). Macrophages and monocytes arise from committed cells in the bone marrow that differentiate in response to the cytokine Monocyte-Macrophage Colony Stimulating Factor (M-CSF) (Abbas, Lichtman and Pillai, 2012). Many tissues have their own long-lived resident macrophages which are derived from yolk-sac or fetal liver precursors such as Kupffer cells (Decker, 1990). Tissue specific

macrophage which have tissue-dependent specialized functions include: alveolar macrophages in the lungs (Joshi et al., 2018) and microglial cells in the brain (Guillemin and Brew, 2004).

Macrophages are involved in innate immunity. They can secrete cytokines such as TNF, several interleukins (IL-1, IL-10, IL-6), and chemokines, that are important for acute inflammatory response (Abbas, Litchman and Pillai, 2012). When stimulated by the cytokines and chemicals produced by T-lymphocytes, macrophages in large numbers are recruited to engage in adaptive immunity to eliminate pathogens (Fujiwara and Kobayashi, 2005).

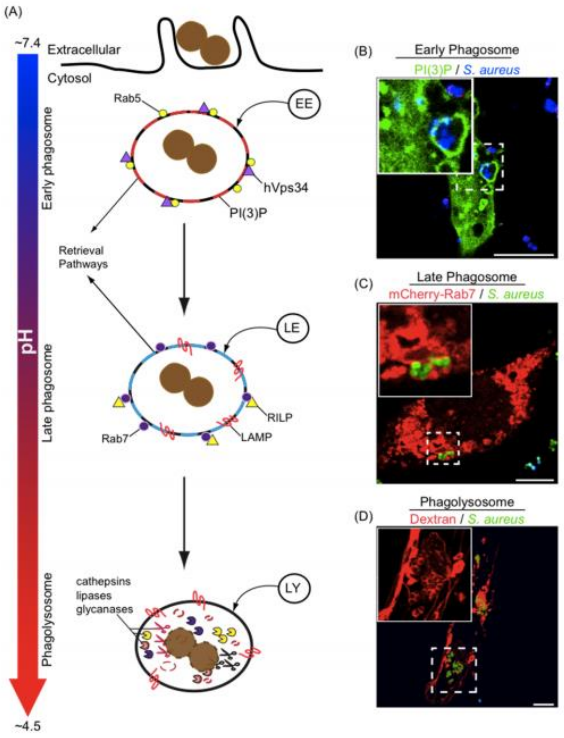

Figure 3. (A) The progression from a nascent, early phagosome to a phagolysosome. During the maturation process biochemical changes take place including the different markers displayed. (B) Laser scanning confocal micrograph images with Staphylococcus aureus (blue stained with eFluor670); (C-D) S. aureus in green (containing GFP). (Image reproduced from Flannagan et al., 2015)

Macrophages populations are classified into two classes: M1- the classically activated, and M2- alternatively activated macrophages (Braga et al., 2015, Mills, 2012). M1 macrophages initiate inflammatory responses involved in eliminating pathogens while the M2 macrophages are known to have anti-inflammatory responses. Classically activated macrophages detect

microbes and foreign antigens using receptors such as Toll-like receptor (TLR) on the cell-membrane which identify Pathogen Associated Molecular Patterns (PAMPs) and Damage Associated Molecular Patterns (DAMPs) (Abbas, Lichtman and Pillai, 2012). In addition, they identify opsonized microbes. Once the pathogen is recognized, macrophages would engulf it through phagocytosis which encases the pathogen in a vacuole (Peterson et al., 1978). This nascent phagosome lacks microbicidal abilities until it matures to a late phagosome during which the pH within the vacuole is lowered by proton pumps to create an acidic environment toxic to pathogens (Figure 3). The mature phagosome then fuses with a lysosome to form phagolysosomes. The phagolysosomes contain reactive oxygen and nitrogen species, glycanases, lipases and proteolytic enzymes that are capable of destroying the microbe (Aderem and Underhill, 1999). Macrophage functions are not only limited to elimination of pathogens, but they are also involved in recognizing and engulfing dead host cells that have undergone necrosis or apoptosis. Macrophage can also stimulate T-cell mediated immunity through antigen presentation. Meanwhile, alternatively activated M2 macrophages are involved in tissue repair, angiogenesis, and fibrosis. Excessive activation of M2 macrophages has been identified to induce asthma and fibrosis (Moreira and Hogaboam, 2011).

1.3.2. Bacterial evasion of phagocytosis

Despite macrophage functioning as sentinels to prevent microbial infection and spread, several microorganisms have developed mechanisms to evade immune responses. Studies have revealed that *Salmonella*, *Shigella* and *Yersinia* are capable of secreting several Type III secretion components that span the bacterial cell-membrane (Kubori et al., 1998, Blocker et al., 1999). This protein is involved in transporting certain chemicals to the extracellular space that could either promote bacterial uptake by host-cells which are not phagocytes (Galan and Collmer, 1999, Cornelis, 2000) or it could altogether prevent phagocytosis as displayed by *Salmonella* (Goosney et al., 1999, Hardt et al., 1998).

Bacteria such as *Staphylococcus aureus, Streptococcus pyogenes* and *Mycobacterium* spp. are able to evade being recognized by the mannose binding receptors and Fc receptors on phagocytes. Typically, these receptors would activate Cdc42 and Rac to initiate a cascade of reactions that would eventually eliminate the phagosome encased bacteria (Joiner et al., 1990, Lefkowitz et al., 1997). Instead, these bacteria subvert the process through binding to complement receptors that do not trigger microbicidal actions of the internalized bacteria (Yamamoto and Johnston, 1984). Other pathogens like *Listeria, Rickettsia* and *Shigella* are able to escape the phagosome utilizing pore-forming proteins across the phagosome membrane. They then enter the cytosol where they continue to replicate while evading destruction by the lysosome fusion (Gouin et al., 1999).

Yet, another mechanism through which some pathogens evade immune responses is observed in *Salmonella* and *Mycobacteria*. Once internalized by a phagocyte, *Salmonell*a secretes yet another Type III protein that blocks the lysosomal fusion with the phagosome (Uchiya et al., 1999). A similar observation was reported by (Kaufmann, 2005) in *Mycobacterium tuberculosis* where the bacilli produce a tryptophan-aspartate containing coat protein (TACO) which prevents the maturation and fusion of the phagosome with the lysosome (Ferrari et al., 1999, Armstrong and Hart, 1971). This mechanism is responsible for the long-term survival of *Mycobacteria* within the alveolar macrophages.

S. agnetis shares a significant number of virulence factors with *S. aureus*, a well-studied pathogen. *S. agnetis* chicken isolate 908 contains 17 recognizable virulence factors with potential to contribute to host immune evasion. Among these are beta-toxins, toxin-A, a gene encoding for a putative hyaluronidase, five superantigen like proteins and seven fibronectin binding proteins (Calcutt et al., 2014, Al-Rubaye et al., 2015).

1.4. Synopsis

BCO is a debilitating bone infection which is an animal welfare issue that costs the broiler industry billions of dollars annually. Previous research had identified *S. agnetis* 908 as a virulent causative agent of BCO lameness in broilers. *S. agnetis* 908 is capable of surviving in and escape from macrophages when compared to a genetically similar cattle isolate. Understanding the mechanisms through which bacteria evade and survive immune responses is critical to development of management strategies to mitigate and control this disease.

This study investigates the role of the deoC1 and deoC2 paralogs of *S. agnetis* 908 which codes for DERA. Specifically, the work focused on identifying whether the A164E substitution is involved in increased bacterial survival and escape from macrophages. Chapter 2 discusses the selection of transformants containing 908 DNA that display macrophage escape and survival through directed genome evolution, while Chapters 3 and 4 explore the effect of *S. agnetis* on the host-cells by utilizing gentamicin protection assays and enzyme protection assays paired with crystal violet staining for cell damage or death.

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Chapter 2

Phagocytosis assays

2.1. Introduction

Macrophages are tissue-associated immune cells that are derived from the myeloid lineage (Murray and Wynn, 2011*,* Abbas, Lichtman and Pillai, 2012*).* They are important in both innate and adaptive immune responses and represent one of the first lines of defense against a pathogen invasion. Macrophages function by producing chemokines and cytokines that activate numerous immune cells. In addition, they trigger the complement cascade involved in pathogen lysis. However, the main function which macrophages are renowned for, is their ability to engulf pathogens and foreign antigens through the process termed phagocytosis (Drevets et al., 2015).

Phagocytosis assays have been developed as a method to study the host interaction with microbes *in vitro* (Caponegro et al., 2021). These assays can be used to evaluate the ability of host immune cells to engulf and internalize a pathogen and determine the bactericidal capacity of the immune cell. For such purposes, the assay often uses fluorescence tagged bacteria or flowcytometry to enumerate the viable intracellular and extracellular bacterial counts. Furthermore, phagocytosis assays are used in determining the pathogen's response to the host cell. Therefore, phagocytosis assays are a great tool to study host-pathogen interactions. The major components of a phagocytosis assay are the host cells, which could range from macrophages to polymorphonuclear neutrophils (PMNs), while the pathogen is most often a bacterial agent (Vonk et al., 2002) .

In our study we use phagocytosis assays to understand the interaction between a chicken-macrophage-like cell line and the Gram-positive bacterium *Staphylococcus agnetis*.

S. agnetis is a coagulase variable, Gram-positive bacterial species originally identified in cattle infected with mastitis (Taponen et al., 2012, Calcutt et al., 2014). *S. agnetis* has more

recently been isolated from chicken diagnosed with bacterial chondronecrosis and osteomyelitis (BCO) (Al-Rubaye et al., 2015, Al-Rubaye et al., 2017). Our study mainly focuses on *S. agnetis* 908, which had been isolated from BCO samples of chicken from the University of Arkansas Poultry Research Farm. Phylogenomics revealed that the chicken isolate 908 was most likely derived from within a clade of cattle isolates (Shwani et al., 2020). However, genomic changes attributed to the transition have yet to be established.

Previously we have identified that *S. agnetis* 908 has the ability to survive and escape chicken and mouse macrophage-like cell lines (Zaki, 2021). In contrast the cattle isolate, *S. agnetis* 1379, is much more susceptible to these phagocytes, despite having a very similar genome (Shwani et al., 2020). To identify the genetic components that contribute to enhanced macrophage survival and escape, these two isolates were used for directed genome evolution (DGE). For our work, 1379 was transformed with 908 DNA and the transformants were passaged through multiple phagocytosis assays consisting of chicken-macrophage-like cells called HTCs. After several passages the survivors displayed the phenotype of isolate 908. Multiple independent DGE transformants were generated and subjected to whole genome sequencing and genome assemblies were compared to 908 and 1379. This led to the identification of specific substitutions in either of two paralogs for deoxyribose-phosphate aldolase (deoC).

Both paralogs of the deoC gene code for a 220 amino acid polypeptide and both are located upstream of a gene coding for phosphopentomutase. The two paralogs can be distinguished by the other flanking genes. The gene upstream of deoC1 is ATP phosphoribosyltransferase regulatory subunit (hisZ) which is transcribed in the opposite direction as deoC1. The gene upstream of deoC2 is tetracycline resistance MFS efflux pump (TRI12) which is transcribed in the same direction as deoC2. Results of tBlastn searches indicate that *S. agnetis* 908 deoC1 is most similar to 1379 deoC2 while the 1379 deoC1 is most

similar to 908 deoC2. Furthermore, the major difference between the two paralogs in 908 vs 1379 is that residue 164 is an alanine in *S. agnetis* 1379, while it is a glutamate for 908.

2.1.1. Directed Genome Evolution

Directed Genome Evolution (DGE) mimics the evolutionary process of selection in a controlled, laboratory setting. Organisms are selected for a desired trait after subjecting the organisms to repetitive rounds of selection processes (Cobb et al., 2013). It has been used as a method to screen for virulence determinants in organisms that are genetically similar but have different phenotypes, as DGE was used to identify the specific amino acid substitution in a porin gene in *Campylobacter jenuni* associated with spontaneous abortion in sheep (Wu et al., 2016).

Previous research in our laboratory had utilized DGE to select for transformants of 1379 that display the phenotype of 908 for escape from and/or survival of HTCs during multiple rounds of phagocytosis assay (Zaki, 2021). This led to the identification of the deoC genes as the most likely determinant for this phenotype. In this chapter we used DGE to select for transformants of 1379 using specific PCR fragments containing the 908 deoC1 or deoC2.

2.2. Materials and methods

2.2.1. Cell cultures

Chicken-macrophage-like cell line (HTCs) was provided by Dr. N. Rath (Poultry Science Center, University of Arkansas, Fayetteville, AR) (Rath et al., 2003). The cell-line was maintained in cell culture flasks incubated at 37^{o}C and 5% CO_2 and passaged in 1:10 ratio every five days. The growth media used contained RPMI-1640 (Corning, Manassas, VA), supplemented with 1 x concentration of antibiotic–antimycotic solution (Invitrogen, Waltham, MA), 10 µg/mL of gentamicin sulphate (VWR, Radnor, PA), 1 mM sodium pyruvate (Invitrogen, Waltham, MA), 1x Glutamax (Invitrogen, Waltham, MA) 50 µM of 2-mercaptoethanol and 10%

low endotoxin fetal bovine serum (FBS) (Gibco, USA) (Rath et al., 2003) The volumes are described in protocol 1 (Supplementary).

The same medium was used in phagocytosis assays except the antibiotic- antimycotic solution and gentamicin was omitted and is referred to as antibiotic-free growth media. Gentamicin growth medium contained 50 µg/mL of gentamicin but did not contain the antibiotic-antimycotic solution.

2.2.2. Bacterial strains

The two main bacterial strains used in this study were *S. agnetis* 908 and 1379. *S. agnetis* 908 was obtained from chicken BCO samples from the University of Arkansas Poultry Research Farms. *S. agnetis* 1379 (MU50.56) was isolated from subclinical mastitis samples provided by Dr. J. Middleton from Department of Veterinary Medicine and Surgery, University of Missouri. The bacterial strains were maintained on Tryptic Soy (TS) agar slant at 4^{o}C.

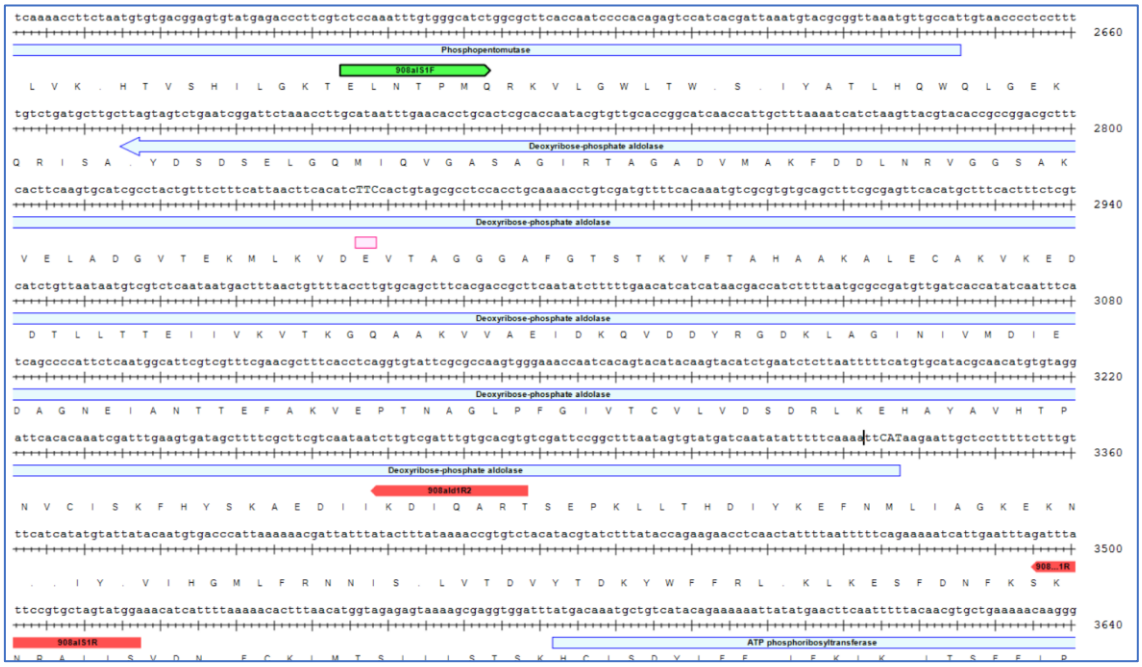

Figure 4. DNA sequence of the region of the *S. agnetis* deoC1 gene with the location of the PCR primers used to amplify specific regions.

Previous work involved transforming 1379 with whole genomic and extrachromosomal 908 DNA. The transformants for the current study were created by electroporation with specific

PCR products. The primer pair 908alS1F x 908ald1R2 partially covered the last half of the upstream gene phosphopentomutase to most of the deoxyribose phosphate aldolase gene (spanning 724 nucleotide bases including the codon that codes for the amino acid residue 164) (Figure 4).

Transformation was performed using electroporation (Shwani, 2021). The transformation with 908 deoC1 was divided into four biological replicates designated 1589A through 1589D. Similarly, the transformation with 908 deoC2 generated DGE products 1590A through 1590D. All stock samples were stored in -80^{o} C in TS broth containing 40% glycerol.

2.2.3. Phagocytosis Assay to determine the effect of multiplicity of infection.

A day prior to the assay, the cell cultures were incubated in antibiotic free growth media overnight. The media was aspirated and prewarmed 1 x Phosphate Buffered Saline (PBS) was added to the cell culture flask. The cells were scraped with a cell scraper and transferred into a 15 mL centrifuge tube. The cells were sedimented by centrifugation at 400 x G for 5 minutes. The pellet was resuspended in antibiotic free growth medium. Countess automatic cell counter (Invitrogen Waltham, MA) was used to enumerate the HTCs in 10 µl of the HTC suspension mixed with 10 µl of Trypan Blue. The HTC suspension was then diluted to achieve a concentration of 3 x 10^5 cell/mL of which 0.5 mL was added to each well of a 24-well tissue culture plate.

Overnight (2 mL, 37^{o}C, shaking at 120 RPM) TS broth cultures of *S. agnetis* 908 or 1379 were diluted 1:100 in antibiotic free RPMI media and their optical densities were measured at 650nm. The Colony Forming Units (CFU) per ml were estimated using standard curves for 908 and 1379 (Supplementary figure 21). The bacterial suspensions were diluted in antibiotic-free medium to achieve a concentration of 3 x 10^5 CFU/mL.

The cocultures were incubated at 37^{o}C, 5% CO_2 for four hours to allow for phagocytosis to take place. Each group had triplicates (n=3) each for every round of phagocytosis assay.

Phagocytosis was stopped by placing the tissue culture plate on ice for 10 minutes. The wells were then washed twice with 0.5 mL of ice-cold PBS to remove non-adherent cells and replaced with gentamicin growth media and incubated overnight (16 to 18 hours) at 37^{o}C, 5% CO_2. The following day, growth media was removed, and the wells were washed twice with 1 x PBS and replaced with 0.5 mL of antibiotic-free growth media. The plate was once again incubated overnight at 37^{o}C, 5% CO_2. Following the overnight incubation, based on phase contrast microscopy observations of the number of extracellular bacteria in the well, estimates were made to determine the dilution factor. The media in the wells were then transferred into 1.5 mL microcentrifuge tubes. This is referred to as the 'supernatant' which refers to the bacteria present outside the HTCs. The remaining HTCs in the wells were lysed by adding 0.5 mL of sterile distilled water and referred to as the 'lysate' which constitutes the cell-associated and bacteria internalized by HTCs. Serial dilutions of the supernatant and lysate were performed using TS broth and 100 µl of the final dilution was plated on TS agar plates which were incubated overnight at 37^{o}C for colony counts.

2.2.4. The effect of various types of sera on bacterial survival

The phagocytosis assay was carried out as described above. The growth media used in the assay, including antibiotic free growth media and growth media supplemented with gentamicin, were prepared as described in the previous section with the exception of the serum used. Instead of 10% low-endotoxin FBS, growth media was supplemented with 10% high-endotoxin FBS, chicken serum and BCO plasma for each of the respective categories tested. Table 1 describes the composition of each type of serum.

Both *S. agnetis* 908 and 1379 were tested for their ability to survive macrophages in each of the four types of growth media. All samples were in triplicates. Two days after coculture the total number of viable bacteria (Both extracellular and intracellular) were enumerated by plating the contents on TS agar and incubating overnight at 37^{o}C.

Table 1. Different sera used in the experiment and their constituents.

Type of serum / plasma	Components
Low-endotoxin Fetal Bovine Serum	Fetal Bovine Serum filtered with triple 0.1 µm filtered (Gibco)
High-endotoxin Fetal Bovine Serum	Fetal Bovine Serum, non-certified for endotoxin (Gibco)
Chicken serum	Chicken serum. Source: New Zealand (Gibco)
BCO plasma	Plasma separated from blood from broilers at the University of Arkansas Research Farms infected with BCO

2.2.5. Directed Genome Evolution

The selection process for DGE used in our study is macrophage survival through multiple rounds of phagocytosis assays. This was used in screening for the transformants (1589A to D and 1590A to D) that display 908's phenotype of macrophage survival. *S. agnetis* 908 and 1379 were used as controls. Phagocytosis assay was carried out as described in section 2.2.3. Two days after coculture, the supernatant and lysate were plated on TS agar and incubated overnight at 37^{o}C.

2.2.6. Molecular analysis

Colony Screening by PCR Sequencing

Individual colonies were picked and transferred on to a master TS agar plate. The plate was incubated overnight at 37^{o}C. The master place streak was sampled with a sterile toothpick and suspended in 100 µl of sterile distilled water in 0.2 mL microcentrifuge tubes. The tubes were thoroughly mixed by vortex and heated to 100^{o}C for 10 minutes followed by 20^{o}C for 10 seconds and stored at 4^{o}C until use.

DNA from colonies was verified by amplification using qPCR-HRM (quantitative PCR High Resolution Melt) with the 16SV2 primers: Bac16Sv2 F 5′-GCGRACGGSTGAGTAACACGTG-3′ and Bac16Sv2 R 5′-CGATCRCCCTYTCAGGTCGGCT-3′

(Zaki, 2021). The PCR mixture contained the following: 2µl of 10X buffer, 0.2 µl of 20 mM dNTPs, 0.2 µl of 10 U/ µl Taq Polymerase, 1 µl of 20 µM forward and reverse primers and 2 µl of the template DNA from the colony boil samples, in a total volume of 20 µl. *S. agnetis* 908 (2 ng/ µl) and 1379 (2 ng/µl) of DNA were used as positive controls while a reaction tube with no template DNA was used as the negative control. The samples were subjected to the following PCR conditions: Initial denaturation of 90^{o}C for 30 seconds, followed by 30 cycles of denaturation at 90^{o}C for 15 seconds, annealing temperature at 60^{o}C for 30 seconds, and elongation at 72^{o}C for 90 seconds, followed by the final elongation at 72^{o}C for 180 seconds.

Following the PCR reaction, amplification was evaluated by electrophoresis in a 1.5% agarose gel. Typhoon^{TM} FLA 9500 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used to scan ethidium bromide-stained gels.

The PCR samples with positive 16S bands were then subjected to PCR with 908alS1Fx908ald1R2 primers. The reaction mixture was prepared as described above. The PCR conditions were as above except the anneal temperature was 59^{o}C.

The PCR products were quantified by using Hoecht staining and fluorimetry with a GloMax Jr according to manufacturer procedures (Promega, Madison, WI) The quantified PCR samples were then cleaned using RapidTips (Chiral Technologies, West Chester, PA). The 908ald1R2 primer was added to the cleaned PCR products and submitted for capillary sequencing by the Eurofins Genomics Company (Louisville, KY). Sequence data was analyzed using SeqMan Pro software (DNAStar version 16, Madison, WI). *S. agnetis* 1379 genome was used as the reference genome (Shwani et al. 2020).

2.2.7. Statistical analysis

Student t-test and one way ANOVA followed by Tukey's post hoc test were used in statistical analysis. All analyses were done using R (R Core Team, 2019) and Microsoft Excel. Significant differences were accepted at $P < 0.05$.

2.3. Results

2.3.1. Multiplicity of infection

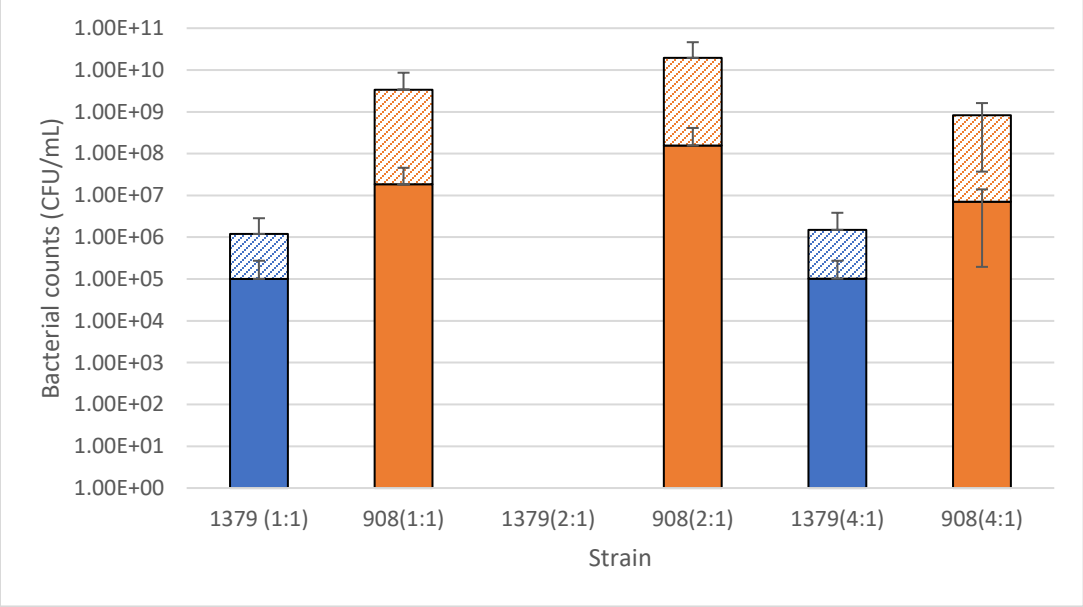

Figure 5. The bacterial counts in Colony Forming Units (CFU/mL) obtained from plating the supernatant (solid fill) and lysate (hatched fill) two days after coculture. *S. agnetis* 1379 in blue, 908 in orange. The error bars represent standard deviations (n=3).

To compare the effect of varying concentrations of bacteria on HTCs, bacteria of concentration 3 x 10^5 cells/mL were added to each well with 0.5 mL of HTC in volumes described in table 2. All samples were run in triplicate.

Table 2. Indicates the volumes of each bacterial suspension of concentration 3 x 10^5 cells/mL that was added to each well.

Ratio	**1**	**2:1**	**4:1**
Volume of Bacterial suspension (mL)	0.5	0.25	0.125
Volume of HTC (mL)	0.5	0.5	0.5

Reducing the concentration of bacteria did not have a significant effect in bacterial survival in both the supernatant and lysate groups ($P > 0.05$). Overall, *S. agnetis* 908 had significantly more survivors both intracellularly as shown by the bacterial counts obtained from

the lysate, and extracellularly as reflected by the bacterial counts of the supernatant (Figure 5). No bacterial growth was observed when 1379 was cocultured with HTCs in 2:1 ratio.

2.3.2. The effect of various types of sera on bacterial survival

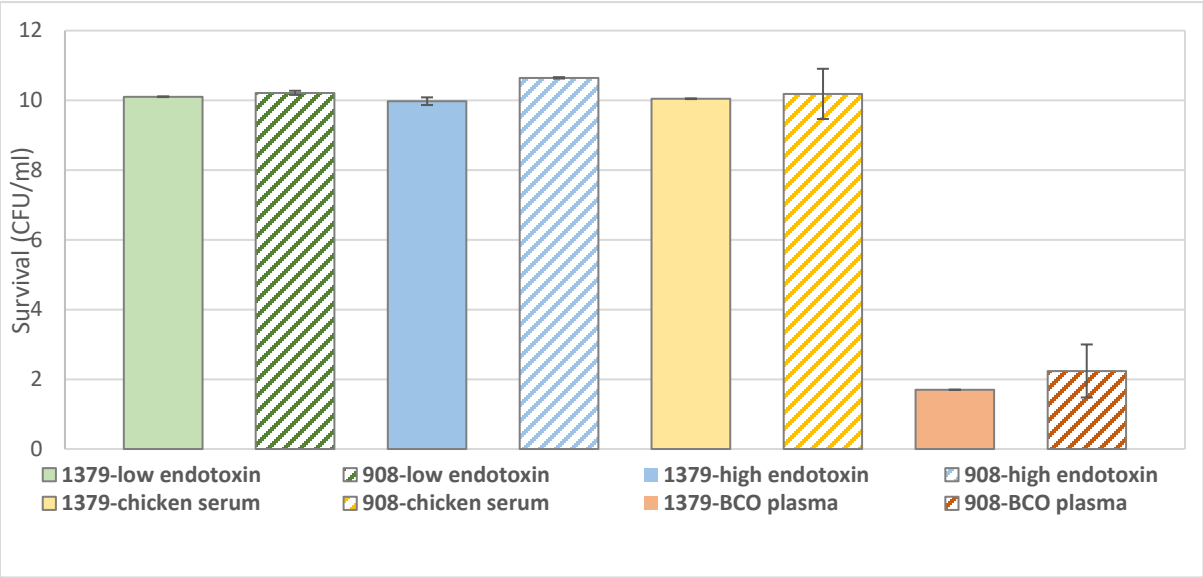

Figure 6. The effect of sera on bacterial survival of *S. agnetis* 908 (hatched fill) and *S. agnetis* 1379 (solid fill). The survivors consist of combined total of the intracellular and extracellular bacterial populations and is measured in log transformed colony forming units (CFU)/mL. Error bars represent standard deviations (n=3)

To test for the effect of different sera used in supplementing the growth media we tested the survival response of *S. agnetis* 908 and 1379 to growth media enriched with four different types of sera: High-endotoxin FBS, low-endotoxin FBS, chicken serum and BCO plasma. BCO plasma was obtained from separating plasma from blood drawn from broilers at the University of Arkansas Research Farm diagnosed with BCO.

The number of viable *S. agnetis* 908 and 1379 significantly decreased when growth media supplemented with plasma from chicken infected with BCO ($P < 0.05$). Meanwhile using high-endotoxin serum or chicken serum did not have profound effects on bacterial survival (Figure 6). Furthermore, there was no significant difference between 908 and 1379 when low-

endotoxin FBS and chicken serum were used. However, 908 showed higher macrophage survival ($4.48x10^{10}$ CFU/mL) when incubated with high-endotoxin FBS (P = 0.013).

2.3.4. Directed Genome Evolution

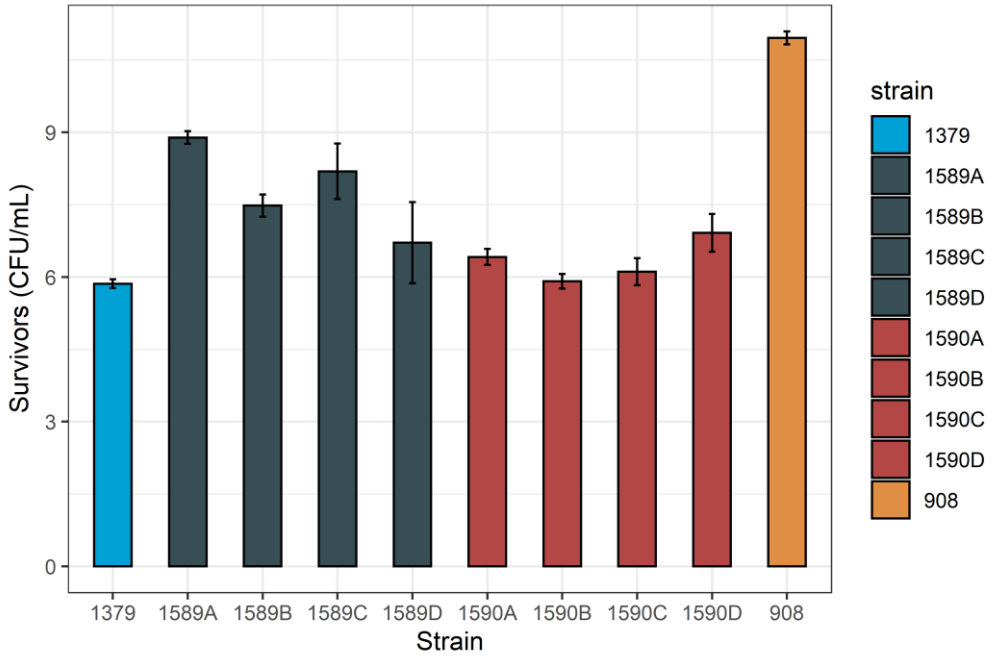

Figure 7. The survivors (both extracellular and intracellular) of the first round of DGE indicated by CFU/mL. The colony counts are log transformed. Error bars represent standard deviations (n=3).

DGE was used to positively select for the transformants that displayed macrophage survival and escape similar to 908. This screening process utilized phagocytosis assays where the transformants were cocultures with HTCs. *S. agnetis* 1379 has the lowest colony counts indicating the least number of survivors with only $6x10^5$ CFU/mL (Figure 7). In contrast *S. agnetis* 908 had colony counts of $8.8x10^{10}$ CFU/mL. The first round of DGE identified 1589A, 1589B and 1589C which were transformed with 908 deoC1 to have significantly higher survivors compared to 1379 ($P < 0.05$) while the transformants of 908 deoC2 did not show significant survival ($P > 0.05$). Transformant 1589A had the highest number of colony counts ($8.05x10^8$ CFU/mL) from all 1589 transformants. Some of these colonies may have incorporated the 908 PCR product by homologous recombination.

Due to lack of reproducibility, the second round of phagocytosis assay was not completed (Described in section 2.4.3).

2.3.5. Molecular analysis

Since the 1589A, B and C transformants displayed phenotypes closest to 908, individual colonies were subjected to DNA sequencing to determine their genetic composition. Following the first round of DGE, PCR was carried out on the surviving 1589A, 1589B and 1589C colonies (See materials and methods).

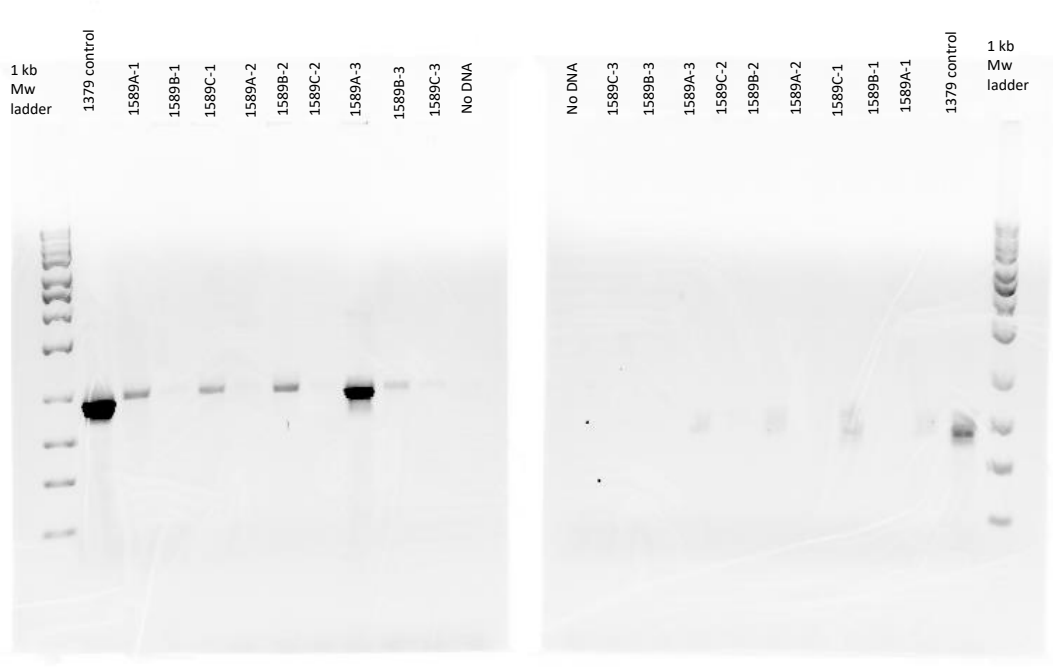

Figure 8. (A) Gel images for PCR with 16SV2 primers. (B) Bands from PCR with 908alS1Fx908ald1R2 primers.

Figure 8 shows the results for the PCR reaction with the initial 16SV2 primers that positively identified and confirmed the presence of bacterial DNA. Following which, those samples were subjected to PCR using the 908alS1Fx908ald1R2 primers. The results show weak bands for two of the 1589A replicates and 1589B replicates and a single replicate for 1589C (Figure). These five samples were submitted for sequencing.

Four of the five samples sent for sequencing – 1589A1, 1589A3, 1589B2 and 1589C1 provided results of good quality. Aligning these sequences with *S. agnetis* 1379 as the reference gene, we identified that bases corresponding with positions 2,765 to 2,850 were different for the transformants relative to 1379 but were identical to 908. However, from 2,660 and 2,621 bases, the transformants had sequences unique to them.

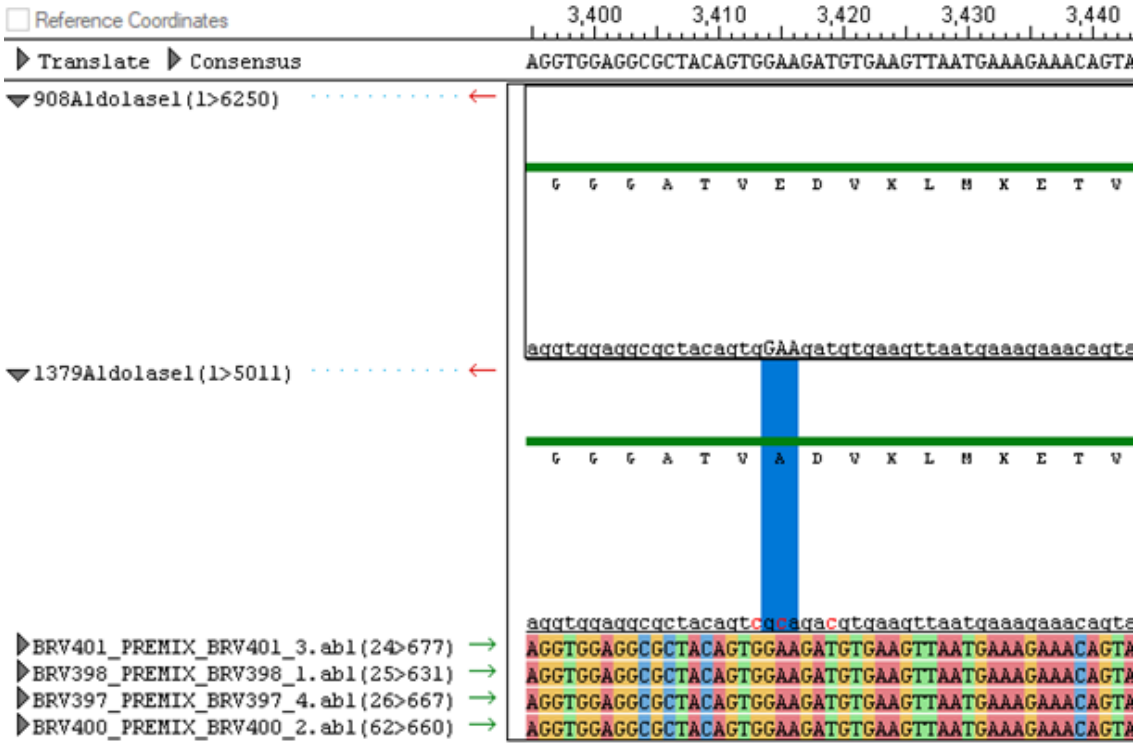

Figure 9. SeqmanPro alignment of the PCR sequence data for DGE transformants aligned with 1379 and 908 reference sequences. Region spans the codon for residue 164 (blue highlight).

Furthermore, bases 2,846 to 2,848 which codes for the 164th amino acid residue was GAA for all transformants which was identical to that of 908. GAA codes for glutamate. Meanwhile the same bases in the reference 1379 gene were GCA which codes for alanine. Thus, it is evident that the transformants that survived macrophages in a manner similar to that of 908 possess the A164E substitution. In addition, the results are indicative of a successful transformation (Figure 9).

2.3.6. Problems with phagocytosis assays

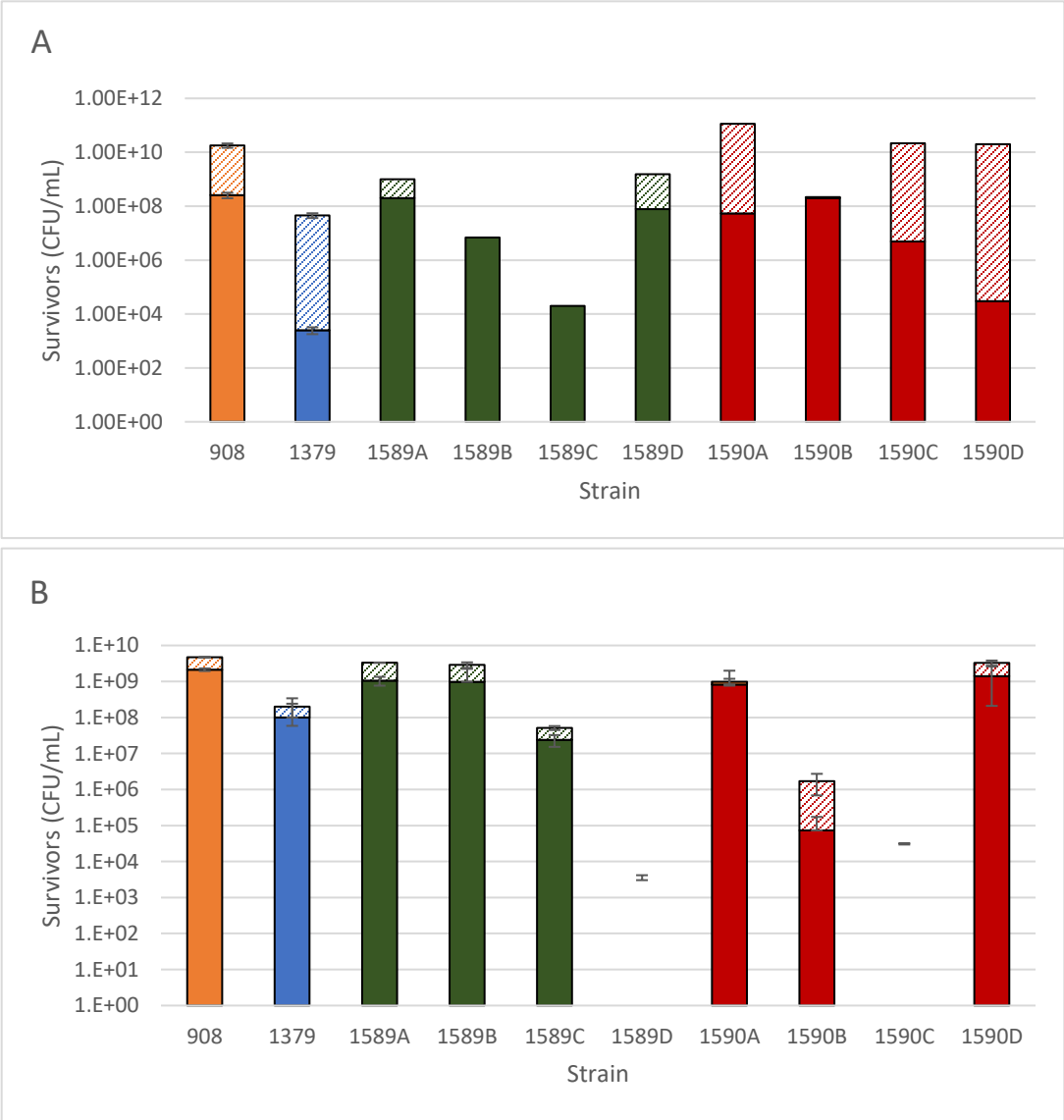

Figure 10. (A) Survivors of round-1 of DGE. *S. agnetis* 908 has the highest number of survivors followed by transformants 1589A, 1589D, and all of 1590A-D and 1379 with the least number of survivors. (B) Round-2 of DGE, with 908 having the highest number of survivors. Only 1589A, 1589B, 1590A and 1590D displayed the survival phenotype. 1589D and 1590C did not contain viable bacteria in supernatant. Supernatant in solid fill, lysate indicated by the hatched fill.

Phagocytosis assays were carried out to study the interaction between *S. agnetis* and the HTCs. Enumerating the total viable bacteria after cocultures provides understanding on the virulence of the bacterial strain. The phagocytosis assay results proved difficult to reproduce. Figure 10 shows results several rounds of phagocytosis assays where the *S. agnetis* 1379

control did not display consistent results in terms of survival. The first round of phagocytosis assay (Figure 10.A), 1379 had a total of $2.28x10^7$ CFU/mL of bacteria surviving macrophages, but only $2.5x10^3$ CFU/mL were isolated from the supernatant. However, when the experiment was repeated by passaging the survivors of round one, $1x10^8$ CFU/mL of 1379 were isolated from the supernatant (Figure 10.B). Yet, the total number of viable 1379 remained constant between $2x10^7$ to $2x10^8$ CFU/mL.

Additionally, the survivors for transformant 1589B and C were less than that of 1379 in the first round of DGE. Furthermore, transformants 1589D and 1590C did not have viable bacteria in the supernatant but when lysate was plated, 1589D had $3.6x10^3$ CFU/mL and 1590C had $3.1x10^4$ CFU/mL of viable bacteria.

2.4. Discussion

2.4.1. Comparison of different multiplicity of infection.

Multiplicity of infection (MOI) is defined as the ratio of infective agents to the target, which in our study is the ratio of *S. agnetis* 908 or 1379 to HTCs (Abedon and Bartom, 2013). Our findings indicate the MOI did not influence the outcome of the phagocytosis assay. The HTCs did not have an advantage when the concentration of bacteria was decreased nor were the bacteria more susceptible to elimination. Others (Hamza and Li, 2014) have reported similar results where the MOI did not have an effect on the number of viable intracellular *Staphylococcus aureus* and cytolysis. Instead, that study reported that the infection time influenced the number of bacteria internalized by macrophages. However, organisms where the MOI influence the immune reaction and cytolysis do exist. *Mycobacterium tuberculosis* is one such bacteria. Several studies describe that increasing the MOI enhances apoptosis of macrophages and ability of the immune cells to eliminate the bacilli, but these findings were not observed with *S. aureus* (Butler et al., 2012, Lee et al., 2006, Wei et al., 2000).

We did not observe viable bacteria when HTCs were cocultured with *S. agnetis* 1379 in a 2:1 ratio. It is possible that this might have been a result of erroneous pipetting leading to inaccurate bacterial or HTC concentrations.

2.4.2. The effect of various types of sera on bacterial survival

Our results indicated that using plasma from chicken diagnosed with BCO drastically decreased the number of both *S. agnetis* 908 and 1379 alike. Plasma, unlike serum, contains clotting factors and apart from differences in electrolyte and chemical composition, both plasma and serum contain immunoglobulins (antibodies) and proteins such as albumin and complement factors (Triglia and Linscott, 1980, Hrubec et al., 2002). The plasma from BCO chickens likely contains antibodies against BCO causing pathogens such as *S. agnetis*. Several studies have identified that opsonization of the pathogen by the antibodies result in an increase in the rate of pathogen elimination. Furthermore, it enhances macrophage oxidative bursts (Cheng et al., 2001). Antibodies against fibrinogen binding protein of *Staphylococcus epidermidis* were more efficient at opsonization of this bacterium resulting in more potent phagocytosis, compared to antibodies against autolysin E (Rennermalm et al., 2004). However, a study on Group B *Streptococci* revealed that the antibodies were able to kill bacteria regardless of the type of serovar (Cheng et al., 2001). At present we do not have data on the type of antibodies present in BCO plasma, but it is consistent with other studies, that the presence of antibodies in plasma or serum would kill pathogens efficiently as reflected by the low bacterial counts for both 908 and 1379.

Our routine growth media are supplemented with 10% low endotoxin fetal bovine serum (FBS). Endotoxin consists of lipopolysaccharides (LPS) and (1→3)-beta-D-glucan (Nikaido and Vaara, 1985). When present in serum used in supplementing cell or tissue culture growth media, it affects the bioactivity of the cell lines which then impacts the outcome of experiments. Therefore, endotoxins are considered a contaminant and confounding component in

media/serum. The presence of endotoxins in cell cultures stimulates the production of Tissue Necrosis Factor (TNF) (Rietschel et al., 1994). TNF production in J774.1 cell line induced by LPS occurs in a dose-dependent manner (Kirikae et al., 1997). Our findings report a significant difference between the number of survivors of 908 and 1379 when growth media supplemented with high-endotoxin FBS is used. However, there is no significant difference between 1379 survivors when incubated with low endotoxin and high endotoxin growth media which suggests that the outcomes are not exclusively the result of the type of serum used. Additionally, there appears to be an overall large number of 1379 survivors regardless of the type of sera used with the exception of BCO plasma. This further proves that the outcome observed is not solely based on the serum used.

2.4.3. Directed Genome Evolution and phagocytosis assays.

DGE requires multiple rounds of phagocytosis assays to select for the transformants with the macrophage survival phenotype of 908. The first round of phagocytosis assay identified three biological replicates of 1589 which were transformed with 908 deoC1 gene that showed macrophage survival closer to 908, but not identical. To ensure the accurate phenotype, the survivors should be passed through HTCs once again. However, the lack of reproducible results confounded the second round of phagocytosis assay.

There are several factors that could contribute to these inconsistent results. We hypothesized that the overnight incubation in 50 µg/mL of gentamicin could have contributed to the inconsistencies. This step is carried out to ensure that all extracellular bacteria are eliminated so that the internalization of bacteria are kept constant by limiting phagocytosis to only four hours. In addition, gentamicin, an aminoglycoside, is supposedly impermeable to eukaryotic cells, therefore it would exclusively eliminate the extracellular bacteria. However, it should be noted that it is important to test for antibiotic susceptibility of bacteria to gentamicin prior to using the gentamicin protection assay. We now believe that an overnight incubation in a

high concentration of gentamicin may have allowed gentamicin to enter the eukaryotic HTCs (VanCleave et al., 2017, Drevets et al., 1994, Ohya et al., 1998, Eze et al., 2000, Hamrick et al., 2003). Internalized gentamicin paired with the possibility of differential sensitivity of 908 and 1379 to gentamicin may have influenced the results of the phagocytosis assay. This is investigated further in chapter three.

Despite the confounding issues with the phagocytosis assay, we were able to select and sequence the DGE survivors for the first round of phagocytosis. The results indicate that 85 nucleotide bases of the transformants with phenotype close to that of 908 macrophage survivors contained the 908 sequence for codon 164 of deoxyribose aldolase phosphate gene. Previous DGE research had identified that the only consistently transferred change in 1379 when transformed with 908 DNA and selected through HTC phagocytosis assays was the substitution of the polar amino acid glutamate for the non-polar alanine for amino acid 164 of deoxyribose phosphate aldolase. When 1379 was transformed with 908 whole genome DNA, the survivors of DGE were sequenced and revealed to have the A164E substitution. This suggested that the A164E substitution had a role in influencing 908's ability to survive or escape macrophages. The importance of the 1589 transformants with the PCR products of 908 deoC1 gene are that the survivors all contain the 908 codon for residue 164. This confirms the function of deoC in macrophage survival. Whereas the previous DGE transformants with 908 DNA contained multiple sequences from the 908 donor DNA (Shwani, 2021). Therefore, the current findings not only confirm a successful transformation but also proves that the A164E substitution is associated with the survival in and escape from macrophages.

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Chapter 3

Gentamicin Protection Assays

3.1. Introduction

Gentamicin Protection Assays (GPA) can be used to evaluate the number of viable bacteria with in a host cell. Gentamicin is an aminoglycoside with bactericidal effects. It has been the drug of choice for treating aerobic Gram-negative bacterial infections espcially those that are caused by the *Enterobacteriaceae* family (Chaves and Tadi, 2021).

Gentamicin requires oxygen to cross the Gram-negative bacterial cell membrane. Once inside the cell, it binds to 16S rRNA at the 30S ribosomal unit (Beganovic et al., 2018). This inhibits translation of mRNA resulting in the production of non-functional, faulty proteins, which are thought to be responsible for cell wall impermeabiltiy. In addition gentamicin would increase the production of reactive oxygen species. It is hypothesized that the combinatory effect of a permeable cell wall and reactive oxygen species results in bacterial cell death (Kohanski et al., 2007).

Gentamicin has been known for its poor membrane permeability thereby, lacks the ability to enter eukaryotic cells. Due to this property, it has been the basis for antibiotic protection assays and has been widely used in studies to eliminate extracellular bacteria (VanCleave et al., 2017).

GPA has been used to study the invasiveness of bacteria such as Shigella by exposing the infected host cells to gentamicin for a short period of time (Sharma and Puhar, 2019). It has also been used in studies which ennumerate the internalized bacteria using techniques such as fluorescence microscopy-based, immunofluorescence and FACSflourescence imaging. In our protocol for macrophage assays, 50 µg/mL of gentamicin was used overnight to eliminate the

exracellular bacteria which was important in order to study the effect of intracellular bacteria on macrophages.

Despite the wide use of antibiotic protection assays, several studies reveal that gentamicin is capable of slowly entering the eukaryotic cell over time and hence influencing the number of intracellular bacteria (VanCleave et al., 2017, Drevets et al., 1994, Ohya et al., 1998, Eze et al., 2000, Hamrick et al., 2003). These studies have highlighted the importance of evaluating the optimal time and concentration for gentamicin exposure.

3.1.1. Crystal violet assay to study the effect of *S. agnetis* on HTCs.

The Directed Genome Evolution (DGE) experiment posed a few problems. Obtaining consistent results were a challenge. Microscopic observations suggested that the HTCs had significant damage the day following the coculture. To study the damage inflicted by each bacterial strain and the 1379 transformant, a staining technique using crystal violet dye was used.

Crystal violet stains the adherent cells by binding to the cell proteins and DNA. Non-viable HTCs and bacteria will detach from the plate surface and are removed. Subsequent staining with crystal violet would indicate only viable cells (Feoktistova et al., 2016). To obeserve the affect of bacterial invasion on the host cells, we used crystal violet to stain viable macrophages. Gentamicin Protection Assay was used to ensure bacteria that escape macrophages would not proliferate extracellularly.

3.1.2. Determining bacterial survival within macrophages

Macrophages are the first line of defense during an infection. Once the bacteria has been engulfed by the macrophage, they are encased with in phagosomes where they are killed with in 15 to 30 minutes by host defense mechanisms such as reactive oxygen species, acidic environment, nutrient depletion and hydrolytic enzymes (Pidwill et al., 2021, Ernst et al., 1999).

Despite being a hostile environment, macrophages are known to be the preferred replication site by most pathogenic bacteria (Thi et al., 2012, Price and Vance, 2014).

Bacteria are constantly evolving to be able to use the host intracellular environment to their benefit. They are capable of altering gene expression to allow for survival and proliferation with in the host (Mekalanos, 1992). For example, *Yersinia pestis* is able to evade phagocytosis, while *Listeria* and *Shigella* species are able to escape phagosomes and enter the cytosol (PizarroCerda et al., 1997). Other bacteria like *Legionella* species and *Mycobacterium tuberculosis* and *Salmonella* are able to prevent the fusion of lysosome and the phagosome and prevent acidification of the phagosome respectively.

To better understand the extent to which *S. agnetis* 908 and 1379 would survive within the HTCs, we lysed the remaining HTCs on day 0, day 1, day 2 and day 3 to determine the number of cell-associated bacteria.

3.1.3. Comparing the effect of heat-inactivated serum on HTC viability

Heat inactivation of serum eliminates complement proteins that are involved in eliciting immune responses in the host. This is important in cytotoxity assays and viral assays where the presence of complement proteins would interfere with results. Several studies highlight the importance of heat inactivating serum. (Fante et al., 2021) states that heat inactivation of human serum promotes human T cell function by reducing C1 inhibitor levels, C3c, C4 and factor B levels while enhancing C1q-IgG and Csd-IgG leves. *Heat-inactivated* serum has also been recommended for growing embryonic stem cells lines (Robertson, 1987) and for insect cell lines (Weiss et al., 1992). Soltis et al., (1979) states that heat inactivation of serum helps avoiding immunoglobin aggregation. In addition, in the past it had served as a method to eliminate mycoplasma and other microorganisms which are known cell culture contaminants. However, at present filteration through 0.1 µm filters eliminate mycoplasma thus heat inactivation is not necessary for this purpose (ATCC - Purpose of heat-inactivating serum-154, 2021).

Fetal bovine serum (FBS) is a common component in cell cultures, serving as a nutrient source and enhances cell proliferation adherence to surfaces. FBS is composed of several complement proteins including 1-3% of human complement levels of C1 and C6 and 5-50% of human levels of other complement proteins with the exception of C3 (Triglia and Linscott, 1980). FBS is completely devoid of fibrin and clotting factors while bovine serum albumin is one of the major consituents of FBS. Other components include growth factors such as hormones, lipids, sugars and protease inhibitors which supports cell growth and provides protection to the cells (Shah, 1999, Even et al., 2006, Kawakami et al., 2016).

Whether serum should or should not be heat-inactivated has been a debate in the scientific community. As of recent, heat inactivation of serum has been discouraged since exposing serum to heat not only destroys heat labile complement proteins but also tend to reduce or destroy serum growth factors essential for cell proliferation and adhesion(Giard, 1987). In their study, Soltis et al., (1979) also reveals that heat inactivation had negatively impacted the growth and proliferation of six cell lines (HBAE, MDBK, Vero, human foreskin fibroblast, MRC-5, and Mv 1Lu).

Therefore, to study the affects and implications of using *heat-inactivated* serum in our cocultures we compared the number of viable HTCs following the infection with *S. agnetis* 908 and 1379 in media supplemented with normal fetal bovine serum (NS) and heat-inactivated FBS (HI).

3.2. Materials and methods

3.2.1. Determining *Staphylococcus agnetis* sensitivity to gentamicin

A 1/100 dilution of an overnight bacterial suspension of 1379 and 908 was made by adding 2 µl of the overnight culture to 2 mL of prewarmed Tryptic Soy (TS) broth. A series of

varying concentration of gentamicin sulphate solutions ranging from 5 µg/mL to 100 µg/mL were prepared as described in the table 3.

Table 3. Volumes of gentamicin sulphate solution added to create a series of varying concentrations to test for sensitivity of *S. agnetis* 908 and 1379 to gentamicin.

	Control	5 µg/mL	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
Volume of 50 mg/mL gentamicin (µl)	0	0.2	0.3	0.6	0.9	1.2	1.5	2.25	3
Volume of RPMI no antibiotics (mL)	1.5	2	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Volume of $1/100^{th}$ dilution of overnight bacterial cultures (µl)	15	20	15	15	15	15	15	15	15

The samples were incubated at 37^{o}C. Optical density readings at 650 nm were taken at each hour for 5 hours. The respective CFU counts were obtained by using the standard curve for each bacterium (Supplementary figure 21).

3.2.2. Crystal violet assay

Preparing the coculture

Bacteria were coculutred with HTCs in 1:1 ratio. Briefly, after an overnight incubation in RPMI 1640 growth media with no antibiotics or antimycotics, HTC cells were scraped and aspirated into a 15 mL conical flask. The HTC cells were sedimented by subjecting to centrifugation at 400xg for 5 minutes. The HTC pellet was resuspended in growth media with no antibiotics or antimycotics. The cell count was obtained using The Countess^{TM} automated cell counter (Invitrogen^{TM}, Carlsbad, California) with 10 µl of HTCs diluted in 10 µl of trypan blue. A

suspension containing $2x10^5$ cells/mL of HTC cells was prepared and 0.5 mL was added into each well of a 24 well tissue culture plate.

Overnight subcultures of *S. agnetis* 908 and 1379 was diluted 1:100 in RPMI 1640 growth media with no antibiotics or antimycotics. The optical density was measured at 650 nm. The colony counts were for each dilution was determined using the standard curves (Supplementary figure 21). A dilution was made to achieve $2x10^5$ cell/mL and 0.5 mL of this suspension was added to each well containing 0.5 mL of $2x10^5$ cell/mL of HTC.

The coculture was incubated at 36° C, 5% CO_2 for 4 hours to allow for phagocytosis to take place after which the plate was placed on ice for 10 minutes to stop phagocytosis. The wells were washed twice with 1 x Phosphate Buffered Saline (PBS) to remove any extracellular unattached bacteria or HTCs.

Crystal violet staining

The wells were stained with 0.5% crystal violet on day 0 (Following 4 hours of phagocytosis), day 1, day 2 and day 3 following coculture. For wells allocated for days 1 to 3, the wells were refed with 0.5 mL of RPMI 1640 with 50 µg/mL of gentamicin following the wash with PBS.

Crystal violet assay was performed as described in (Feoktistova et al., 2016) with slight modifications. In brief, 200 µl of 0.5% Crystal Violet was added to the PBS washed wells and allowed to incubate for 20 minutes at room temperature on a bench rocker with a frequency of 20 oscillations per minute (University of Kansas Medical Center, 2021). Crystal violet was then removed, and the wells were washed four times with distilled water ensuring that cell are not washed away. The plate was then allowed to dry for four hours. The stain was solubilized by adding 600 µl of methanol and incubating the plate for 20 minutes at room temperature on a bench rocker with a frequency of 20 oscillations per minute. Two-hundred 200 µl of methanol solubilized crystal violet stain was transferred on to a 96 well flat bottom microtiter plate and absorbance was measured using ELx808 Absorbance Reader (Bio-Tek Instrument,Inc,

Winooski, Vermont) at 570 nm. Each sample was run in triplicate and three empty wells stained with crystal violet was used as the blank.

3.2.3. Determining bacterial survival within macrophages

Bacteria and HTCs were cocultured as described in the above section. The gentamicin protection assay was carried out over a three day period during which each day, the wells were replaced with 0.5 mL of RPMI 1640 growth media supplemented with 50 µg/mL of gentamicin and allowed to incubate overnight with the exception of wells allocated for day 0. These wells were subjected to only a two-hour incubation with gentamicin supplemented growth media and then lysed to determine the cell associated bacterial counts. The only variation was the introduction of 1589A which is a DGE transformant of *S. agnetis* 1379 (Described in Chapter 2).

The wells to be processed were washed with 1 x PBS four times and 0.5 µl of sterile distilled water was added in order rupture the remaining HTCs. The lysate was aspirated into a 1.5 mL microcentrifuge tube. 1/10th dilution of the lysate was plated on Tryptic Soy (TS) agar plates and incubated overnight in 37^{o}C. This would provide the cell-associated bacteria for each day.

3.2.4. Comparing the effect of heat-inactivated serum on HTC viability

The coculture and gentamicin protection assay was performed as described in the previous section. For the heat-inactivated group, RPMI 1640 growth media was enriched with heat-inactivated fetal bovine serum (FBS). In brief, heat-inactivated FBS was prepared by incubating low-endotoxin FBS (Gibco) at 56^{o}C for 30 minutes, shaking every 10 minutes (Johnson, 2012, ATCC - Purpose of heat-inactivating serum-154, 2021). Heat-inactivated FBS was added to growth media containing sodium pyruvate (Invitrogen, Waltham, MA), 2-mercaptoethanol (Invitrogen, Waltham, MA), L-GlutaMAX^{TM} (Invitrogen, Waltham, MA) and RPMI-1640 (Corning, Mansaas, VA) prepared according to protocol 1 (Supplementary) to make

up to 10% of the total volume of media. Growth media enriched with normal FBS was also prepared in a similar manner. Each sample was run in triplicates.

Crystal violet assay was performed on each day, for three days following the coculure as described in the previous section. Sampling was done on day 0 (4 hours after coculture), day 1, day 2 and day 3. 0.5 mL of growth media containing 50 µg/mL of gentamicin was added to each well each day. The optical density at 570 nm was measured using the microtitre plate reader.

3.3. Results

3.3.1. Results of *Staphylococcus agnetis* sensitivity to gentamicin

The OD and CFU data indicate that 908 is susceptible to all concentrations of gentamicin sulphate after 2 hours of incubation (Figure11). However, 1379 does not appear to be affected by gentamicin sulphate (Figure 12). Interestingly, all gentamicin sulphate treated samples are similar to the untreated control 1379.

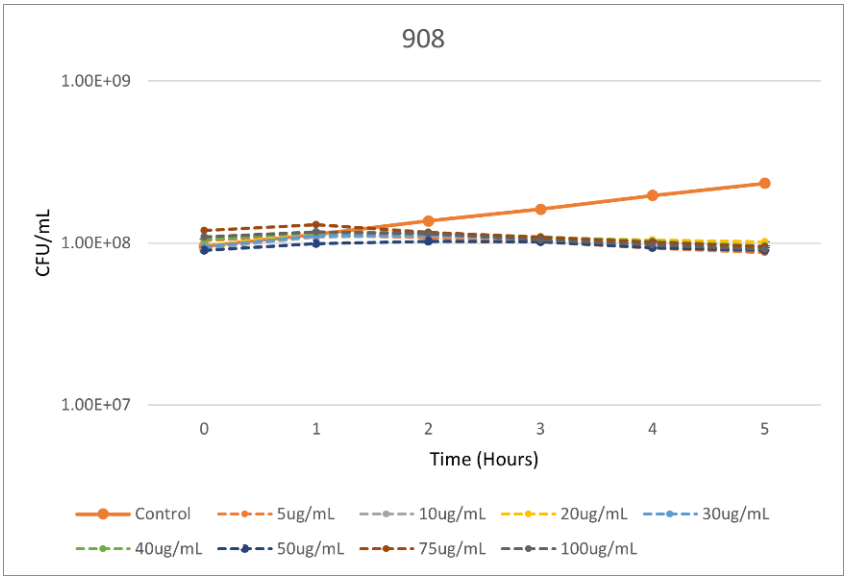

Figure 11. Computed colony forming units of *S. agnetis* 908 following treatments with different concentrations of gentamicin sulphate over a 5-hour period. The control has not been treated with gentamicin and is indicated with a solid orange line.

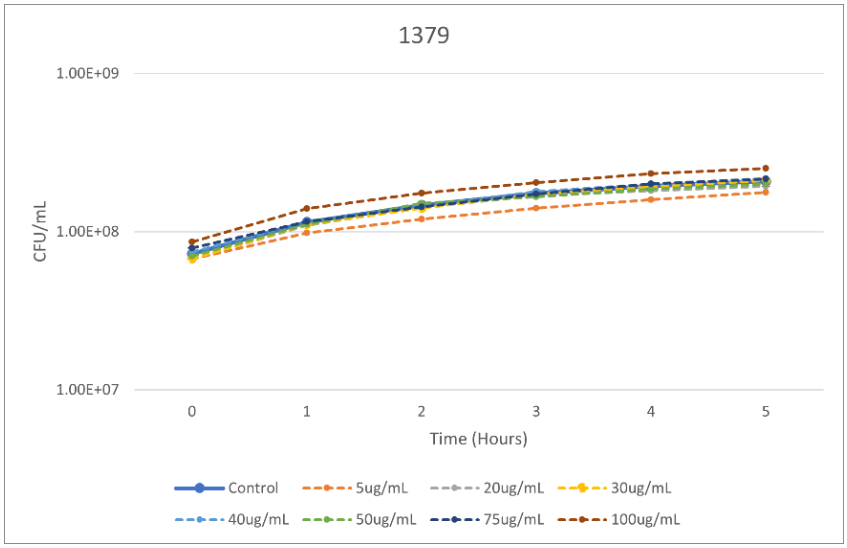

Figure 12. Computed colony forming units of *S. agnetis* 1379 following treatment with different concentrations of gentamicin sulphate over a 5-hour period. The control has not been treated with gentamicin and is indicated with a solid blue line.

3.3.2. Results of crystal violet assay

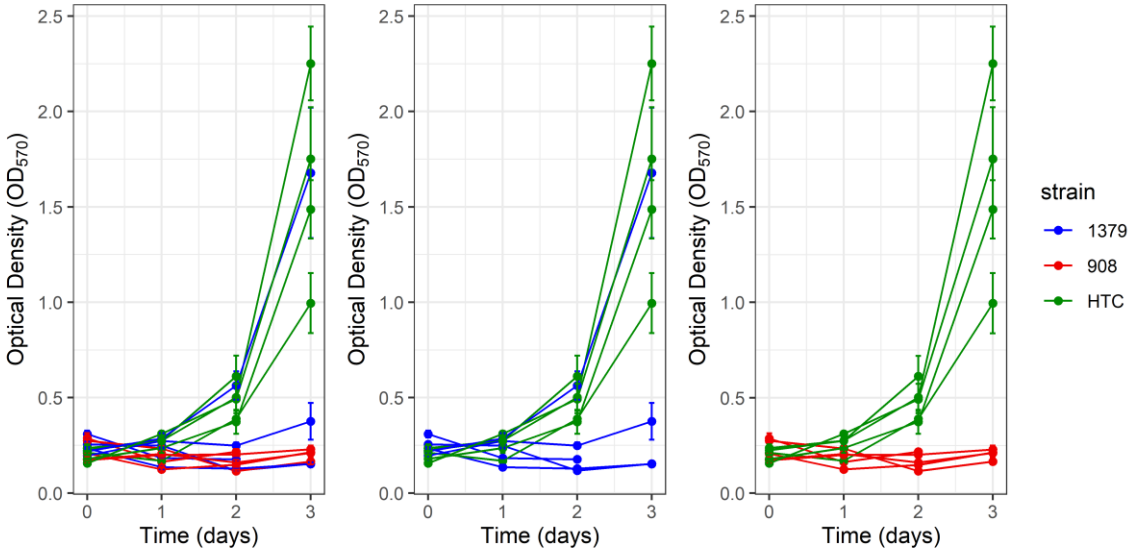

Figure 13. Compilation of data from multiple trials of crystal violet assays. Each sample was run in triplicates. **(Left)** Mean optical density readings of HTCs infected with *S. agnetis* 908, 1379 and HTC control; **(Centre)** OD readings of *S. agnetis* 1379 infected HTCs compared with HTC control; **(Right)** OD readings of *S. agnetis* 908 infected HTCs compared with HTC control. Error bars represent standard deviations (n=3).

The optical density readings correlate to the number of viable HTCs in each sample. The HTCs on the control wells proliferated exponentially during the three day period while the HTCs in wells infected with *S. agnetis* 908 did not show exponential growth (Figure 13). Instead there

is a slight decrease in the number of viable HTCs on the first day following coculture. Interestingly, the wells infected with 1379 show more variabilty in their optical density readings unlike 908. In some experiments the 1379 wells have OD readings similar to that of the control wells indicating proliferation while in other experiments the OD values were similar to that of the 908 infected wells.

The HTC control wells continued to proliferate exponentially since they were uninfected and were supplied with ideal growth conditions. The 908 infected HTC cells declined in number due to death caused by bacterial invasions. It is clear that during an infection, the HTCs are unable to proliferate.

3.3.3. Results of determining viable cell-associated bacteria.

Day 0 provides the colony counts of the cell-associated bacteria 4 hours after coculture. The results show that approximately 90% of the bacteria that were introduced into the coculture had survived the two-hour incubation in gentamicin growth media which suggests that the colony counts we obtained by plating the lysate are the intracellular and cell-associated bacteria.

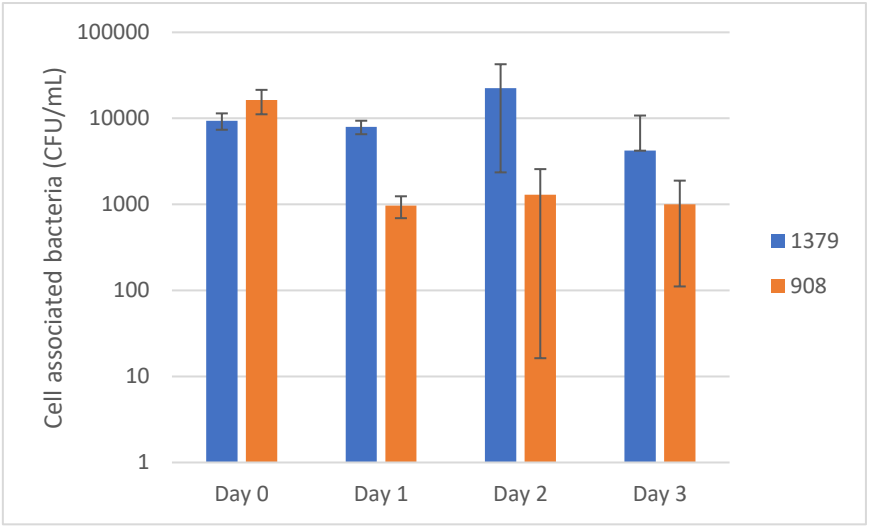

Figure 14. Colony forming units (log scale) determined for the cell-associated bacteria over a three-day period. Error bars represent standard deviations (n=3)

There is no significant difference between the cell-associated colony counts for 908 and 1379 (P>0.05). However, after being exposed to an overnight incubation in gentamicin supplemented growth media, the number of cell-associated 908 have significantly decreased (P = 0.00675) and continues to remain constant during the next few days while, the cell associated bacterial counts for 1379 remain unchanged (Figure 14). It is evident that *S. agnetis* 1379 has more cell associated bacteria remaining at the end of the three-day period (45%) when compared to 908 (6%) (Figure 15).

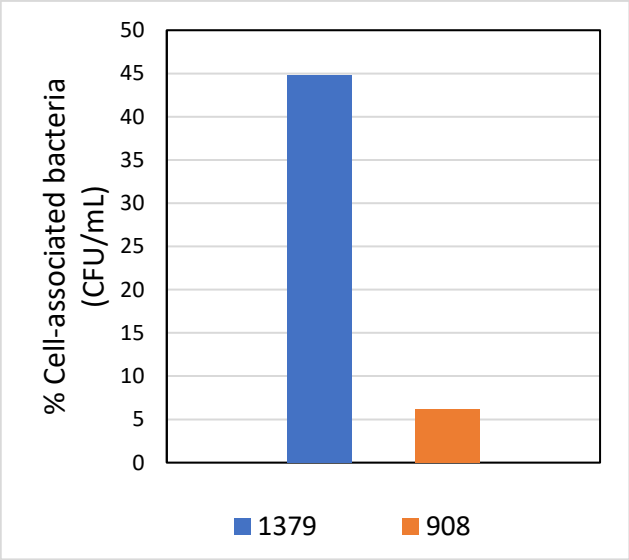

Figure 15. Percent cell associated bacteria for *S. agnetis* 908 (Orange) and 1379 (Blue) three days after coculture.

3.3.4. Results of heat-inactivated serum on the crystal violet assay

The results show that the HTCs in the control wells grow exponentially regardless of the type of sera used. Growth rate is highest between day 2 and day 3 (Figure 16). There is no significant difference between the heat-inactivated and normal sera groups (P>0.05). The infected HTCs lack proliferation and exponential growth, but rather show a reduction on day 1 following the coculture and there on, remains constant. The only divergent result observed is in a single trial where 908 infected HTCs in the normal sera group show an increase in optical density values by day 3.

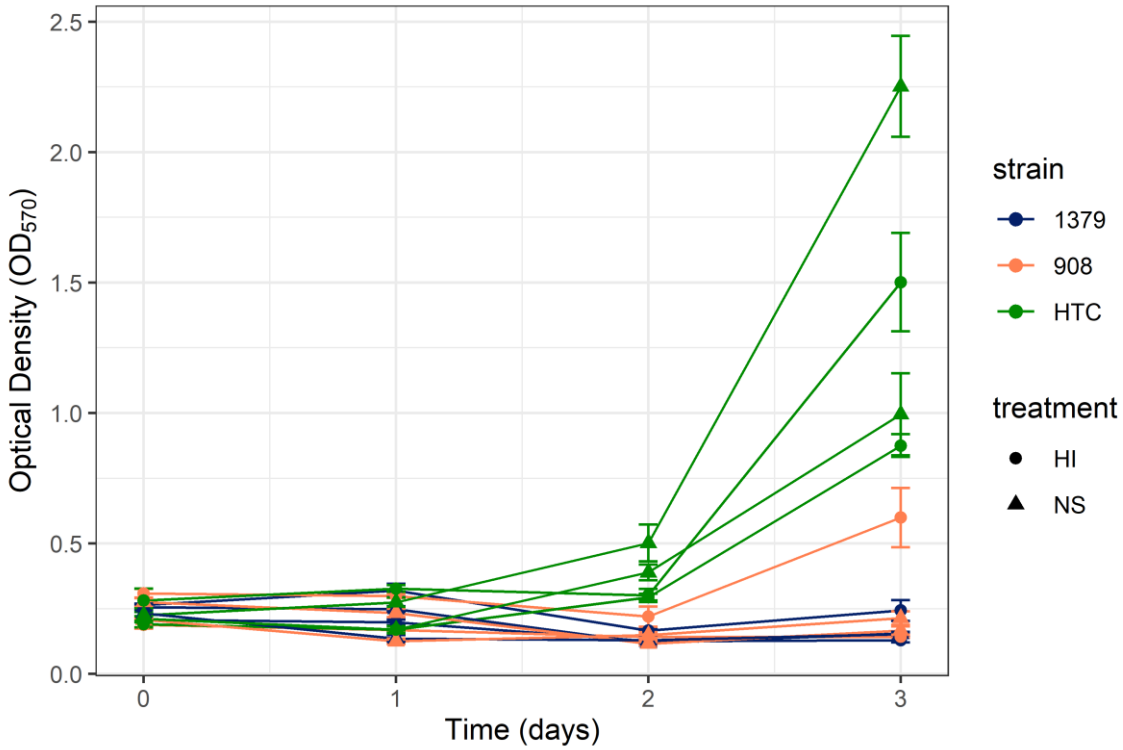

Figure 16. Affect of heat-inactivated serum on the viability of HTCs. The mean optical density values at 570nm corressponding to HTC control (Green), *S. agnetis* 908 (Orange) and *S. agnetis* 1379 (Blue). Heat-inactivated serum samples are indicated with triangles while normal sera are marked with a circle. HI – Heat-inactivated; NS – Normal Serum Error bars represent standard deviations (n=3)

3.4. Discussion

Present day microbiology requires fast and efficient methods to determine bacterial counts. A few novel methods used include turbidity, flow cytometry, microscopic methods, and impedance measurements (Rasch, 2004). Measure of turbidity or optical density is one common method used in many studies. Determining CFU counts through OD readings can be done is a variety of ways such as fitting a primary growth curve model to OD data (Dalgaard and Koutsoumanis, 2001, Horakova et al., 2004), using calibration curves based on the linear relationship between OD and CFU counts based on Beer-Lamberts law to recalculate OD data to CFU counts (Jorgensen and Schulz, 1985, Lack et al., 1999) or using logarithmic transformed OD and CFU counts to normalize variation and fit to a linear regression curve (Hudson and

Mott, 1994). The latter has been used in our study to make the standard curves for 908 and 1379.

But do optical density values correlate to viable cell counts under stressful conditions? The optical density values of 1379 treated with varying concentrations of gentamicin appear to have a similar pattern to that of the untreated control group. Therefore, OD values alone would not be sufficient to determine the susceptibility of a bacterium to an antibiotic. This is because OD readings are not always an accurate representation of viable cell counts especially when the bacteria are subjected to stress. Antibiotics such as gentamicin used in our experiment, induce stress in bacterial cells. Exposure to stressful conditions has been shown to cause modifications in bacterial cell structure (Cefali et al., 2002). When this is the case, the relationship between logarithmic transformations of OD and CFU/mL can result in discrepancies (Francois et al., 2006, Jorgensen and Schulz, 1985, Chorin et al., 1997). Instead, it could reflect the size and shape of the bacterial isolates as well (Udekwu et al., 2009). We have observed that *S. agnetis* 1379 undergoes autolysis during storage in 4^{O}C. We hypothesize that this could be a response to cold stress. However, we have not tested this. It is possible that stress brought by the antibiotic could affect 1379 in a manner that cell death does not correlate to a decrease in OD readings. Instead, it could potentially have similar cell densities to the control 1379 that has not been treated with antibiotics. Since we obtained respective CFU data using the standard curve it is impossible to interpret the MIC for *S. agnetis* due to discrepancies between the OD readings and CFU counts. This emphasizes the need to determine the viable bacterial counts through the traditional plating method in order to obtain accurate and reliable results especially when testing the susceptibility of a bacterium to antibiotic.

We do not observe exponential growth in our experiment. According to CLSI guidelines (Wilker, 2005), the requirements to determine the minimum inhibitory concentration (MIC), consists of media inoculated with cultures adjusted to yield $\sim 5 \times 10^5$ cells/mL, allowed to grow exponentially in the optimal temperature and conditions and the MIC should be determined

during a short period using optical density (Udekwu et al., 2009). However, the starting number of bacteria in our experiment was approximately $1x10^8$ CFU/mL. Furthermore, our bacterial populations were at their stationary phase during the experiment and as a result, we are unable to observe an exponential growth making it difficult to come to interpret antibiotic sensitivity results.

The results of our optimized crystal violet assay show that staining eukaryotic cells such as HTCs with 0.5% crystal violet is an effective and efficient method to quantify the viable eukaryotic cells. All infected HTCs decrease in number on day 1 (one day following coculture) and remain at a constant level as indicated by the optical density values. This decrease in HTCs could be caused by by cell death induced by the bacterial invasion. Cell death can be caused by apoptosis, programmed necrosis, and pyroptosis (Mitchell et al., 2016) however, our experiment does not provide data to determine the mechanism of cell death. Through our results it is clear that the bacterial infected HTCs undergo cell death during the first day following infection as observed by the reduction in optical density values on day 2, and are unable to recover and proliferate. This suggests that the HTCs are possibly battling viable intracellular bacteria.

Additionally, this assay has also highlighted the downsides of antibiotic protection assays. Antibiotic protection assays allow for the study of intracellular bacterial virulence mechanisms by eliminating and limiting extracellular bacteria (Tabrizi and Robinsbrowne, 1993). The antibiotic selected for this purpose should be sufficiently potent to kill the extracellular bacteria but also be impermeable to the host cell. Gentamicin which we have used in our assay and streptomycin has been widely used in antibiotic protection assays by many studies because they possess these abilities.

However, recent literature have raised concerns on the use of antibiotic protection assays due to findings that the antibiotics such as gentamicin could in fact penetrate eukaryotic cells over time and accumulate enough to kill intracellular bacteria (VanCleave et al., 2017, Drevets et al., 1994, Ohya et al., 1998, Eze et al., 2000, Hamrick et al., 2003). This is reflected

through the variability in the results of our experiment. Through out the various trials, the OD values reflecting the viable HTCs in the wells infected with 1379, show fluctuations. One possible explanation for this is the prolonged exposure to a high (50ug/mL) of gentamicin.

According of Drevets et al. (1994) and Hamrick et al. (2003), intracellular bacteria compartmentalized with in vacuoles such as phagosomes are more susceptile to being inhibited by aminoglycosides. When exposed for a prolonged duration, aminoglycosides such as Gentamicin in the extracellular space is taken up by the eukaryotic cell through pinocytosis. The antibiotic, now contained within the vescicle is more likely to fuse with the other vacuoles such as phagosomes resulting in the death of bacteria contained within it (Flannagan et al., 2016).

Bacteria such as *Staphylococcus agnetis* are engulfed by macrophages and contained with phagosomes where they are killed by hydolytic enzymes, acidic pH, Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), lack of nutrients and Adenosine Monophosphate (AMPs) (Pidwill et al., 2021). It is possible that majority of the *S. agnetis* in our study are contained within these phagosomes as well. Thus, we speculate gentamicin had enetered the HTCs during the course of our three day experiment and implemented their bactericidal effects on the internalized bacteria.

Furthermore, many studies suggest the use of a low concentration of gentamicin such as 5µg/mL for a prologed duration or a higher concentration for a shorter duration. In their study Hamrick et al. (2003) describes that despite the use of 5µg/mL of gentamicin for 4 hours, gentamicin had managed to enter the eukaryotic host cell and kill intracellular bateria. Our study used 50µg/mL of gentamicin for three days which would definitely have caused gentamicin to enter HTCs. This would have influenced the intracellular baterial counts which could explain the variability of our results especially in HTCs infected with *S. agnetis* 1379.

The current data is not sufficient to explain why 1379 is more susceptible to inconsisttent results. It is possible that *S. agnetis* 1379 could be more susceptible to internalized gentamicin more than 908. We are yet to study the mechansim of phagocytosis and the location of *S.*

agnetis with in a macrophage. It is also possible that the location of the engulfed bacteria would influence their susceptibility to gentamicin based on whether they are within phagosomes or have managed to evade phagosomes and remain in cytoplasm. Further studies such as observation of fluorescent labelled bacteria are required to understand the bactericidal affects of internalized gentamicin on the different strains of *S. agnetis* in order to explain the inconsistent resuts observed for *S. agnetis* 1379.

It is possible that intracellular bacteria contributes to the majority of the cell-associated bacteria since the gentamicin supplemented growth media should kill any extracellular bacteria. Following this assumption, it appears that 1379 persists with in the intracellular space of the macrophage as reflected by it's unchanging colony counts. In contrast, *S. agnetis* 908 seem to be susceptible to the killing mechanisms of the macrophage.

However, it should be noted that the downsides of a prolonged exposure to gentamicin may have an affect on these results. As mentioned before, it is possible that gentamicin had entered the intracellular space of the HTCs and influenced the colony counts. If *S. agnetis* 1379 is less susceptible to gentamicin it could have contributed to 1379's intracellular survival. Furthermore, the location of 1379 with in the macrophage would too have contributed to the final result since gentamicin is known to enetr cells via pinocytosis which would mean the pinocytostic vescicle has a greater chance of fusing with the phagosome. If 1379 escapes phagosomes and remain in the cytosol it would be less susceptible to the affects of intracellular gentamicin.

The HTCs in the control wells were provided with ideal growth conditions that supported their survival and proliferation as reflected by the increasing optical density values. However, it is evident through our results that heat inactivating FBS does not have an affect in which HTCs grow and proliferate. In contrast to several studies (Soltis et al., 1979, Giard, 1987) using heat-inactivated FBS did not negatively or positively impact HTC adherence or proliferation. Additionally , we do not observe a noticeable affect on the bacterial infected HTCs as well.

Normal FBS contain several complement proteins that would enhance host immune responses. This could lead to the assumption that using heat-inactivated serum would in fact cause more host cell damage due to the lack of complement proteins and immunoglobulins. Yet, our results reveal that the damage inflicted on HTCs by the bacteria are not different between the heat-inactivated FBS group and normal FBS group. This supports studies that suggest heat inactivating serum is not a requirement to maintain cell cultures nor would it interfere with coculture results. It does not appear to have a benefit in supporting bacterial survival as reflected through the number of viable HTCs.

However, in one of our trials, HTCs infected with *S. agnetis* 908 supplemented with heat-inactivated FBS showed increasing OD readings two days after infection. This could be an anomaly and can be due to the negative effects of prolonged exposure to a high concentration of gentamicin. As explained in the previous section, even though gentamicin has been thought to be impermeable to eukaryotic cells, several studies have revealed that it does in fact enter eukaryotic cells and could kill intracellular bacteria (VanCleave et al., 2017, Drevets et al., 1994, Ohya et al., 1998, Eze et al., 2000, Hamrick et al., 2003). It is possible that the vvariations observed in our results are due to the downsides of gentamicin protect assays.

3.5. References

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Chapter 4

Enzyme Protection Assay

4.1. Introduction

Antibiotic Protection assays make use of the bactericidal properties of antibiotics such as that of the aminoglycoside gentamicin, to eliminate extracellular bacteria, to assess survival of only intracellular bacteria. Due to their large size and negative charge (Tulkens, 1991), it is believed that these antibiotics cannot permeate eukarytoic cells. However, recent studies have showed that gentamicin is capable of penetrating eukarytoic cells and affecting intracellular bacterial viability (VanCleave et al., 2017, Drevets et al., 1994, Ohya et al., 1998, Eze et al., 2000, Hamrick et al., 2003). Furthermore, it is possible that gentamicin may fail to completely eliminate extrcellular bacteria depending on the antibiotic sensitivity of the particular bacterial species. This may compromise the accuracy of gentamicin protection assays.

To overcome these drawbacks, an alternative technique using cell wall degrading enzymes, termed as Enzyme Protection Assay (EPA), was investigated.

4.1.1. What is an Enzyme Protection Assay?

EPA makes use of enzymes that are lethal to the bacteria of interest. The enzymes used for EPA should not be able to cross the eukaryotic cell membrane thereby, it would only eliminate extracellular bacteria. The principle is similar to that of Gentamicin Protection Assays (GPAs) (Sharma, 2019; Vancleave, 2017)

Lysostaphin is one such enzyme commonly used in studies that involve *Staphylococcus* species. Lysostaphin was first discovered by (Schindler and Schuhardt, 1964) during transduction experiment involving *Staphylococcus aureus*. They observed a white colony srrounded by a zone of inhibition on a lawn of *S. aureus* bacteria. Upon further experimentation they identified the strain termed as K-6-WI, to be a Gram-positive coccus of the *Staphylococcus*

genus that had been exposed to Blair type phage no. 6. Through experiments the bacteriolytic substance produced by K-6-WI had been isolated and revealed to be the potent bacteriolytic agent, lysostaphin. Due to its rapid rate-of-kill exceeding that of viruent bacteriophages, and ability to destroy many phage-types of *Staphylococcus aureus*, lysostaphin has not been categorized as a bacteriophage lysis enzyme. Nor is it categorized as a lysozyme of bacterial origin, since it does not display the same antibacterial properties against the Gram-positive *Micrococcus lysodeikticus* (Salton, 1952) or Gram-negative organisms that are generally susceptible to lysozyme action (Warren et al., 1955). In addition, it is potent against all *Staphylococci* regardless of their coagulase production whereas a lysozyme it would only be potent against coagulase negative strains (Kern et al., 1951)

Lysostaphin is a a glycylglycine endopeptidase, which acts on *Staphylococci* and other gram-positive bacteria by cleaving the pentaglycine cross bridges of the peptidoglycan cell wall (Wu et al., 2003). The enzymatic activity of lysostaphin is highly efficient, where 2 U (units) of lysostaphin for 5 minutes is sufficient to emilminate several methicillin-sensitive and methicillin-susceptible *S. aureus* strains (Kim et al., 2019).

Lysostaphin has been identified for having strong bactericidal properties. It is potent against more than 50 strains of *Staphylococcus* species. Yet, does not have an affect on 27 other species including *Streptococcus faecalis, Escherichia coli* and *Klebsiella pneumoniae* (Schindler and Schuhardt, 1964). The potency and strength of it's bacteridical effect has been described as more powerful than long term exposure to high concentrations of oxacillin, vancomycin and clindamycin. Furthermore, it was efficient enough to disrupt biofilms of *S. aureus* and *Streptococcus epidermidis* (Wu et al., 2003).

4.1.2. Advantages of EPA over GPA

EPA has yielded better results in accurately enumerating the internalization capacity of *S. aureus*, and also determining the intracellular bactericidal capacity RAW264 when compared

to GPA (Sharma and Puhar, 2019, VanCleave et al., 2017). EPA had an advantage especially when measuring bacterial kinetics where the bactericidal agent should be able to eliminate bacteria with in a short time. Since gentamicin takes a longer time to kill the extracellular bacteria, GPA results may not provide consistent results. However, the biggest advantage of the EPA is that it does not alter or influence the intracellular bacterial counts. Even though lysostaphin may permeate eukarytotic host cells. It has been identified that any internalized lysotaphin is inactivated due to the enzymes unstable nature (Kim et al., 2019). The host cell such as macrophages are capable to degrading and modifying the internalized lysostaphin, thereby preventing it from killing intracellular bacteria.

One of the few disadvantages of the EPA is that it involves an enzyme, therefore the physiological conditions of the assay should be controlled to maintain an optimum pH and temperature (Yokogawa et al., 1975).

4.2. Materials and methods

4.2.1. Measuring sensitivity of *Staphylococcus agnetis* 908 and 1379 to lysostaphin

We selected 2 U/mL of lysostophin as the concentration for our experiment (Kim et al). In brief, $2x10^5$ cells/mL of *S. agentis* 908 and 1379 were prepared in RPMI 1640 growth media supplemented with sodium pyruvate, 2-mercaptoethanol and Glutamax. Lysostaphin (BioVendor, LLC, Asheville, NC) was added as 0.64 ul of 3 mg/ml (12,411 U/ml) stock solution to the bacterial suspensions to achieve 2 U/ml of lysostaphin. The bacterial suspensions were then incubated for two hours with OD_{650} recorded every 30 minutes and viable colony counts assessed by plating 100ul of the bacterial suspensions on Tryptic Soy (TS) agar plates.

4.2.2. Crystal Violet Assay to determine viable HTCs.

To compare the efficacy of the Enzyme Protection Assay (EPA) we used the Crystal Violet (CV) assay on *S. agnetis* 908, 1379 and transformant of 1379: 1589A, which was confirmed to contain the A164E substitution through sequence data (see Chapter 2).

The wells were stained with 0.5% crystal violet according to the CV assay protocol described in Chapter 3 to determine the viable HTCs after coculture. To ennumerate the cell-associated bacteria, the HTCs were lysed with 0.5mL of sterile distilled water. The lysate was then plated on TS agar plates and incubated overnight in 37^{o-}C.

The experiment was carried out over a three day period. Each sample was performed in triplicates. Day-0 samples were processed six hours post coculture (four hours of phagocytosis and two hour incubation time in lysostaphin growth media). Wells allocated for days 1 to 3 were replaced with 0.5 mL of fresh RPMI growth media with 2 U of lysostaphin each day.

4.3. Results

4.3.1. Measuring sensitivity to lysostaphin

The colony counts reveal that it takes, two hours for 2 U of lysostaphin to fully eliminate 908 while 1379 is killed with in 30 minutes of exposure to lysostaphin (Figure 17) indicating that 1379 is more susceptible to killing by lysostaphin. But overall, it is evident that both *S. agnetis* 908 and 1379 are susceptible to 2 U of lysostaphin.

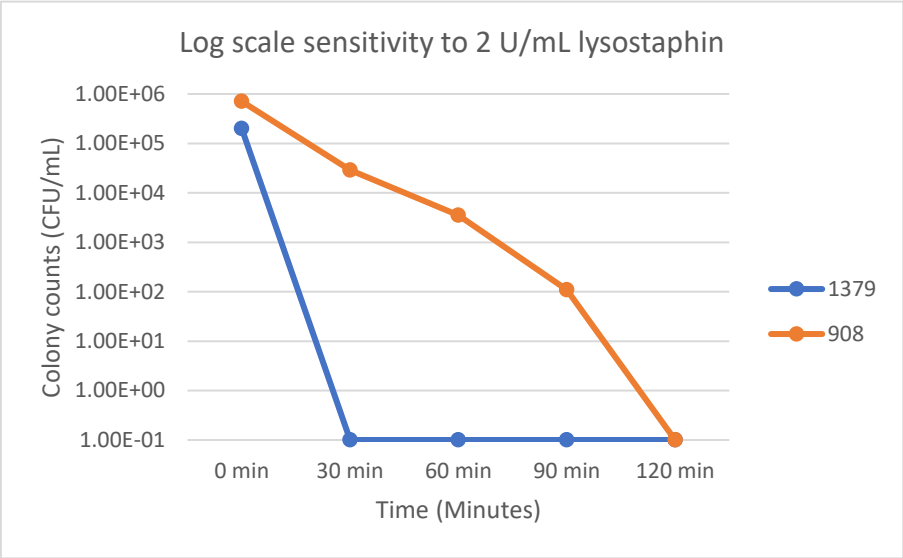

Figure 17. Susceptibility of *S. agnetis* 908 and 1379 to 2 U/mL of lysostaphin measured during a two-hour period. The colony counts are in log scale.

In order to determine whether a higher lysostaphin concentration would decrease time taken to eliminate *S. agnetis* 908, we repeated the experiment using 6 U/mL of lysostaphin (Figure 18). However, increasing the concentration of lysostaphin did not reduce the killing time as shown by figure. Instead similar to results with 2 U/mL concentration, it took 6 U lysostaphin two hours to eliminate 908 which indicates that 908's susceptibilty to lysostaphin is concentration independent.

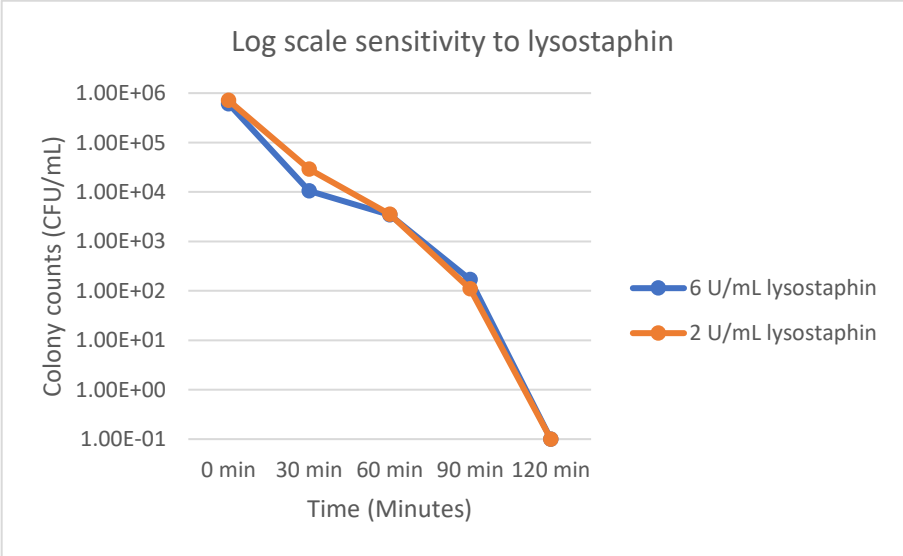

Figure 18. Susceptibility of *S. agnetis* 908 to 2 U (orange) and 6 U(blue) of lysostaphin as determined through colony counts given here in log-scale.

4.3.2. Crystal Violet Assay paired with EPA.

In Chapter 3, CV assay with GPA revealed that HTCs infected with *S. agnetis* 908 and the transformant 1589A did not proliferate during the three-day period while the HTCs infected with *S. agnetis* 1379 proliferated in a manner similar to the uninfected HTC control (Figure 19). However, when CV assay was paired with EPA the results were different. The OD readings revealed that all three strains affected the HTC viability in a similar manner. HTC proliferation was observed only in the uninfected control group.

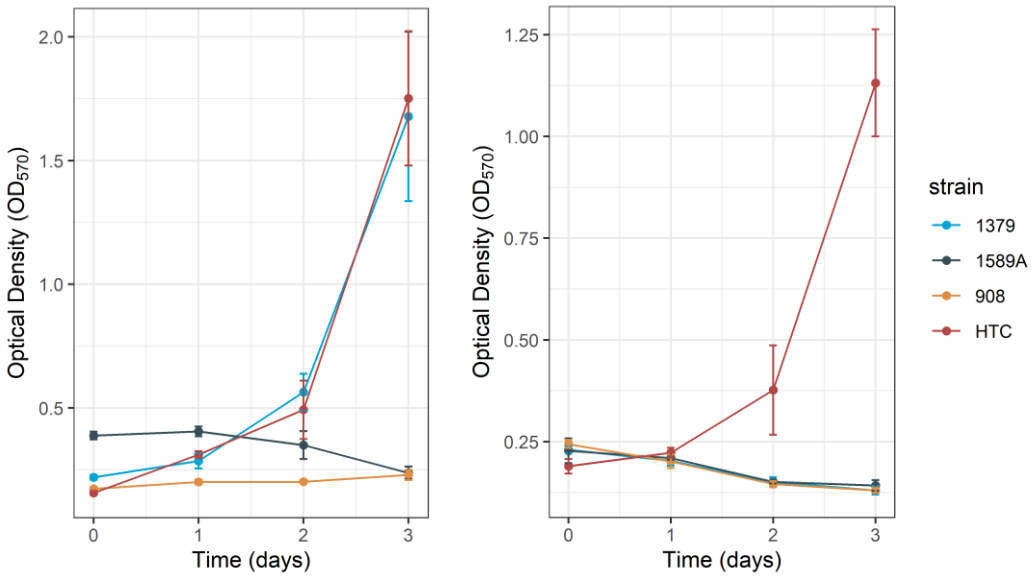

Figure 19. Comparison of CV assay results obtained through GPA (left) and EPA (right). Error bars represent standard deviations (n=3).

To further confirm these results, we lysed the HTCs on day-3 to enumerate the cell-associated bacteria. The results obtained through the GPA revealed that *S. agnetis* 1379 has the highest number of cell-associated bacteria while 908 and the transformant have fewer bacteria three days post-infection (Figure 20). However, when EPA was used, only the transformant showed a significant decrease in bacterial cell counts on day 3, with no significant differences between *S. agnetis* 908 and 1379 ($P>0.05$).

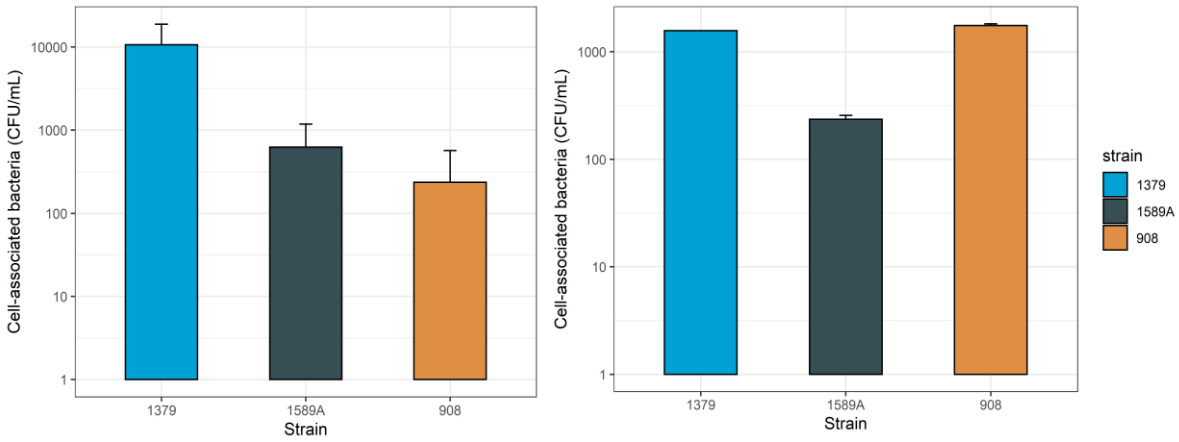

Figure 20. Cell counts of cell-associated bacteria in Colony Forming Units (CFU/mL) of the three strains 1379, 908 and 1589A on day-3 of the GPA (Left) and EPA (Right). Error bars represent standard deviations (n=3).

4.4. Discussion

4.4.1. Measuring sensitivity to lysostaphin

Previous literature have identified almost 50 strains of Staphylococci including methicillin resistant strains are susceptible to lysostaphin (Schindler and Schuhardt, 1964, Kim et al., 2019, Easmon et al., 1978). However, to our knowledge, no study had previously determined the sensitivity of *Staphylococcus agnetis*. Our findings have shown that both strains of *S. agnetis*, 908 and 1379, are susceptible to lysostophin. However, in order to eliminate *S. agnetis* 908 completely, a two hour incubation in 2 U of lysostaphin was required.

The results of our time-kill assay revealed that the sensitivity of 908 to lysostaphin was concentration independent. This contrasts with Kim et al. (2019) where three methicillin-resistant strains of *S. aureus* were eliminated rapidly when incubated with 2 U (17.6 nM) of lysostaphin instead of 1 U (8.8 nM). It is possible that 908 could be rapidly eliminated using even higher lysostphin concentration than 6 U/mL, but we have not pursued that aspect further.

4.4.2. Crystal Violet Assay paired with Enzyme Protection Assay

The findings of our study corroborate previous literature that describe the advantages of EPA over GPA (Kim et al., 2019). The CV assay reflects the benefits of the EPA clearly with the consistent results. As explained in the previous chapter, CV assays that were carried out using GPA yielded inconsistent results with 1379 showing variability. When we compared results using EPA, it was clear that gentamicin had influenced the CV assay results by permeating the host cell. We observed that *S. agnetis* 1379 infected HTCs show recovery and proliferation when paired with GPA. It is possible that 1379 resides in the cytosolic space of macrophages thereby managing to evade the bactericidal effects of gentamicin encased in vacuoles (Flannagan et al., 2016). This would have provided 1379 with a safe niche to remain viable within the macrophages as indicated by the higher colony counts for 1379.

The reduction in the number of HTCs are caused by damage inflicted to macrophages by bacteria, during which the bacteria would rupture the macrophage either through programmed cell death or simple escape, resulting in a reduction in viable HTCs (Mitchell et al., 2016, Ross, 2001, Lamkanfi and Dixit, 2010). Through previous work we have found that 908 is capable of surviving or escaping macrophages better than 1379 (Zaki, 2021). The phagocytosis assays used in the study involved an overnight incubation in 50 µg/mL of gentamicin supplemented growth media where gentamicin could have permeated the HTCs. The results we observed following the gentamicin incubation were identical to the results we quantified through CV assay with more viable HTCs present in 1379 while less viable HTCs in 908 infected wells. It is possible that gentamicin is influencing 1379 escape from macrophages. However, we have not tested for this assumption. But this could explain why 908 continued to damage HTCs resulting in reduced OD readings in the CV assay and low colony counts obtained on day 3. Further studies using fluorescent labelled bacteria and HTCs would help understand this phenomenon better.

The EPA has provided evidence that all three strains of *S. agnetis* including the transformant 1589A, cause equal damage to the host HTCs. Therefore, we conclude that this experiment does not provide sufficient data to support the hypothesis that A164E in the deoC gene is responsible for the increased escape and survival observed in 908. Furthermore, our results seem to indicate that *S. agnetis* 908 and 1379 may have differential response to gentamicin which was reflected through the phagocytosis assays paired with GPA. However, further studies are needed to test this hypothesis.

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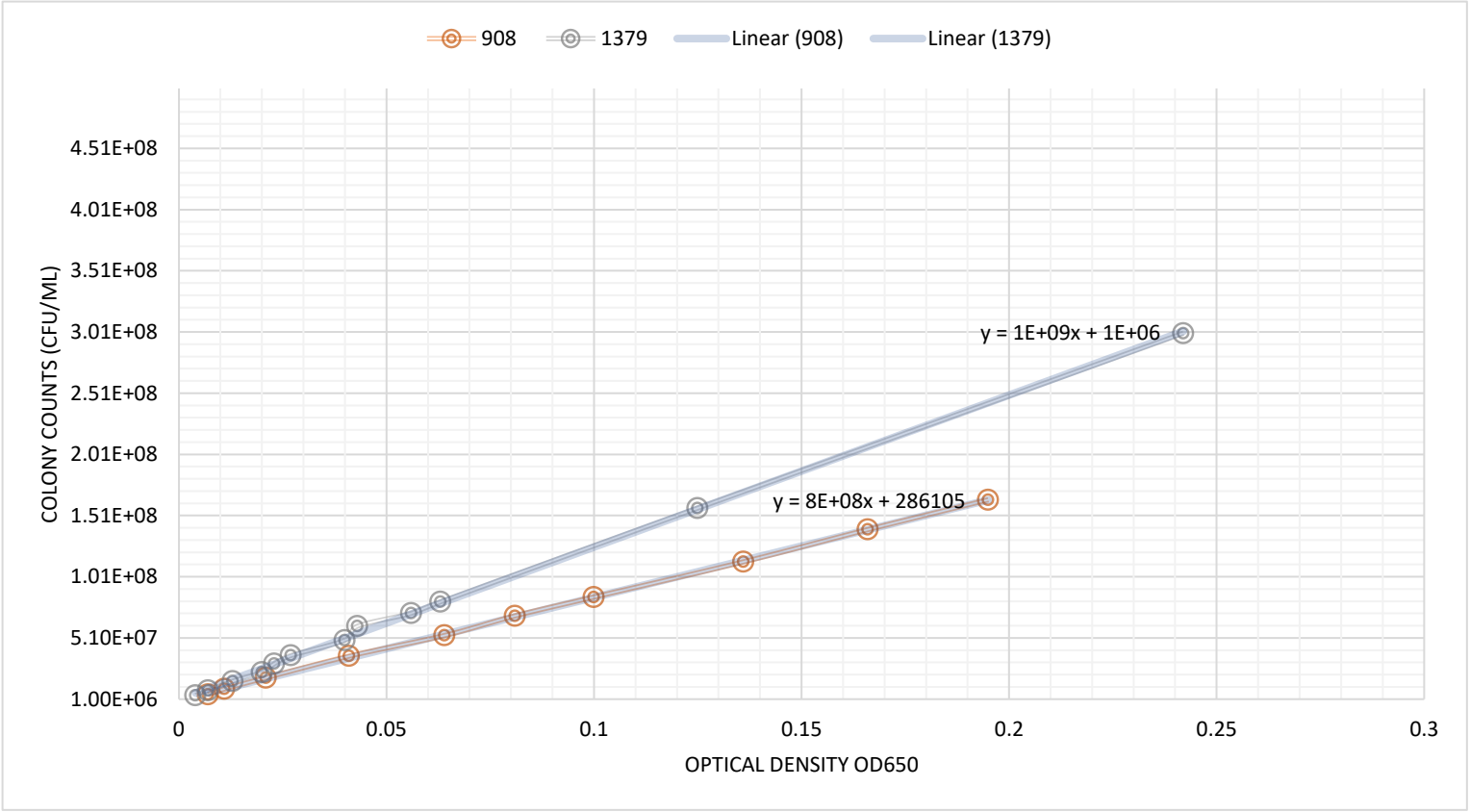

Figure 21. The standard curve used in determining the colony counts for *S. agnetis* 908 and 1379 based on their optical density values

Protocol 1

RPMI 1640 growth media supplemented with antibiotics and antibiotics.

(All volumes for adjusted for 250 mL of media):

- 2.5 mL Sodium pyruvate 100x
- 227 µl Beta-mercaptoethanol (sterile)
- 2.5 mL L-Glutamax 100x
- 25 mL 10% fetal bovine serum (low-endotoxin)
- 217 mL RPMI 1640 (Without L-glutamine)
- 2.5 mL 100x Antibiotic-antimycotic solution
- 50 µl of gentamicin sulphate solution

Appendix

UNIVERSITY OF ARKANSAS

Office of Research Compliance

April 17, 2015

MEMORANDUM

TO: Dr. Douglas Rhoads

FROM: W. Roy Penney
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 15017

Protocol Title: "Bacterial causes of lameness in chickens – Phase II"

Approved Project Period: Start Date: April 9, 2015
Expiration Date: April 8, 2018

The Institutional Biosafety Committee (IBC) has approved Protocol 15017, "Bacterial causes of lameness in chickens – Phase II" You may begin your study.

Ensure all laboratories and farm facilities used for these experiments include BSL2 signs, prior to any experimental activities.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

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UNIVERSITY OF ARKANSAS

Office of Research Compliance

January 27, 2020

MEMORANDUM

TO: Dr. Douglas Rhoads

FROM: Ines Pinto, Biosafety Committee Chair

RE: Protocol Modification

PROTOCOL #: 15017

PROTOCOL TITLE: Bacterial pathogenesis associated with bacterial chondronecrosis with osteomyelitis in broilers

APPROVED PROJECT PERIOD: **Start Date** April 9, 2015 **Expiration Date** April 8, 2021

The Institutional Biosafety Committee (IBC) has approved your request, dated December 24, 2019, to modify Protocol # 15017, "Bacterial pathogenesis associated with bacterial chondronecrosis with osteomyelitis in broilers".

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

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