2022-12-13

The Role of Chitinase A in Mastitis-Associated Escherichia coli Pathogenesis

Weston D. Hutchison
Brigham Young University

The Role of Chitinase A in Mastitis-Associated *Escherichia coli* Pathogenesis

Weston D. Hutchison

A thesis submitted to the faculty of
Brigham Young University
in partial fulfilment of the requirements for the degree of

Master of Science

David L. Erickson, Chair
Joel S. Griffitts
Eric Wilson
Bradford K. Berges

Department of Microbiology and Molecular Biology

Brigham Young University

ABSTRACT

The Role of Chitinase A in Mastitis-Associated
Escherichia coli Pathogenesis

Weston D. Hutchison
Department of Microbiology and Molecular Biology, BYU
Master of Science

Bovine mastitis is a common disease among dairy cattle characterized by the inflammation of the udders and loss of milk production. Mastitis-associated *Escherichia coli* (MAEC) are frequent causes of the disease, but the features that distinguish them from other *E. coli* strains remain enigmatic. MAEC infections can range from sub-clinical to severe, acute cases that can be fatal. Historically, the severity of mastitis has been attributed to host factors but more recently, a few bacterial genes have been shown to contribute to virulence in mastitis infections. In a large-scale genomic analysis of >100 MAEC isolates the gene for Chitinase A (ChiA) was positively associated with robust growth in the mammary glands during a mouse model of mastitis. This correlation suggests the hypothesis that ChiA contributes directly to MAEC fitness.

The regulation of *chiA* has not been documented in contexts relevant to bovine mastitis. In the lab strain K-12, *chiA* is not expressed during aerobic growth in rich media. However, previous work with enterotoxigenic *E. coli* strain H10407 indicated that expression may be induced by hypoxic environments and the presence of bile salts. To measure expression of *chiA,* I created a *chiA*-GFP reporter plasmid and measured changes in fluorescence using flow cytometry. My results indicate promoter activity of *chiA* in MAEC is significantly increased in hypoxic conditions and the presence of bile salts, but not both. Adhesion to host tissues is an important characteristic of successful pathogens. Since ChiA facilitates adhesion between adherent-invasive *E. coli* and intestinal epithelial cells, I investigated its role in adhesion to bovine mammary epithelial cells in four MAEC strain backgrounds. Isogenic mutants lacking *chiA* were made in 2 mild (M45 and M93) and 2 severe (M111 and G1) clinical isolates. Loss of *chiA* resulted in significant reduction of adherence of M45, M93, and G1 to epithelial cells, but not M111. Wild type levels of adhesion were restored upon reintroduction of *chiA* into mutants through a plasmid vector. Additionally, the genomes of each MAEC isolate were analyzed for the presence of genes that could possibly influence the adhesion and virulence. Strain M111 contained genes for 2 distinct fimbriae that were not present in the other MAEC strains, possibly reducing its reliance on ChiA.

The interaction of ChiA with mammary epithelial cells in MAEC could possibly offer an advantage for certain strains to be better suited to colonize and persist in mammary glands. Increased understanding of the regulation of *chiA* and its role in adherence can lead to novel targets for more effective treatment and prevention of bovine mastitis.

Key words: mastitis, adherence, chitinase, *Escherichia coli*, epithelial cell

ACKNOWLEDGMENTS

First and most importantly, I could never thank my wife, Hollee, enough for all the support she has given me and sacrifices she has made to help me achieve my dreams. She truly was the most supportive and understanding person I have had the privilege and pleasure of having by my side.

I would also like to acknowledge the contributions and mentoring of my advisor, David Erickson. There is little doubt in my mind that I could not have finished without his help. His constant guidance kept me focused on my goals and taught me a great deal about how to be an effective, intelligent researcher. I would also like to thank Eric Wilson for being the initial interaction I had with this graduate program. An experience that made me feel welcome and piqued my curiosity about the world of bacterial pathogenesis.

The remaining members of my committee, Joel Griffitts and Brad Berges, were also instrumental in my success and I cannot thank them enough. Joel was a significant source of reassurance and helped me to learn to follow my curiosity and follow the data instead of the dogma I had cooked up in my mind. Brad and his lab members were a significant source of help in troubleshooting various issues I had run into, which directly resulted in the success I have seen so far.

Finally, I would like to thank all the undergraduate, graduate students, and staff that helped me: Tyler Brown, Shalee Carlson, Benjamin Ogilvie, Andres Silva, Nick Christman, Rachel LeBaron, Jacob Fairholm, and Logann Smith for all of their help during my time here at BYU.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SECTION 1: INTRODUCTION

1.1 Bovine Mastitis

Mastitis is a common condition in dairy cattle, defined as an inflammatory response in the mammary gland, usually as a result of a bacterial infection. It is characterized by swelling and redness of the udders, milk that is watery or contains pus, as well as an increased somatic cell count (SCC) of leukocytes, neutrophils, and macrophages in milk [1]. Bovine mastitis can be characterized in 3 classes: clinical, sub-clinical, and chronic. These classes are based on the symptoms and level of inflammation in the cow [2]. Mastitis is commonly diagnosed by a SCC in milk above 200,000 cells/mL with the level of inflammation being proportional to the SCC [3].

Clinical mastitis is characterized by a sudden onset of pain and inflammation in the cow's udder. Their milk may also present with clots, flakes, or a watery consistency. Clinical mastitis can be further subdivided into per-acute, acute, and sub-acute, dependent on the level of inflammation and severity [4]. Sub-clinical mastitis is characterized by the lack of visible symptoms in the milk or udder but decreased milk production and an increased SCC. This is often unnoticed or misdiagnosed making it a higher risk condition for older cows [4]. Sub-clinical mastitis is estimated be 15 to 40 times more prevalent than clinical mastitis [5] and therefore cause a more significant economic impact. One study found a prevalence of sub-clinical mastitis to be up to 42.5% in cows on medium sized farms [6].

Chronic mastitis involves recurrent symptomatic episodes, often as a result of incomplete clearing of a previous case of mastitis. Biofilms make the bacteria more recalcitrant to the host immune response and antimicrobial treatment, which likely

contributes to the development of chronic mastitis [2, 3, 7]. Incomplete clearing of a mastitis infection or over use of antibiotics can lead to the emergence of antibiotic resistance in these pathogens [8], an issue recognized as a significant risk to global health.

Any type of mastitis infection puts a cow at risk for damage to their mammary gland which can lead to decreased milk production and further health complications [9]. This can lead to a persistent decrease in milk production for the cow and even lead to an earlier culling of cows that produce less milk or have chronic mastitis [4].

1.1.1 The Impacts of Bovine Mastitis Worldwide

Bovine mastitis is mostly caused by bacterial infections which can be classified based on their epidemiological origin: contagious and environmental. The former is mainly caused by bacteria like *Staphylococcus aureus, Streptococcus spp.,* and *Mycoplasma spp*. These often spread from an infected cow to an uninfected one through worker's hands, towels, or equipment from milking [10]. *S. aureus* is the predominant causative agent of contagious mastitis. Not only has *S. aureus* been well-studied in general, but it is also arguably one of the most well characterized mastitis pathogens [11]. The incidence of bovine mastitis caused by *S. aureus* can reach 100% in dairy herds whose regular prevalence ranges from 30-50% [12]. In a wide range of countries worldwide, *S. aureus* is the most common bovine mastitis isolate [11, 13-18]. This high incidence has led to concern of zoonotic spillover of methicillin-resistant *S. aureus* (MRSA) strains into human populations with some studies showing that bovine isolates of MRSA are genetically similar to human isolates [12, 14], indicating this spillover is already occurring.

Environmental infections are mostly caused by *Escherichia coli, Enterobacter aerogenes,* and *Klebsiella spp*. commonly originating in the environment around the cow such as their bedding material, soil, manure, feces, and stagnant water [10]. *E. coli* is the most frequently isolated environmental pathogen. While mastitis-associated *E. coli* (MAEC) does not have many well-understood virulence factors like other extra-intestinal pathogenic *E. coli,* MAEC is the most common cause of recurrent mastitis, or mastitis caused by the same bacterial strain in multiple instances. Döpfer et al sampled dairy cows from 300 herds and found that 11% of all mastitis cases caused by *E. coli* were considered recurrent [3]. In a separate study of over 20,000 cows in the Netherlands, 30% of diagnosed cases were caused by *E. coli* [19], making it one of the most common and costly pathogens in bovine mastitis.

The cost of mastitis can range from USD$200-450 per case [20, 21] when product loss, treatment, diagnosis, and other costs are considered. This results in almost $2 billion just in product loss every year in the United States [21, 22]. The largest contributor to these losses is sub-clinical mastitis cases, which can be caused by both environmental and contagious pathogens. In a recent study done in Ecuador, the estimated economic impact of sub-clinical mastitis in over 12,000 cows across 55 farms was USD$800,000 per year in the region with this impact being most noticeable in small to medium sized farms [6]. A separate study in Bangladesh estimated the regional impact of sub-clinical mastitis to be USD$2.1 million per year [23].

It is estimated that worldwide consumption of dairy products will increase by 1% each year between 2019-2028 [24], meaning dairy production will need to increase to meet the coming demand. The European Union (EU) has projected that dairy

production will increase by 0.2% per year in New Zealand, 1% per year in the United States, and <3% per year in developing countries [25], making mastitis a significant concern when it comes to the current and future health of dairy animals as well as a worldwide economic concern. This highlights the need for better understanding of mastitis pathogens and more effective diagnostic and treatment methods.

1.1.2 Diagnosis of Bovine Mastitis

The diagnosis of bovine mastitis is a critical step in prevention, treatment, and control [26-28]. Early diagnosis reduces the risk of permanent damage to the mammary gland, recurring infections, chronic mastitis, and loss of milk production. Unfortunately, due to the variety of presentations of bovine mastitis, it can be very difficult to diagnose in a timely manner.

Diagnosis can be made through multiple different methods, each with their own advantages and drawbacks. Most commonly, the SCC is used as the primary indicator, absent any obvious physical or behavioral symptoms exhibited by the cow. SCCs are determined by the number of white blood cells in milk which are primarily, lymphocytes, polymorphonuclear neutrophils, and macrophages [29]. An SCC of 200,000 cells/mL is considered to signal a case of mastitis [30], though this number varies depending on the normal SCC of the cow, the pathogen, and the measurement method. Measurement of certain biomarkers such as N-acetyl-β-D-glucosaminidase and lactate dehydrogenase can also be used for a diagnosis. Finally, culturing and/or identifying the causative organism is another common method of diagnosis.

1.1.3.1 Diagnosis Through Somatic Cell Count and Biomarkers

The California Mastitis Test, developed by Schlam and company in 1957, is one of the most used methods for diagnosing bovine mastitis through SCC [31]. This test works by lysing somatic cells in the milk. The resulting cell lysate reacts with reagents in the kit to create two indicators of SCC: the formation of a gel and the appearance of a purple color. Sodium alkyl aryl sulfonate is used to lyse cells and facilitate the formation of a gel [32]. As a result of the sudden lysis, the pH of the solution can change, making it more alkaline; this is indicated by the presence of bromocresol purple. In addition, released proteins and DNA will react with the sodium alkyl aryl sulfonate, increasing the viscosity of the solution. The level of viscosity and varying shades of purple in the sample can be used to estimate the SCC, though it is not nearly exact.

This test can be used at the side of the cow and is relatively affordable, with 350 tests being available for ~USD$12 [33]. However, despite its relative ease of use and low price, it is not widely available or accessible in relatively low-income areas and countries [34]. It can also lead to false positive results if a cow naturally has a higher SCC than the kit determines as negative as well as due to low sensitivity and difficult or variable interpretation of results [33].

Other methods are useful to determine SCC such as direct microscopic counting, Coulter counting, fluoro-optic electronic cell counts, or flow cytometry. Directly counting cells with stains such as methylene blue provides a more accurate SCC and can be done relatively quickly. Directly counting somatic cells can be challenging though, since it can be difficult to differentiate between cytoplasmic particles and cells [35]. Coulter counting is done by fixing somatic cells with formaldehyde, adding a lysis buffer

to remove fat cells, and counting them using a machine that detects the changes in an electrical current as the cells pass through. Alternatively, flow cytometry is a reliable method that provides very accurate and precise counts. It works well on fresh and preserved samples of milk, requiring little to no treatment beforehand. When combined with fluorescent markers, flow cytometry provides a high-throughput, relatively affordable method for determining SCC [36]. However, all of these methods require skilled labor and specialized equipment, making them inaccessible to many farms around the world.

An alternative method of diagnosing bovine mastitis is by quantifying biological markers that are present in milk during clinical cases. Comparative proteomics between healthy and diseased cows provides a highly specific (~100%) and sensitive (~75%) diagnostic method [37, 38]. Recently, milk amyloid A was found to be strongly correlated to SCCs in both clinical and sub-clinical mastitis cases [39]. Other enzymes such as alkaline phosphatase and milk arginase have also been identified as potential diagnostic markers [40-42]. The levels of these enzymes and markers are most affordably and accurately measured with immunological assays such as enzyme-linked immunosorbent assays (ELISA) [43].

1.1.3.2 Diagnosis by the Identification of Causative Organisms

Isolating the causative organism in a mastitis infection is one of the most accurate methods of diagnosis and leads to more targeted treatments for the cow. This can be done through traditional methods such as growing and isolating colonies of the bacteria or through genetic testing such as polymerase-chain reaction (PCR) and sequencing.

Differential and selective media such as mannitol salt agar for *Staphylococcus spp.,* eosin-methylene blue agar for *E. coli*, or pleuropneumonia-like organism (PPLO) medium for *Mycoplasma spp*. are used for more accurate identification with growth. The sensitivity of selective media is comparable to PCR in the identifying pathogens that are often difficult to culture like *Mycoplasma spp* [44]. In conjunction with isolation and growth of the organism, PCR can be used to further characterize the causative organism.

PCR testing is a very quick, sensitive, and specific method to diagnose mastitis and can be combined with sequencing for genes such as 16s rRNA [44]. Results can be available within 1-2 days, reducing time to treatment. The sensitivity and specificity can range from 76.9%-100% and 63.3%-98.7%, respectively [44-46], depending on the targets of the PCR. Multiplex PCR tests have been developed that offer advantages over traditional 16s rRNA PCR and sequencing, such as ease of use, sensitivity, and specificity [47]. In addition to accurate identification of bacterial species in mastitis, PCR has also been used to great effect to identify antibiotic resistance genes in these pathogens allowing for more targeted treatments [45].

Determining microbial etiology for mastitis, especially sub-clinical mastitis, can increase positive outcomes for treatment and the health of the animal [48]. However, 10-40% of milk samples of clinical and sub-clinical mastitis cases show no growth with bacteriological examination [49]. A multitude of explanations could be given as to why this is: *Mycoplasma spp*. require special culturing methods and media [50], growth of the pathogen may take too long, or one bacteria may suppress the growth of a competing bacteria in culture. While culturing of causative microorganisms is considered the gold

standard for diagnosing bovine mastitis, false negative rates between 27%-50% have been reported[13, 51-53].

1.1.4 Antimicrobial Treatments of Bovine Mastitis

The proper treatment of mastitis is a vital part of controlling the condition and preventing future health issues for the animals. Historically, antimicrobial therapy has been the most common method of treatment for mastitis, dating back to soon after the discovery of antimicrobials [54-57]. However, its effectiveness today can be extremely limited, due to the overuse of antibiotics in agriculture and livestock [58-61]. The success of treatment may also be reduced by the administration route (intramammary infusion vs systemic), the type of antibiotics used, and the causative agent [26]. Antimicrobials such as macrolides (e.g., erythromycin), tetracyclines, and trimethoprim-sulfonamides exhibit reduced activity in milk, making them less effective when administered via intramammary infusion [54, 62, 63]. Systemic administration of antimicrobials is thought to be more effective, theoretically being able to more broadly penetrate mammary tissue [64]. However, systemic administration can inadvertently lead to an increase in antimicrobial resistance of bacteria in the gastrointestinal tract, an issue that is of growing concern in public and global health [8, 12, 57, 61, 65, 66].

Antimicrobial resistance reduces the effectiveness of current treatments. Rasheed et al. showed that over 14% of *E. coli* isolated from food sources such as milk, beef, and poultry, were resistant to one or more commonly used antibiotic [67]. Similarly, MRSA was found on 29.6% of sampled cows in China [15]. Issues like these can also lead to increased prevalence of chronic and sub-clinical mastitis. Antimicrobial residues can also persist in milk following treatment [68], highlighting the need for a better

understanding of the pathogenesis of mastitis-associated bacteria as well as investigation into more effective treatments.

1.2 Extraintestinal Pathogenic and Mastitis-Associated E. coli

E. coli is one of the most ubiquitous gram-negative organisms in the world. It is a member of the family *Enterobacteriaceae* and can be found in the intestinal tract of most mammalian species and birds. *E. coli* bacteria mostly behave as a commensal organism though many strains are considered pathogenic. Most pathogenic *E. coli* strains cause intestinal illness, but many other strains are especially suited for causing infection outside of the intestines. These are called extraintestinal pathogenic *E. coli* (ExPEC).

ExPEC can cause a wide variety of illnesses. They are the leading cause of urinary tract infections [69, 70], second most common cause of neonatal meningitis [71, 72], and the most prevalent cause of sepsis from gram-negative bacteria [73]. These ExPEC infections lead to increased healthcare costs, morbidity, and lost productivity [74]. Despite a large impact on global health, ExPEC has not reached the public eye as much as intestinal pathogens, likely due to a relatively constant endemicity in most areas [75]. This has led to a lack of awareness and less research on ExPEC strains than other areas of *E. coli* pathogenesis.

E. coli strains can be placed into phylogroups according to the presence or absence of four specific genes (Groups A, B1, B2, C, D, E, F, Clade I). Phylogroup analysis is of limited value in predicting the pathogenic potential of *E. coli* strains including those that cause mastitis. However, ExPEC tend to be from different phylogenetic groups than their intestinal pathogenic relatives. Intestinal pathogens are

predominantly from phylogroup A, B1, or D [76] while ExPEC isolates are mostly grouped into phylogroup B2 and followed by group D [77-84]. This is in contrast to commensal strains of *E. coli*, which are predominantly in phylogroup A and B1 [82, 85]. Most isolates of MAEC are also part of phylogroup A and B1 [86-92] with phylogroup A being heavily associated with pathogenic strains in animals [88].

E. coli is the most frequently isolated Gram-negative organism in bovine mastitis [2-4, 20, 22]. For a long time, the severity of mastitis was thought to be mainly dependent on host factors such as immune response, lactation stage, and anatomy [93]. Colonization of the udder by *E. coli* most commonly occurs in early stages of lactation and during calving [94] and during the dry period, a time when the cow is most at risk for coliform mastitis [60]. Doses as small as 50 colony-forming units (CFUs) can induce an immune response [95].

Following infection, milk can begin to appear clotted and thick [96], the animal can start to feel pain at the site of infection and develop an acute fever [97]. Typically, after 48 hours from the initial onset, symptoms can disappear or reduce to unnoticeable levels if the inflammation was mild. However, some severe inflammatory responses can cause endotoxic shock resulting in permanent health issues or even death of the cow [93]. Even following successful treatment, the udder may sustain permanent damage from an acute MAEC infection.

1.2.1 Putative Virulence Factors Found in MAEC are Poorly Understood

E. coli has a wide variety of virulence factors that allow it to cause a broad range of infections among many different hosts (Table 1-1). Some MAEC also carry these genes including those that code for biofilms [98], capsule synthesis [99], and serum resistance

[100, 101]. Unfortunately, very few studies have tested their contribution to virulence in mastitis models of infection [102]. Lehtolainen et al. analyzed the genomes of over 150 MAEC strains isolated from clinical mastitis cases in Finland and compared the prevalence of each putative virulence factor found with the clinical presentation of each strain. They showed that some virulence factors (P and S fimbriae, CNF1 and CNF2) were strongly correlated with the phenotype of persistent cases of clinical mastitis, while none were correlated with the severity of the infection [102]. Capsule production, zinc uptake, and iron scavenging through the ferric dicitrate receptor all directly influence the ability of MAEC to grow in mouse mammary glands [99]. However, very few putative virulence factors have been tested for their role in experimental mammary gland infections, leaving their impact in MAEC virulence largely enigmatic.

Table 1-1: Virulence Factors Found in MAEC

Virulence Factor	**Role**	**Reference**
Fimbriae/Afimbriae (AfaE-8†, P and S fimbriae**, LPF1†, LPF2†, Type 1 fimbriae)	Adhesion	[101, 103-108]
Intimin† (*eae*)	Adhesion	[103, 105, 109]
Aerotaxis Receptors†	Detection of O_2 niches	[110]
Biofilm associated genes† (*fliC, fimA, csgA, luxS*)	Protection against host defenses and antimicrobials	[111, 112]
Polysaccharide Capsule (Group III)*	Protection from phagocytosis and destruction by macrophages	[99]

Iron Transport systems (yersiniabactin†, enterobactin†, sitABCD†, fecABCDE*)	Iron acquisition and transport to *E. coli*	[101, 113, 114]
Zinc Uptake (*znuABC**)	Zinc acquisition and protein function	[99, 115]
Shiga toxins† (Stx1, Stx2)	Induction of apoptosis of mammary cells	[116-118]

A review by Nawel Zaatout, 2022 contains more information about these virulence factors [119].

*These genes have been experimentally shown to contribute to colonization of mammary glands
†These genes have been verified as important for the virulence of other pathogenic *E. coli* but have not been experimentally shown to contribute to colonization of mammary glands

1.2.1.1 Biofilms are Thought to Contribute to MAEC Persistence

Biofilms are a community of bacteria that produce a polymeric extracellular matrix consisting of saccharides, DNA, or proteins [98, 120-123]. The process of forming a biofilm can be divided into 4 steps: reversible adhesion, irreversible adhesion, biofilm maturation, and dispersion [124]. Once the biofilm is formed, it can act as a protective barrier against many different environmental pressures such as antimicrobials [125], phagocytic host immune cells [126, 127], and oxidative stress [128].

The initial adhesion of *E. coli* to a biotic or abiotic surface can be facilitated by many different factors. Hydrodynamic and electrostatic forces of the environment and surface are overcome using flagellar propulsion allowing the bacterium to loosely or reversibly bind to the target surface [129, 130]. This reversible binding is usually caused by forces like van der Waals interactions, temperature, steric hindrance, or hydrophobic forces [130, 131]. The magnitude of these forces and ability of the bacterium to overcome

them largely depends on the surface. If the surface exhibits more repulsive forces towards the bacterium, it will not be able to attach effectively.

Following the reversible adhesion to the surface, *E. coli* can irreversibly bind to the surface using pili, fimbriae, or surface proteins [129]. Soon after, the bacterium can begin to produce components of the extracellular matrix such as autotransporters [132] and extracellular polymeric substances [133]. Other molecules such as quorum sensing autoinducers like N-acyl-homoserine lactones will also be produced and exported into the surrounding environment to modify gene expression of the bacterial community at large [134]. This can lead to increased expression of motility and virulence factors such as fimbriae and heat-labile toxins [135], which have been described in many MAEC strains (Table 1-1).

In an *in vitro* model of bovine mammary epithelial cells, non-adherent *S. aureus* was markedly more sensitive to antimicrobial treatment than adherent staphylococci [136], suggesting that biofilm formation is beneficial to the survival of bacteria during treatment. Biofilm formation occurs in MAEC strain P4 during both mouse and bovine models of infection, however, its role in pathogenesis and virulence is not yet understood but likely contributes to persistence in the mammary gland [137].

1.2.1.2 Polysaccharide Capsules Contribute to MAEC Virulence

Capsules are produced by a wide variety of both non-pathogenic and pathogenic bacteria [138-141]. In *E. coli*, capsules typically consist of polysaccharides that are produced by the cell and exported to the space around the cell. The primary components of the extracellular polysaccharides (LPS, O antigens, and capsular or K antigens) are serotype specific. The serotype of these antigens can be used to predict virulence of the

bacterium [142]. There are over 150 serogroups of O antigens and more than 80 serotypes of K antigens (see the Complex Carbohydrate Structure Database, https://ccrc.uga.edu/) [143].

Some MAEC strains make capsules that are vital to their success as a pathogen. M12 is a strain of MAEC that was found to make a Group III capsule. Olson et al. found that when they disrupted capsule synthesis by deleting *kpsC* and *kpsS* the virulence of M12 was completely eliminated in *Galleria mellonella* and slightly reduced in mouse mammary gland model of infection [99]. In subsequent work, this capsule was found to be indispensable in causing sepsis and colonization of kidneys in a urinary tract model of infection [138].

1.2.1.3 Adhesion is Important for Persistent MAEC Infection

Adhesion and invasion of epithelial cells has long been described as an important factor for the success of many pathogenic *E. coli* strains [144]. The ability to adhere to host cells allows the bacterium to not only colonize host tissues but persist in areas that it may not otherwise be able to. Pili and fimbriae are the most well-understood adhesins. Fimbriae are rod-like structures 5-10 nanometers in diameter and can reach lengths of greater than 10 micrometers [145]. In MAEC, genes encoding P and S fimbriae have been significantly correlated with strains that cause chronic mastitis [102], suggesting that adhesion mammary epithelial cells is important for continuous infection of a mammary gland.

Proteins other than fimbriae also contribute to bacterial adhesion and invasion of host cells. NlpI is an outer membrane lipoprotein found in all *E. coli* that is important in meningitis and intestinal infections caused by pathogenic *E. coli* [146, 147]. Intimin is

another adhesion factor that has been found in several different strains of MAEC [103, 105, 109] (See Table 1). It is expressed in both enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) [148]. Intimin binds an epithelial cell surface protein, Hp90, and the translocated intimin receptor and allows the bacterium to strongly adhere to the cell surface [149]. Genes encoding for both intimin and translocated intimin receptor have been found in MAEC but any possible contribution to virulence has yet to be investigated [150].

Persistent bacterial infections frequently involve adhesion, invasion, and intracellular replication of the pathogen in host cells [151]. Döpfer et al demonstrated that some MAEC possesses the ability to invade and persist in mammary epithelial cells [152]. MAEC strains isolated from persistent mastitis infections display much higher rates of invasion of bovine mammary epithelial cells than their transient counterparts (10.6-fold difference) and similar rates of adhesion [153], suggesting that these initial steps of colonization are important in the progression of the disease.

Olson et al. analyzed the genomes of >100 MAEC isolates after identifying which isolates were more successful in both mouse and bovine models of infection [115]. Isolates that were more prevalent in later stages of infection were significantly more likely to possess a gene called *chiA*, a putative periplasmic chitinase, adjacent to a type 2 secretion system (T2SS) [99]. A separate analysis of over 800 publicly available genomes was performed on commensal and pathogenic *E. coli* strains. Commensal strains were isolated from the intestines of healthy avian (chicken or turkey) or bovine hosts while pathogenic strains were isolated from lesions of disease such as bovine mastitis or avian colibacillosis. Pathogenic isolates were much more likely to have *chiA*

(formerly *yheB*), a putative bifunctional lysozyme/chitinase, and its T2SS in their accessory genome than the commensal strains (79.9% vs 47.0%, respectively) [Olson et al., Unpublished data]. These findings indicated *chiA* might play a role in MAEC pathogenesis.

1.3 Chitinase A and its Possible Role in MAEC Adherence to Host Cells

Chitin is a biopolymer consisting of a repetitive chain of β-(1,4)-N-acetyl-D-glucosamine units. It is found in arthropods [154], fungi [155], and cephalopods [156] and is the second most abundant biological molecule after cellulose [157, 158]. While mammals do not synthesize chitin, they possess a family of proteins called number-18 glycoside hydrolases. This protein family consists of 2 subfamilies: chitinase proteins and chitinase-like proteins [159, 160]. The former can both bind and digest chitin molecules via hydrolysis while the latter binds chitin molecules but lacks the ability to digest them. Many bacteria also possess genes for chitinase proteins. The soil-dwelling gram-negative bacterium, *Serratia marcescens*, effectively degrades chitin and using three unique number-18 chitinases, ChiA, ChiB, and ChiC [161, 162]. In some species, chitinases can degrade other N-acetyl glucosamine containing polysaccharides like peptidoglycan [163], suggesting these proteins may serve multiple purposes.

E. coli cannot fully digest and metabolize long chains of chitin. However, it can grow on the disaccharide, N,N'-diacetylchitobiose ($GlcNAc_2$) [164]. ChiA degrades $GlcNAc_2$ and *N, N',N"*-triacetylchitotriose into singular GlcNAc units [165]. In the lab strain K-12, ChiA is negatively regulated by the nucleoid-structuring protein, H-NS [165, 166]. While ChiA is regularly found in the periplasm, it is exported outside of the cell, a

characteristic common among bacterial chitinases [167], via the T2SS that is frequently encoded adjacent to it [165].

Chitinases are increasingly being recognized as important virulence factors in both enteric pathogens [168-170] and non-enteric pathogens [171-173]. In *Salmonella enterica* serovar Typhimurium, chitinases promote adhesion and invasion of intestinal epithelial cells through modification of the host glycoproteins [174]. Specifically, *Salmonella* chitinase cleaves the terminal sialic acid and Gal-β-1,4 acetyl glucosamines to make the mannose residues more accessible to the bacteria, which are targets of type I pili. These chitinases also increase immune evasion by up-regulating production of reactive nitrogen and oxygen species in intracellular bacteria to reduce the host immune response in phagocytes by causing down regulation of MHC-II, preventing the antigen presentation by both activated macrophages and dendritic cells [175].

Legionella pneumophila chitinases are required for colonization of the lungs due to their ability to degrade mucin [173, 176]. *L. pneumophila* secretes its chitinase protein (LP-ChiA) through a T2SS in a similar manner as *E. coli*. Following secretion, LP-ChiA associates with the outer leaflet of the outer membrane [176]. ChiA from MAEC shares a high level of homology with both *L. pneumophila* and *Salmonella* chitinases (see Supplementary Figure 12). This homology is mainly concentrated in the regions of MAEC ChiA that have been previously described as chitin-binding domains (residues 25-567) [177].

MUC1 is a glycoprotein found on the surface of mammary epithelial cells in cows [178]. MUC1 is highly polymorphic and extensively glycosylated [179]. It also binds to *E. coli* strain K-12, preventing adhesion to mammary epithelial cells. MUC1 transcription is

upregulated in response to the presence of LPS, indicating that it is part of the innate immune response in coliform mastitis [179]. It is possible that ChiA binds to or digests portions of MUC1 preventing it from inhibiting adherence to mammary epithelial cells, similar to how other species of pathogenic bacteria use chitinase to degrade mucin or glycoproteins to promote colonization [174-176] though this has not been investigated.

Adherent-invasive *E. coli* (AIEC) strain LF82 was isolated from the chronic ileal lesion of a Crohn's patient [180]. Low et al. showed that in the absence of ChiA, LF82 does not adhere well to human intestinal epithelial cells. In addition, N-glycosylation was critical for the adhesion of LF82 to epithelial cells [177]. They showed this interaction was due to binding to the host protein, Chitinase 3-like-1 (CHI3L1). In addition to its function in intestinal cells, CHI3L1 is also expressed on the surface of mammary and in milk as a consequence of inflammation from coliform mastitis [159]. CHI3L1 is found in a wide range of mammalian species [181], suggesting an important role in defense of mucosal epithelial cells. Whether ChiA acts as an adhesive factor during MAEC infection has not been investigated.

1.4 Summary

The wide range of severity and clinical presentation of mastitis infections by MAEC is impressive. Recently, work has begun to characterize the putative virulence factors of MAEC and find correlations or relationships with severity or presentations of mastitis. However, these studies are in their infancy, with few directly testing the role of these factors in the pathogenesis of this disease.

The role of ChiA in MAEC has not yet been investigated. The *chiA* gene is present at significantly higher rates in MAEC strains that are more competitive during

experimental infection [115] as well as in pathogenic strains of both avian pathogenic and mastitis-associated *E. coli*. suggesting it plays an important role in the ability of MAEC to colonize and persist in mammary glands. Further characterizing the expression of ChiA and identifying its role in adhesion to mammary epithelial cells is the focus of this thesis. Understanding the role ChiA plays in the pathogenesis and progression of mastitis would lead to a better understanding of this disease, potentially identifying a novel target for antimicrobial therapies, treatments, and vaccines.

SECTION 2: MATERIALS AND METHODS

2.1 Bacterial strains and media

All bacterial strains were routinely grown in Luria-Bertani (LB) (Tryptone 10 g/L, NaCl 10 g/L, Yeast Extract 5 g/L, if applicable agar 15 g/L) media at 37°C unless otherwise stated. Liquid cultures were grown shaking at 220 rpm unless specified otherwise. Antibiotics were added to media as required for growth of mutants and maintenance of plasmids (chloramphenicol 10 μg/mL, ampicillin 100 μg/mL, gentamicin 10 μg/mL). The donor *E. coli* strain MFDpir was grown in LB plus di-aminopimelic acid (DAP) (Sigma, #D1337) at 330 μM concentration. Super-Optimal Broth (SOB) (tryptone 20 g/L, yeast extract 5 g/L, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$) [182] was used for growth of strains prior to transformation. Transformations were recovered in SOB with catabolite repression (SOC) (SOB with 20 mM glucose).

Each strain used in this thesis, their clinical presentation, location each MAEC strain was isolated, Clermont Type, and multilocus sequencing type (MLST) is listed in Table 2-1.

Table 2-1: List of Strains used in this Study

Name	**Genotype or Main Characteristic**	**Clermont Type**	**MLST**	**Location Isolated/Source**
M45	Mild, clinical MAEC	C	ST23	Ontario, Canada [99]
M93	Mild, clinical MAEC	A	ND*	Ontario, Canada [99]
M111	Severe, clinical MAEC	A	ST10	Alberta, Canada [99]
G1	Severe, per-acute clinical MAEC	B2	ST95	Idaho, USA This Study

MFDpir	MG1655 RP4-2-Tc::(ΔMu1::*aac(3)IV*-Δ*aphA*-Δ*nic*35-ΔMu2::*zeo*) Δ*dapA*::(*erm-pir*) Δ*recA*			[183]

*ND=Not determined

2.2 Generation of Knock-out Mutants in MAEC Strains

2.2.1 Creation of pAX1 Allelic Exchange Plasmid

Primers to make the construct to knock-out *chiA* were designed as described by Wiles et al. [184]. First, a region of ~500 bp upstream and a region of ~500 bp downstream of *chiA* was amplified using PCR. Protocols for these PCR reactions for these regions are described in Table 2-2 as PCR1. The forward upstream primer (primer 003) contained a SalI recognition site (SalI: GTCGAC) on the 5' end and the reverse downstream primer (primer 006) contained an AvrII recognition site (AvrII: CCTAGG) on its 5' end for future insertion into the pAX1 plasmid. The reverse upstream (primer 004) and forward downstream primers (primer 005) each contained tails of the reverse complement of the other primer. This provides an area of overlap to allow the upstream and downstream sequences to be "stitched" together. All primers used for this are listed in Table 2-4.

After both the upstream and downstream sequences were amplified, these two products were "stitched" together using the PCR2 protocol laid out in Table 2-2. The resulting PCRs were separated on a 0.8% agarose gel. Bands at the correct size (~1,000 bp) were excised and DNA was isolated using Zymoclean™ Gel DNA recovery kit (Zymo Research, #D4001) according to the manufacturer's instructions.

Next, the isolated stitch PCR product was digested using SalI-HF (New England Biolabs, #R3138) and AvrII (New England Biolabs, #R0174) according to the

manufacturer's recommendations. The pAX1 plasmid was digested using the same restriction enzymes and protocol. The digested stitch PCR product was isolated using Monarch© PCR & DNA Cleanup Kit (New England Biolabs, #T1030). The digested pAX1 plasmid was separated on a 0.8% agarose gel, then the band was excised and isolated using Zymoclean™ Gel DNA recovery kit (Zymo Research, #D4001).

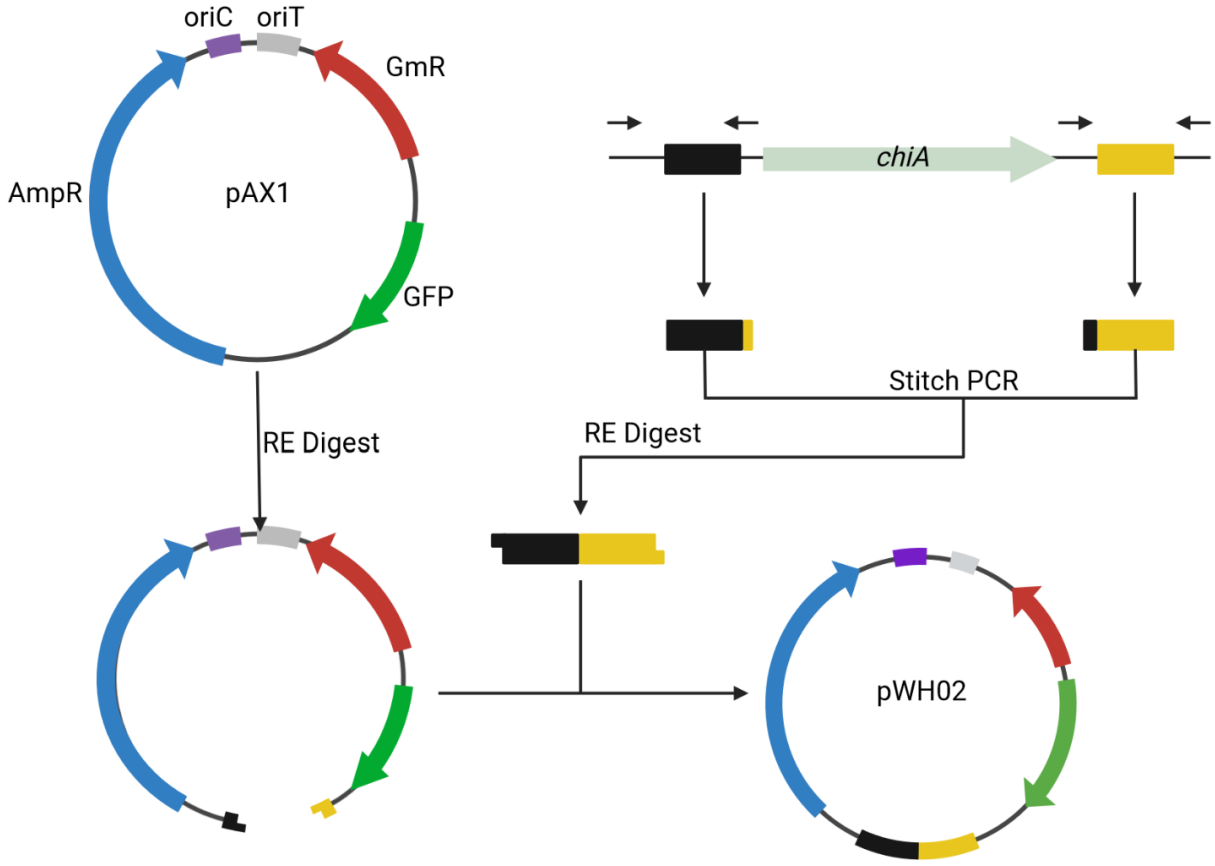


Figure 2-1: Creation of pWH02 from pAX1

Finally, the digested stitch PCR product and digested pAX1 were ligated together using T4 DNA Ligase (New England Biolabs, #M0202) according to the manufacturer's suggested protocol in a 20 µL reaction carried out at room temperature for 60 minutes. This resulted in pWH02. Then 2 µL of the reaction was transformed via electroporation into the donor *E. coli* strain MFDpir. Transformants were selected for by plating on LB+Amp+DAP. This resulted in strain MFDpir1.

Table 2-2: Upstream and Downstream Region Amplification for pAX1 Plasmid

25 µL PCR1 Reaction:	**PCR1 Protocol:**
12.5 µL Q5 Master Mix 1 µL genomic *E. coli* DNA 1.25 µL Primer 003/005 1.25 µL Primer 004/006 9 µL mgH_2O*	1) 98°C for 30 sec 2) 98°C for 10 sec 3) 60°C for 15 sec 4) 72°C for 15 sec 5) Go to Step 2—30X 6) 72°C for 5:00 7) Hold at 4°C
25 µL PCR2 Reaction:	**PCR2 Protocol:**
12.5 µL Q5 Master Mix 1.25 µL Primer 003† 1.25 µL Primer 006† 1 µL Upstream PCR1 product** 1 µL Downstream PCR1 product** 8 µL mgH_2O*	1) 98°C for 30 sec 2) 98°C for 10 sec 3) 55°C for 30 sec 4) 72°C for 30 sec 5) Go to Step 2 – 10X 6) Add primers to 500 nM 7) 98°C for 10 sec 8) 60°C for 15 sec 9) 72°C for 30 sec 10) Go to Step 7 – 20X 11) 72°C for 5:00 12) Hold at 4°C

*mgH_2O=molecular grade H_2O
† primers should not be added to the reaction until Step 6. This allows for the two PCR products to stitch together and amplify as a single amplicon
** both upstream and downstream products should be added to the reaction in equimolar amounts. Adjust mgH_2O as needed.

2.2.2 pAX1 Mating Reactions and Mutant Screening

Overnight cultures of both the target strain and donor strain MFDpir containing pWH02 were sub-cultured into fresh media at a 1:1,000 dilution and incubated at 37°C and 30°C, respectively, until an OD_{600} of 0.4-0.5. After this, 750 µL of the target strain and donor with were each combined in a 1.5 mL microcentrifuge tube and cells were pelleted by centrifugation at 10,000xG for 1 min. Supernatant was discarded and cells

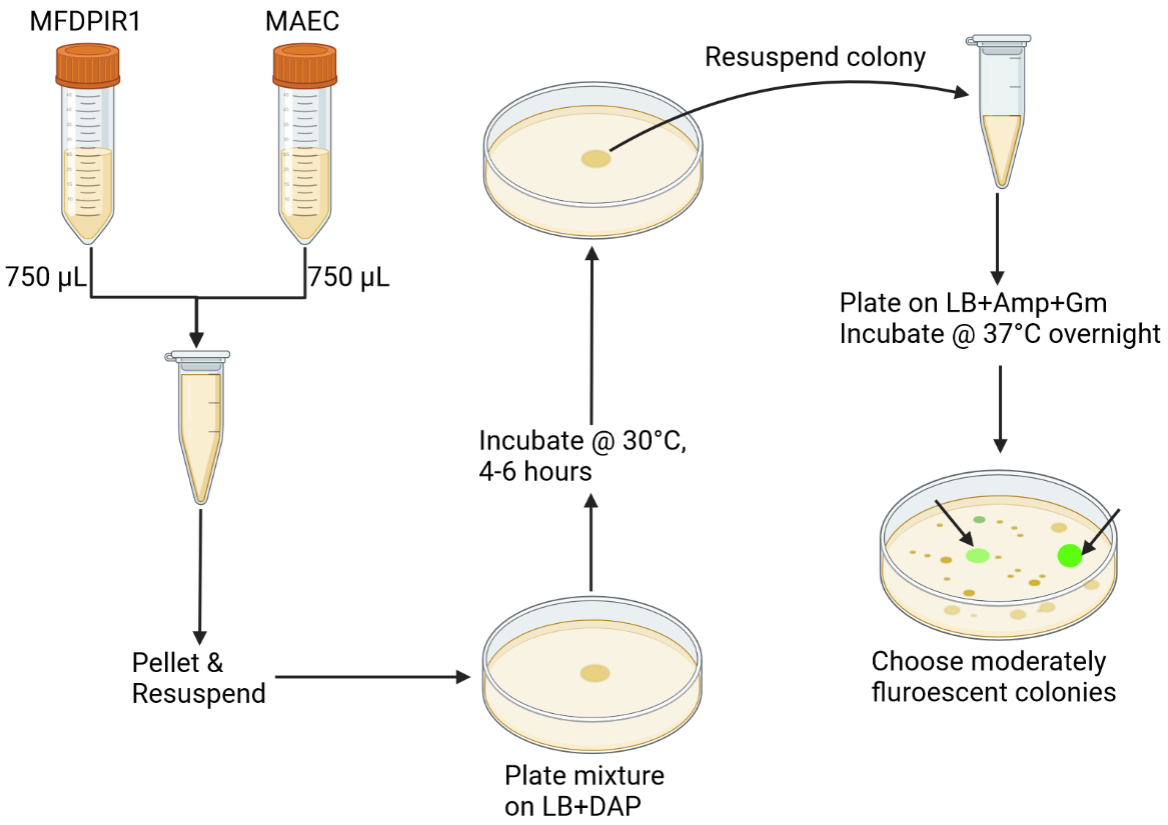


Figure 2-2: Mating Reactions with MFDPIR+pWH02 to Create Merodiploids

were resuspended in 50 µL of LB+DAP, then placed on a 25 mm filter disk with 0.45 µm pores. This was incubated at 30°C for 4-6 hours.

Following the co-incubation, the bacteria were dislodged from the filter disk by vortexing in 500 µL sterile PBS. 100 µL of this mixture and 100 µL of a 1:10 dilution was plated on LB agar with ampicillin and gentamicin and incubated overnight at 37°C to select for insertion of the plasmid into the chromosome. Colonies that exhibited mild fluorescence were considered potential merodiploids. Merodiploid colonies were grown in LB broth for 6 hours at 37°C, serially diluted, and plated on LB agar. Single colonies that had lost any noticeable fluorescence were chosen and analyzed with PCR to verify deletion of *chiA* using primers 001 and 002 (internal) or primers 003 and 006

(external). Gels of each wild type and Δ*chiA* mutant with these primers are shown in Figures 2-3 and 2-4.

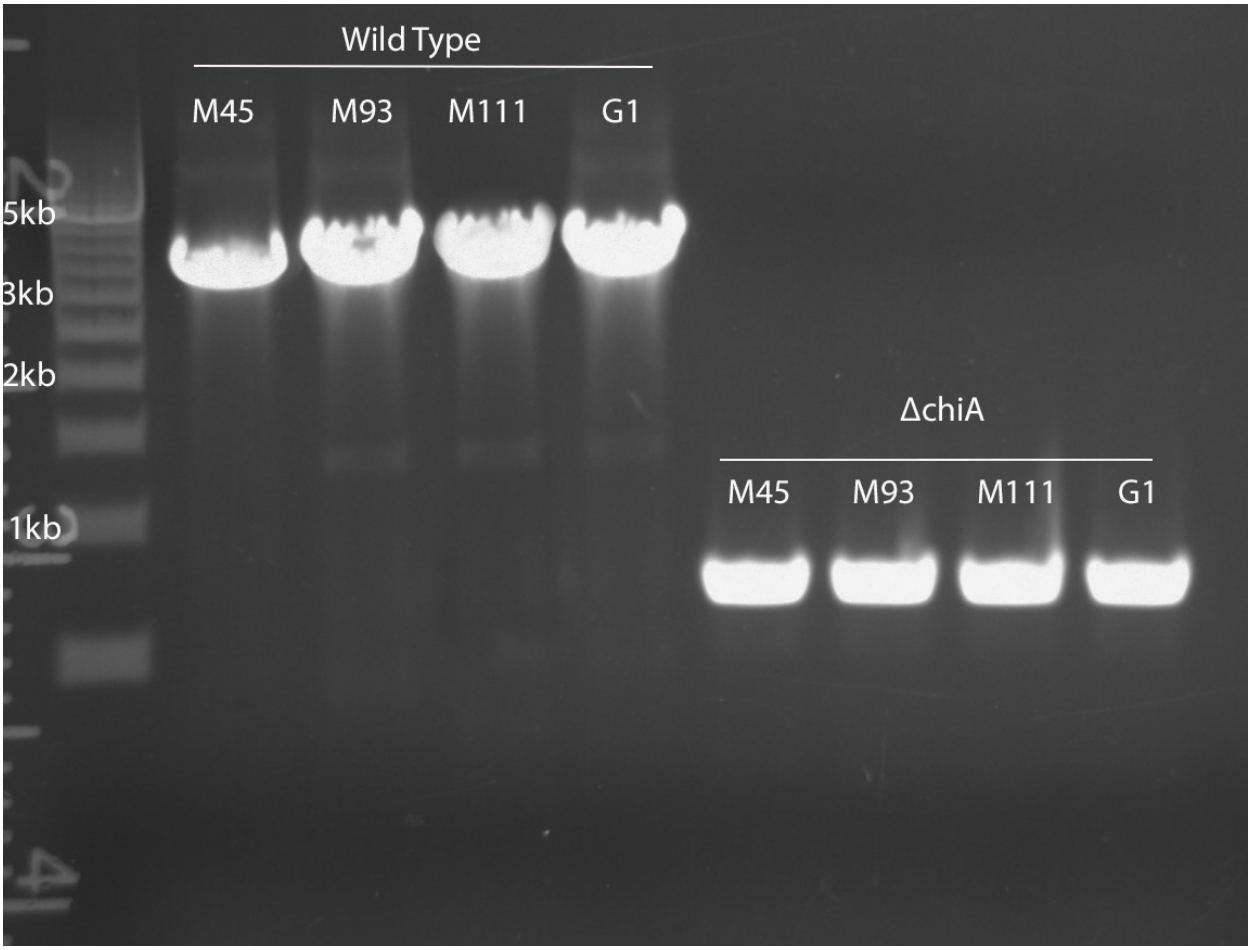


Figure 2-3: Gel of Wild Type and ΔchiA Mutants Tested with External Primers.
Δ*chiA* mutants were screened for using primers 003 and 006. The wild type strains gave bands ~3.7 kb while Δ*chiA* mutants gave bands ~800-900 bp.

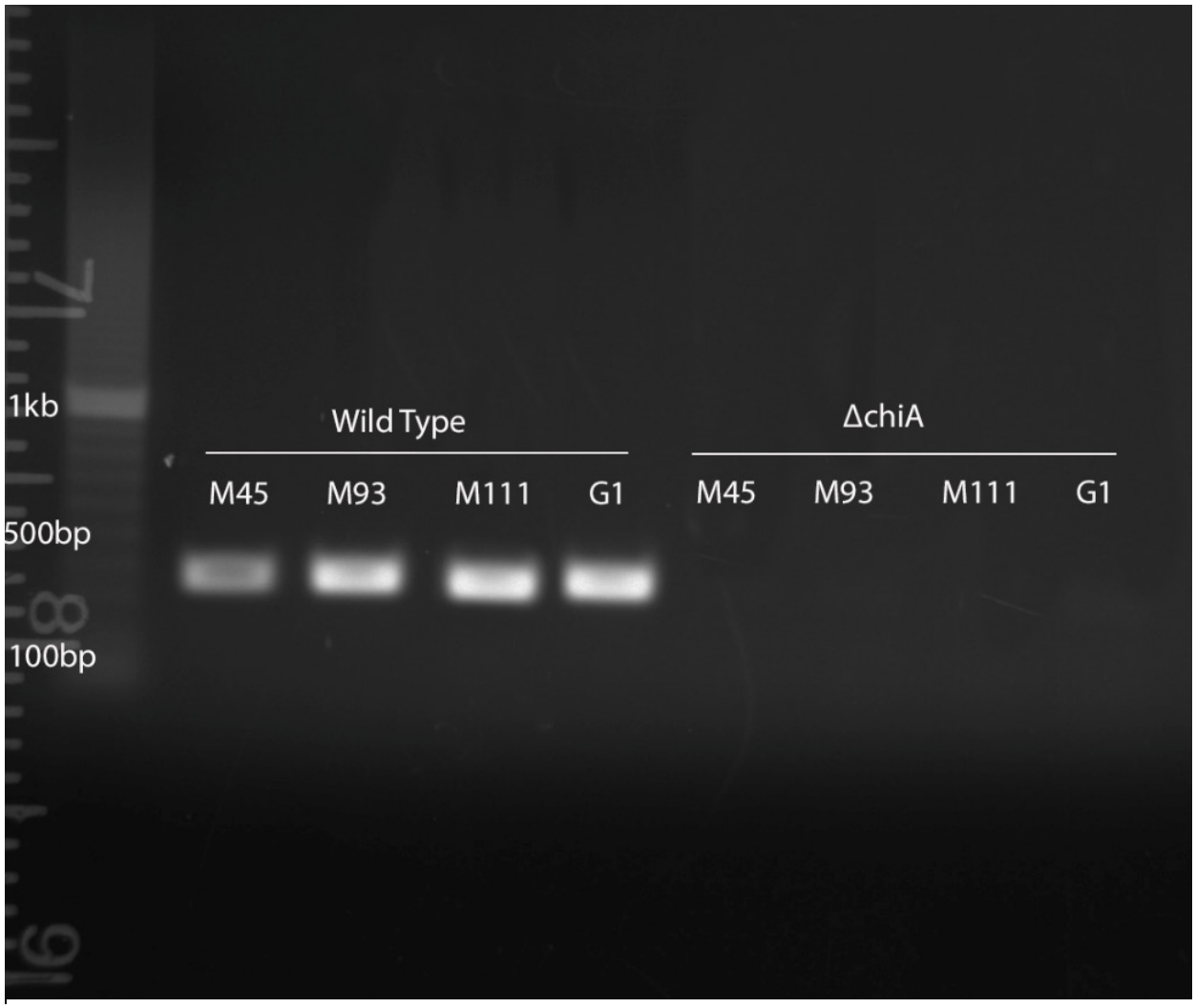


Figure 2-4: Gel of Wild Type and ΔchiA Mutants Tested with Internal Primers.

Δ*chiA* mutants and wild type MAEC strains were screened using internal *chiA* primers 001 and 002. Wild type strains showed a band ~450 bp while no band was present for Δ*chiA* mutants.

2.3 Transformation of Plasmids

Plasmids were transformed into bacteria using either heat-shock or electroporation. Heat-shock was used to propagate cloning plasmids before being re-isolated and electroporated into target strains. For heat-shock, NEB© 5-alpha Competent *E. coli* (New England Biolabs, #C2987H) was thawed on ice for 5 minutes. Then, 1-5 µL containing 1-100 ng of plasmid was added to cells and mixed by flicking. This mixture was placed on ice for 45 minutes before heat-shocking in a 42°C heating

block for 30 seconds. The mixture was then placed back on ice for 5 minutes before adding 950 µL of SOC. The culture was then incubated shaking (220 rpm) at 37°C for 1 hour before plating on LB with the appropriate antibiotic for selection of transformants.

For electroporation, an overnight culture of cells was diluted 1:1,000 in fresh SOB and grown at 37°C to an OD_{600} of 0.4-0.5 (2-3 hours). 1 mL of culture was pelleted by centrifuging at ≥16,000 rcf for 1 minute at 4°C. The supernatant was discarded, cells were washed in 1 mL of ice-cold 10% glycerol, and pelleted again. This was repeated for a total of 3 washes. Following the final wash, cells were resuspended in 75 µL of ice-cold 10% glycerol. Then, 10-500 ng of plasmid was added to the cells and they were electroporated using a MicroPulser Electroporator (Bio-Rad, #1652100) on the Ec1 setting (1 pulse at 1.8 kV, time constant 2.5s) in an ice-cold 1mm cuvette (Genessee, #40-103). Cells were recovered with 900 µL of fresh SOC and incubated at 37°C for 1 hour with shaking (220 rpm) and 100 µL was plated on LB with the appropriate antibiotic to select for transformants and incubated at 37°C overnight.

2.4 Complementation of Knockout Mutants

The *chiA* gene was reintroduced into each knockout mutant by cloning the gene into pJET1.2 (ThermoFisher, #K1231) This was done by amplifying *chiA* including 300 bp upstream to include the putative promoter using primers 007 and 008 (Table 2-4) and ligating the resulting product into pJET1.2 according to the manufacturer's instructions. This created plasmid pWH01. This was then transformed by heat-shock into NEB© 5-alpha Competent *E. coli* (New England Biolabs, #C2987H) and plated on LB with ampicillin overnight. Successful transformants were screened for using colony PCR with primers pJET1.2 forward and pJET1.2 reverse. Colonies containing the

plasmid with the correct insertion were grown overnight in Terrific Broth (Tryptone 12 g/L, yeast extract 24 g/L, 0.4% glycerol (v/v), potassium phosphate monobasic 2.3 g/L, potassium phosphate dibasic 16.4 g/L) with ampicillin overnight. pWH01 was then isolated using ZR Plasmid Miniprep kit (Zymo Research, #D4016) and transformed via electroporation (see section 2.3).

2.5 MAC-T Cell Culture and Media

Bovine mammary alveolar epithelial cells (MAC-T cells) were generously sent to our lab by Dr. Janos Zempleni (University of Nebraska-Lincoln). They were grown in T-75 flasks with 40% (v/v) Dulbecco's Modified Eagle Medium (DMEM), 40% (v/v) Ham's F12 Medium (Corning, #10-092-CM), and 10% (v/v) FetalPURE™ bovine serum (FBS) (Genessee, #25-525). FBS was heat-inactivated prior to use in media by incubating in a 56°C water bath for 30 minutes with periodical mixing. This was supplemented with bovine insulin, 5 µg/mL (Millipore-Sigma, #I0516), hydrocortisone, 1 µg/mL (Sigma, #H0888), HEPES buffer, 23 mM (Sigma, #H3375), sodium bicarbonate, 2.2 g/L (Fisher Scientific, #S233-500), and L-glutamine, 40 mM (Sigma,#G6392). Penicillin, Streptomycin (100 U/mL and 100 µg/mL, respectively) (ThermoFisher, #15140122) and Amphotericin B (2.5 µg/mL) (Sigma, #A2942) were added into the media for routine growth. MAC-T cells were grown at 37°C and 5% CO_2 (v/v).

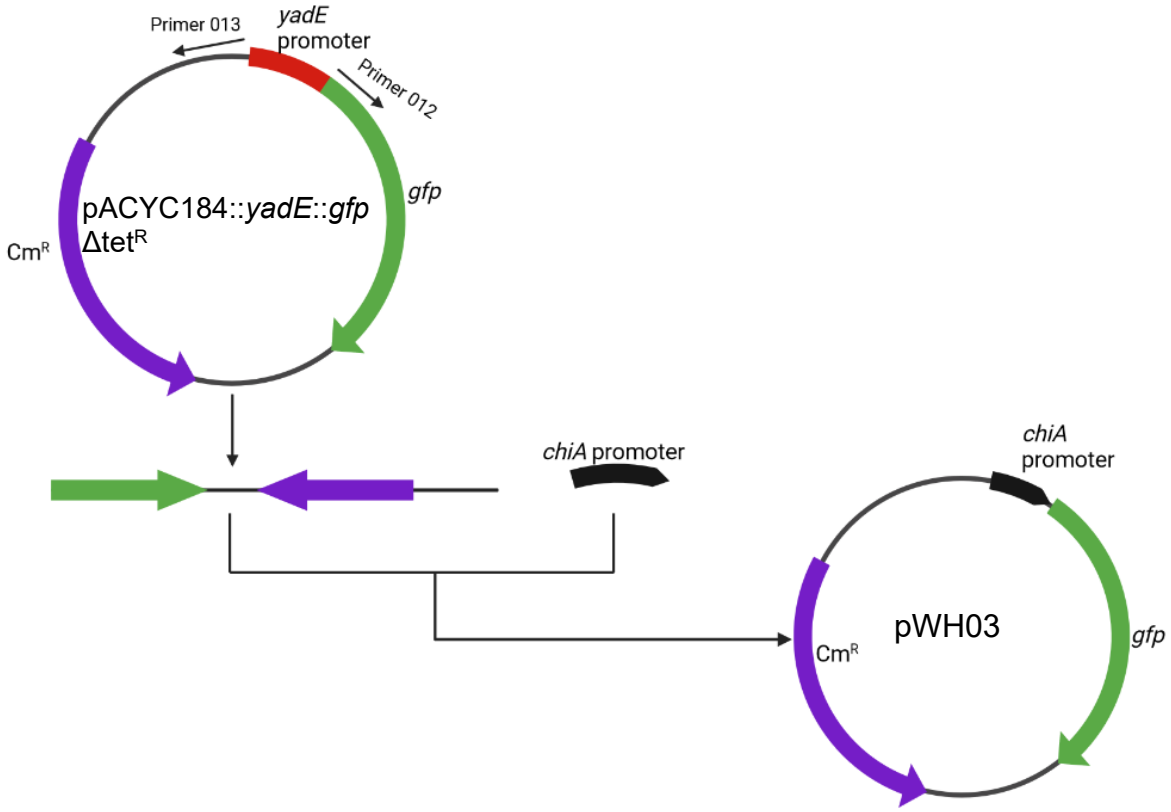


Figure 2-5: Creation of pWH03 Using PCR

2.6 Flow Cytometry and GFP Expression Reporter

pWH03 is a GFP reporter plasmid that was created by amplifying a previous plasmid made in lab (pACYC184::yadE::GFPΔ*tet*) [185] using primers 012 and 013 (Table 2-5) and inserting the sequence for the *chiA* promoter region in front of the GFP. This PCR reaction is detailed in Table 2-3 under "Plasmid linearization PCR". The PCR reaction was treated with DpnI to remove residual plasmid by adding 20 U of DpnI (New England Biolabs, # R0176) directly to the PCR reaction and incubating at 37°C for 1 hour. This reaction was inactivated by incubating at 80°C for 10 minutes. The promoter sequence for *chiA* was amplified with primers 010 and 011 which both contained overlap with the reporter plasmid. This PCR reaction is detailed in Table 2-4 under "Promoter PCR". Both reactions had their respective products cleaned and

isolated using Monarch© PCR & DNA Cleanup Kit (New England Biolabs, #T1030). These two PCR products were then ligated together using overlap-extension PCR. The protocol for this PCR reaction is detailed in Table 2-3 under the "Overlap-PCR" protocol. This was then transformed via heat-shock into NEB© 5-alpha Competent *E. coli* (New England Biolabs, #C2987H) as outlined in section 2.3. G1 was transformed with pWH03 using electroporation to create G1/PWH03.

For hypoxic conditions, a 5.0 L anaerobic jar (BD Biosciences, #260672) with 1, 2.5 L anaerobic generation sachet (ThermoFisher, #68061-10SATCHETS-F) was used. Samples were also grown in the presence of bile salts at 1.5% (w/v) in LB broth. Aerobic conditions were created by shaking samples at 220 rpms. All samples were incubated at 37°C overnight (16-18 hrs) before being analyzed. 50 μL of the cultures were suspended in 950 μL of sterile PBS.

The suspension of bacteria was then measured through the FL-1 channel on a BD Accuri™ C6 Flow Cytometer (BD Biosciences). 10,000 events were measured and backgated gated to only include singlets with a fluorescence measurement greater than 1. The observed fluorescence was normalized to the fluorescence G1/pWH04 which contained a plasmid with no promoter sequence in front of the *gfp*. This was done 2 times with each sample being measured in triplicate.

pWH04 was created according to the "Plasmid Linearization PCR" protocol listed in Table 2-3. The resulting linear construct was then digested using SalI (New England Biolabs, #R3138L) and DpnI. The resulting digested product was then cleaned and reisolated. This was ligated together without an insert using T4 Ligase and the reaction was transformed into G1 measured via electroporation to create G1/pWH03.

Table 2-3: PCR Reactions to create pWH03 and pWH04

Plasmid Linearization PCR	**Plasmid Linearization PCR Protocol**
25 µL reaction	1) 98°C for 30 sec
12.5 µL Q5 Master Mix	2) 98°C for 10 sec
1 µL pACYC::yadE::GFP::Δ*tet*	3) 58°C for 15 sec
1.25 µL primer 011‡	4) 72°C for 1:00
1.25 µL primer 012‡	5) Go to Step 2—30X
9 µL mgH_2O*	6) 72°C for 5:00
Promoter PCR	**Promoter PCR Protocol**
25 µL reaction	1) 98°C for 30 sec
12.5 µL Q5 Master Mix	2) 98°C for 10 sec
1 µL genomic *E. coli* DNA	3) 58°C for 15 sec
1.25 µL primer 009	4) 72°C for 10 sec
1.25 µL primer 010	5) Go to Step 2—30X
9 µL mgH_2O*	6) 72°C for 5:00
Overlap PCR	**Overlap PCR Protocol**
25 µL reaction	1) 98°C for 30 sec
12.5 µL Q5 Master Mix	2) 98°C for 10 sec
2 µL Promoter PCR product†	3) 72°C for 2:00
1 µL Plasmid Linearization PCR product†	4) Go to Step 2—20X
9.5 µL mgH_2O*	5) 72°C for 5:00

*mgH_2O=molecular grade H_2O
†The promoter PCR product should be present at 2x the molar amount than the plasmid linearization PCR product. Adjust MgH_2O as needed.
‡Primers are replaced with 013 and 014 when creating pWH04

2.7 Adhesion assays

MAC-T cells were seeded into a 12-welled plate and grown to ≥95% confluency. The density of epithelial cells was determined by trypan blue exclusion and counting using Cell Counter model R1 automated cell counter (Olympus). Approximately $6x10^5$ MAC-T cells were present in each well. The number of cells and viability did not vary

throughout the assays as determined by trypan blue exclusion. On the day of each assay, spent media was removed from each well and MAC-T cells were washed 3x with 1 mL of sterile PBS to remove residual antibiotics from media and unhealthy cells. 1 mL of media without antibiotics was added into each well and MAC-T cells were allowed to incubate for ≥2 hours prior to inoculation with bacteria.

Overnight cultures of bacteria were diluted in sterile PBS to an OD_{600} of 0.5. Bacteria was added to a multiplicity of infection (MOI) of 10 bacterial cells to 1 MAC-T

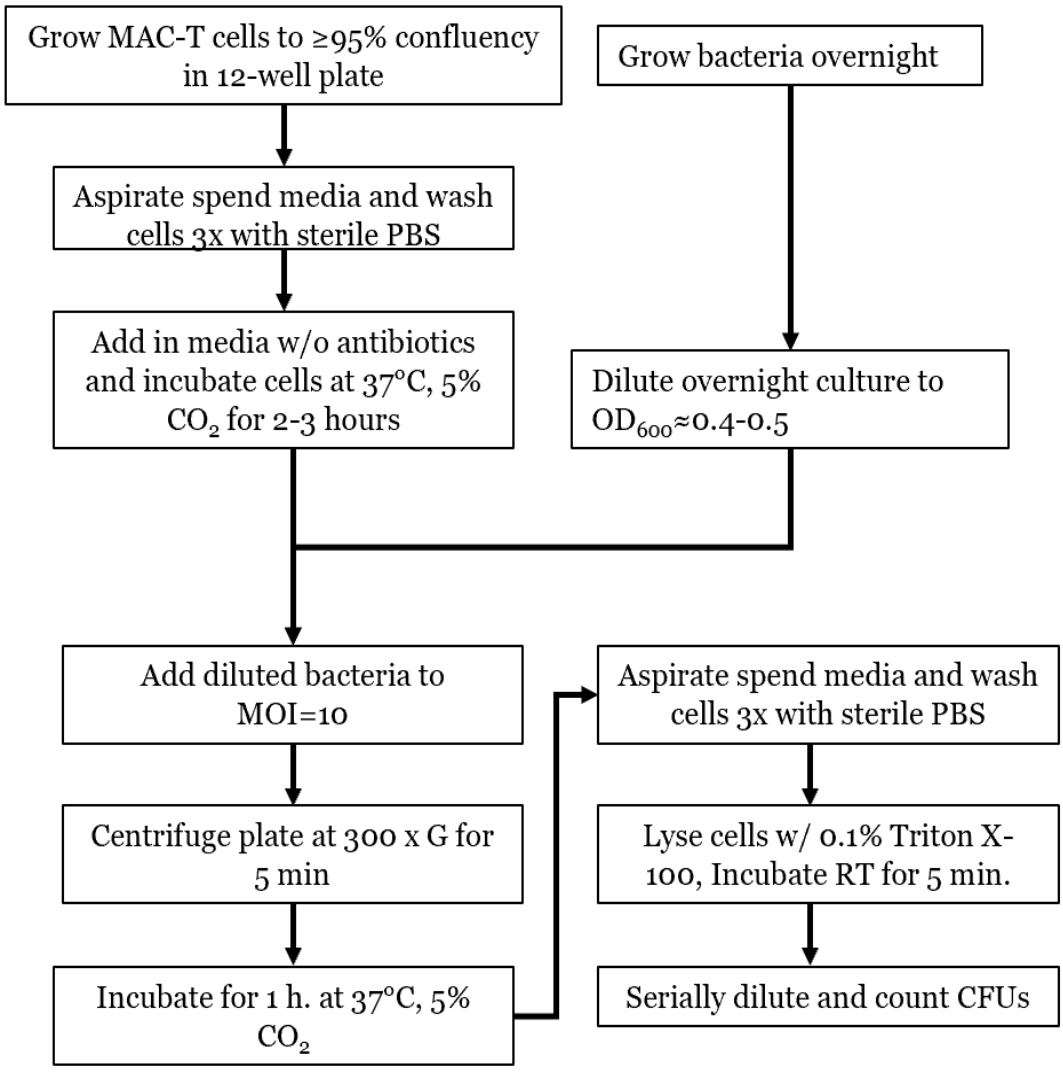


Figure 2-6: Workflow of Adhesion Assays.

cell (approx. $6x10^6$ bacteria, MOI=10:1). The 12-well plates were then centrifuged at 300xG for 5 min at room temperature in order to synchronize contact of bacteria with MAC-T cells. Plates were then incubated at 37°C and 5% CO_2 (v/v) for 1 hour. Following incubation, media was aspirated out of the wells and washed 3 times with 1 mL of sterile PBS to dislodge weakly adhered bacteria. 500 µL of 0.1% Triton X-100 (Sigma, #X100) in PBS was then added into each well and incubated at room temperature for 5 minutes to lyse MAC-T cells. The resulting suspension of bacteria was then serially diluted and plated on LB overnight and CFUs were counted. This was repeated two times and measured in triplicate.

2.8 Primers and Plasmids

All primers and plasmids used are listed in Tables 2-4 and 2-5, respectively.

Table 2-4: Primers used in this Study

Primer	**Sequence**	**Function**
001	GGTTGCGGATCAGGCATCTA	Forward internal primer for *chiA*
002	GCGTCAATTTCTGCATCGCT	Reverse internal primer for *chiA*
003	gcaggtcgacTAATGCCGGGCGACAACAT	Upstream forward of *chiA* with SalI site for construction of pWH02
004	gcggcgatactggaaggtattCCCTTGTGACGTAAAAACTGC	Upstream reverse of *chiA* for construction of pWH02
005	gcagtttttacgtcacaagggAATACCTTCCAGTATCGCCGC	Downstream forward of *chiA* for construction of pWH02
006	cagtcctaggTTGAGACGTTTGAGACCCCAG	Downstream reverse of *chiA* with AvrII site for

		construction of pWH02
007	CGTTTCGCAATCCGTGAAGG	Forward primer to amplify *chiA*
008	GCGGCGATACTGGAAGGTAT	Reverse primer to amplify *chiA*
009	tatctcttcaaatgtagcacTGCATTTGTTGGCTGTATATC	Forward primer for *chiA* promoter to create pWH03
010	agttcttctcctttacgcattATAATCCCTTGTGACGTAA	Reverse primer for *chiA* promoter to create pWH03
011	ttacgtgcacaagggattatAATGCGTAAAGGAGAAGAACT	Forward primer to linearize pACYC184::yadE::GFPΔ*tet* to create pWH03
012	gatatacagccaacaaatgcaGTGCTAACATTTGAAGAGATA	Reverse primer to linearize pACYC184::yadE::GFPΔ*tet* to create pWH03
013	gcaggtcgacAATGCGTAAAGGAGAAGAACT	Forward primer for null reporter plasmid pWH04
014	gcaggtcgacGTGCTAACATTTGAAGAGATA	Reverse primer for null reporter plasmid pWH04
pJET1.2 Forward	CGACTCACTATAGGGAGAGCGGC	Forward primer for verification of insertions into pJET1.2
pJET 1.2 Reverse	AAGAACATCGATTTTCCATGGCAG	Reverse primer for verification of insertions into pJET1.2

Table 2-5: Plasmids used in this Study

Plasmid	**Description**	**Source**
pJET1.2/blunt	pBM1, Amp^R, *eco47IR,* T7 promoter	ThermoFisher (Cat# K1231)

pWH01	pJET1.2/blunt with *chiA* cloned into position 369	This Study
pAX1	pSC101, Rep101(Ts), Gm^R, Amp^R, Tet^R, GFP	[184]
pWH02	pAX1, Δ*chiA*	This Study
pACYC184::yadE::GFPΔ*tet*	pACYC, Cm^R, GFP, *yadE* promoter	[185]
pWH03	pACYC, Cm^R, GFP, *chiA* promoter	This Study
pWH04	pACYC, Cm^R, GFP, no promoter	This Study
pKD46	repA101, Amp^R, araBAD, λgam/bet/exo,	[186]

2.9 Identification of Virulence Factors

Virulence factors were identified by using Virulence Factor Database 2.0 (Institute of Pathogen Biology, Beijing, http://www.mgc.ac.cn/VFs/) [187]. The assembled draft genomes were submitted to the database in Genbank format.

2.10 Multisequence Alignment of Glycosyl Hydrolase Family 18 Proteins

Genes encoding glycosyl hydrolase family 18 (GH18) proteins from *Salmonella enterica* serovar *Typhimurium* strain 14028S, *S. enterica* serovar *Typhi* strain CT18, and *Legionella pneumophila* strains C9_S and 130b were aligned to the protein sequence for ChiA from MAEC strain G1 using constraint based multiple alignment tool (COBALT) on the NCBI website. The alignment was viewed and edited using Jalview [188].

2.11 Statistical analysis

All statistical analysis was done using Prism9 (GraphPad). Differences in adhesion assays were analyzed using a one-way analysis of variance (ANOVA) with Tukey's correction for multiple comparisons. Differences in *chiA* expression from flow cytometery and plate reader values from pWH06 were determined using a Mann-

Whitney U test. A p-value less than or equal to 0.05 was considered statistically significant.

SECTION 3: RESULTS AND DISCUSSION

Based on previous work demonstrating the role of ChiA in adhesion to intestinal epithelial cells, I hypothesized that ChiA would play a similar role in adhesion to bovine mammary epithelial cells. I also hypothesized that expression of *chiA* would be increased in hypoxic environments, such as the milk of a cow with mastitis. Oxygen levels in milk have been found to be up to 90% lower in cows with mastitis and high somatic cell counts [189, 190]. Milk from mastitis cases also contains bile salts, which increase over the course of the infection [191], possibly as a result from increased serum uptake in the mammary gland [192]. MAEC also originates from fecal matter of cows, where bile salt concentrations can reach 1.1% [193]. As such, I predicted that expression of *chiA* would increase in the presence of bile salts. To determine the effect of environmental conditions on expression of ChiA, fluorescence of strain G1 containing pWH03, a GFP reporter plasmid with the *chiA* promoter, was measured in hypoxic conditions and in the presence of bile salts.

The role of ChiA in adhesion to mammary epithelial cells was investigated by creation of isogenic mutants and then measuring levels of adhesion observed in an *in vitro* environment. Four strains were investigated: M45, M93, M111, and G1. M45 and M111 were both successful in bovine mammary glands in a competitive experimental model of infection (John Lippolis and Michael Olson, unpublished data). M45 and M93 were isolated from mild cases of clinical mastitis. M111 was isolated from a severe case of clinical mastitis. G1 was isolated from a case of severe, gangrenous mastitis and caused significant clinical symptoms in the affected cow.

3.1 Expression of chiA is Increased in Hypoxic Environments and in the Presence of Bile Salts, but not both

To explore environments that could lead to changes in expression of *chiA*, I used a reporter plasmid that contained a GFP marker with the putative *chiA* promoter sequence directly upstream (pWH03). The putative promoter was amplified based on previous primer extension analysis that defined the transcriptional start site as approximately 35 base pairs upstream of *chiA* to be the promoter, with the -35 and -10 boxes being discernable as well [166]. As such, I amplified an area of 241 bp immediately upstream of *chiA* using primers 013 and 014 (see Table 2-4) for creation of pWH03. This plasmid was transformed into strain G1 via electroporation. A control plasmid lacking a promoter sequence upstream of *gfp* (pWH04) was also created and transformed into G1.

G1 containing either pWH03 or pWH04 were incubated in LB broth in aerobic and hypoxic conditions as well as in the presence of varying concentrations (0.5%, 1.0%, and 1.5% w/v) of bile salts. These environments were chosen due to recent data that indicated *chiA* transcription increased in LB with 0.5% (w/v) bile salts or with low oxygen in enterotoxigenic *E. coli* (ETEC) [194]. Cultures were grown overnight (16-18 hrs) and diluted 20-fold in sterile PBS for measurement by flow cytometry. The median fluorescence of G1 containing pWH04 was subtracted from the values of pWH03. Results are presented in Figure 3-1. Histograms showing representative samples from 2 different measurements are shown in Figure 3-2.

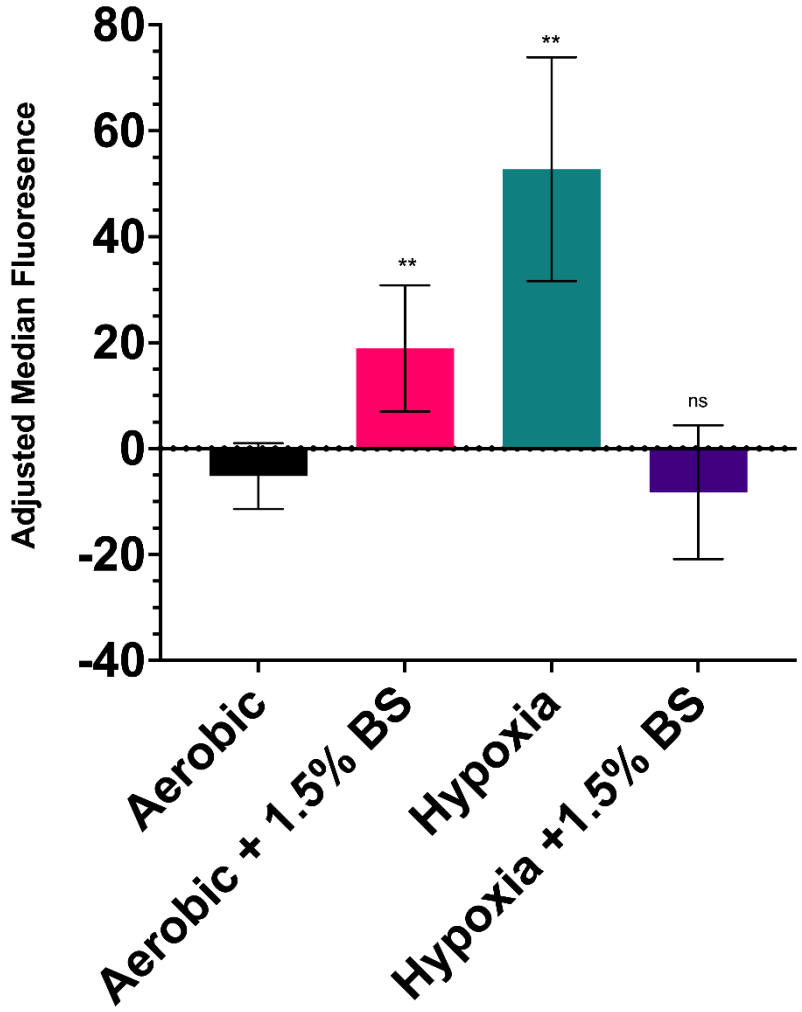


Figure 3-1: Adjusted Median Fluorescence of G1 with pWH03 in Hypoxia and/or Bile Salts. MAEC strain G1 was incubated conditions with hypoxia, 1.5% (w/v) bile salts, and a combination of both. The average adjusted median fluorescence is shown with error bars indicating a 95% confidence interval. Significance was determined by a Mann-Whitney U-Test. Each sample was compared to standard aerobic conditions (black). **p<0.05. BS=Bile Salts

When grown in the presence of 1.5% bile salts, G1 showed increased promoter activity. Standard aerobic conditions had an average adjusted median fluorescence of -5.18 while 1.5% bile salts were 18.92. This was considered significantly different than under ($p=0.022$). In addition, when G1 was cultured in hypoxic conditions, promoter activity was further increased. Hypoxic conditions had an average adjusted median

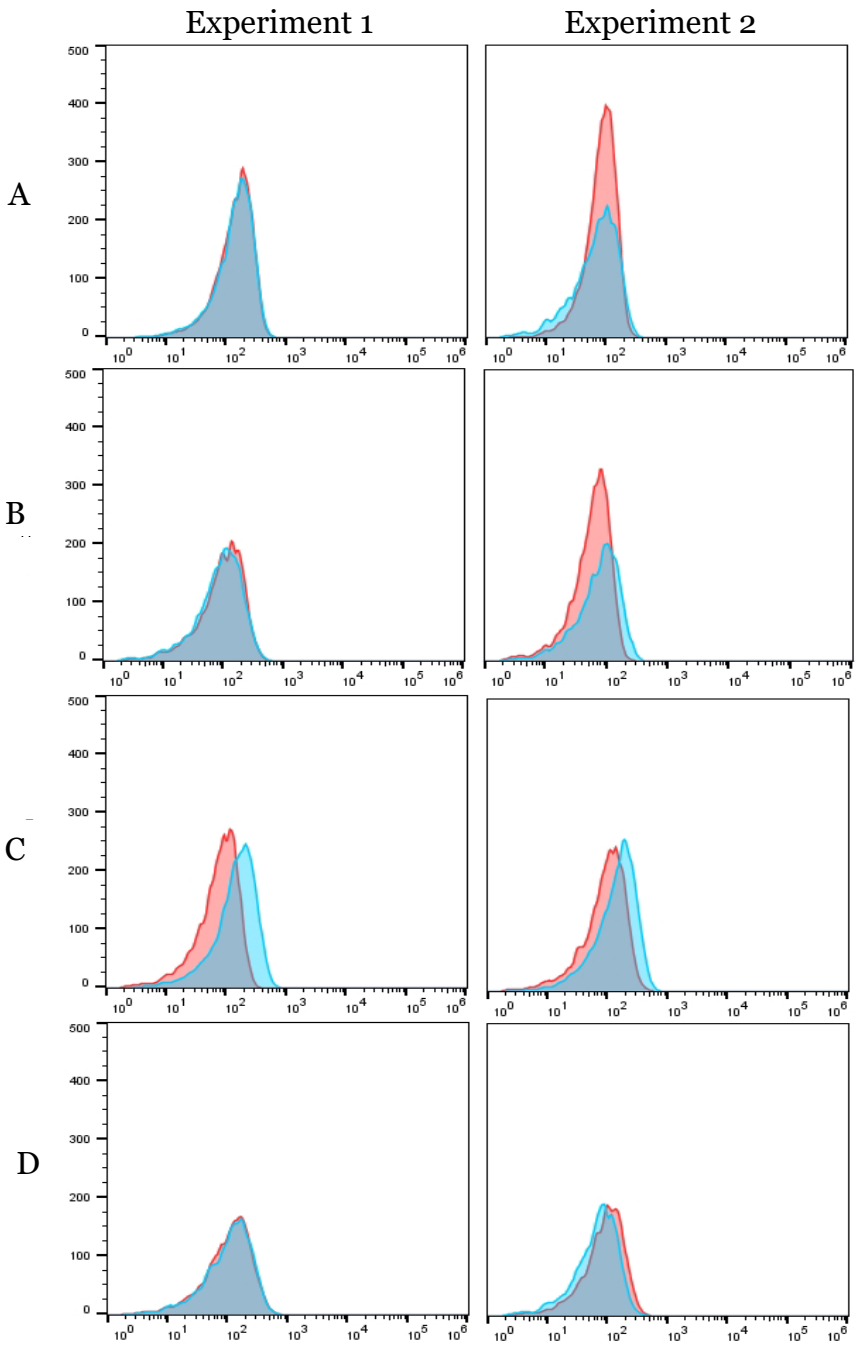


Figure 3-2: Histograms of Fluorescence Values of G1 with pWH03 or pWH04. Histograms with representative samples from 2 separate experiments showing the fluorescence values of G1 with pWH03 (blue) or pWH04 (red) in aerobic conditions (A), 1.5% (w/v) bile salts (B), hypoxic conditions (C), and hypoxic conditions with 1.5% bile salts (D). The number of events is shown on the y-axis and the fluorescence value is shown on the x-axis.

fluorescence of 52.77. This was also significantly different than aerobic conditions (p=0.022).

Surprisingly, the combination of hypoxia and 1.5% bile salts did not change promoter activity of *chiA*. These conditions had an average adjusted median fluorescence of approximately -8.22. There was no statistical difference between aerobic conditions and hypoxia with 1.5% bile salts (p=0.776).

While I showed that promoter activity of *chiA* is increased in both hypoxic conditions and in the presence of 1.5% bile salts, this assay does have limitations. Bile salts are made of up multiple different types of molecules which are present in different concentrations depending on the location in the intestinal tract. In *Vibrio parahaemolyticus*, a two-component signaling system is able to bind bile salts that pass through the outer membrane, altering gene expression and inducing expression of certain virulence factors such as a type III secretion system [165, 177]. This also only occurs when certain bile salts are bound [195]. It is possible this may be true for MAEC since *E. coli* contains similar systems [196]. This assay used a premade mixture of bile salts which may not be representative of the bile salt components present in the mammary gland during mastitis.

This assay is also limited in the fact that it does not provide a direct measurement of *chiA* transcript or ChiA protein levels, so any post-transcriptional or post-translational regulation that may occur is not apparent. Other approaches such as qPCR and Western blotting would help confirm the results presented here. It may also be beneficial to establish whether the H-NS protein represses *chiA* in multiple MAEC strains as previously described in lab strain K-12 [165, 177]. If so, I would predict

conditions leading to repression of H-NS such as over expression of Lon protease or PhoP mediated-displacement would lead to increased expression of *chiA* [197].

3.2 ChiA plays a Significant Role in Adhesion to MAC-T cells in Certain MAEC Strains

To test the role of ChiA in adhesion to epithelial cells, I created markerless deletion mutants using the pAX1 plasmid (See Section 2.2) [184]. Bovine mammary alveolar epithelial (MAC-T) cells were used as an *in vitro* model of infection. MAEC strains M45, M93, M111, and G1 were grown overnight, diluted in PBS, and used to inoculate MAC-T cells at a multiplicity of infection (MOI) of 10 (approx. $6x10^6$ bacterial cells per well). The cultures were incubated at 37°C for 1 hour before weakly adherent

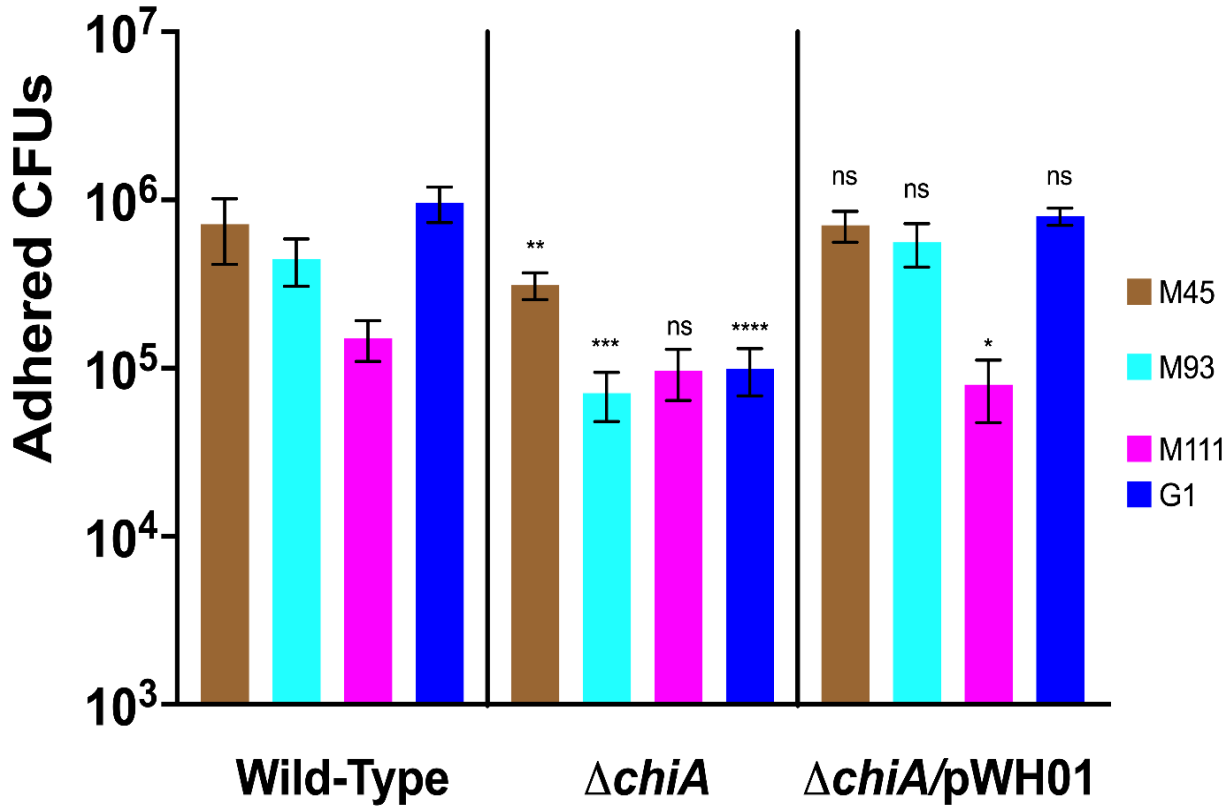


Figure 3-3: Adhered CFUs of each MAEC strain. M45 (brown), M93 (cyan), M111 (pink), and G1 (blue) were incubated with MAC-T cells and the adhered CFUs were enumerated. The wild type of each strain is shown on the left, the Δ*chiA* mutants in the middle, and Δ*chiA* mutants containing pWH01 on the right. One-way ANOVA with Tukey's correction was used to determine statistical differences. Displayed statistics are the comparison to the wild type of each respective strain. *p=0.05, **p>0.05, ***p=0.0001, ****p>0.0001.

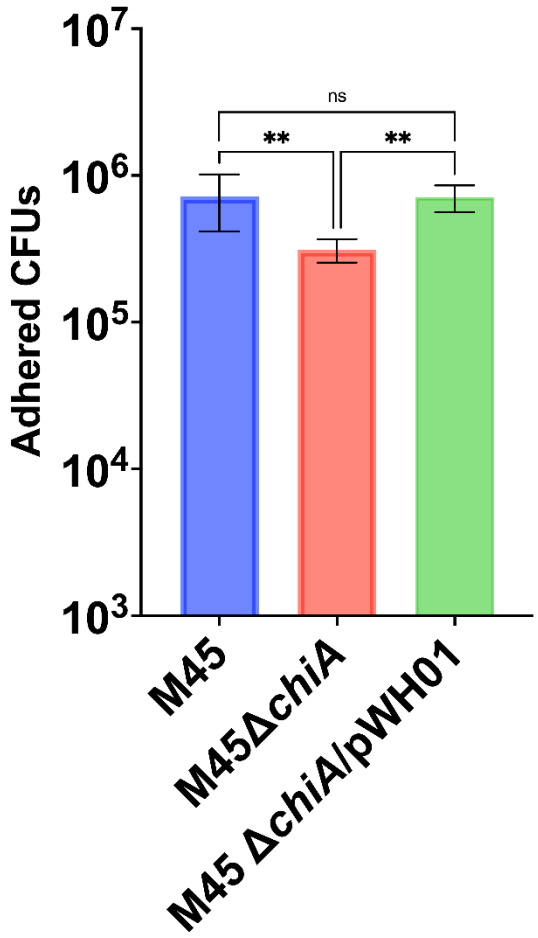


Figure 3-4: Adhered CFUs of M45 Following Incubation with MAC-T Cells for 1 hour**.** WT=wild type. ** p≤0.01 as determined by One-way ANOVA with Tukey's correction

bacteria were washed off using PBS, and the MAC-T cells were lysed with 0.1% Triton X-100. The solution was then serially diluted and plated on LB agar to enumerate the colony-forming units (CFUs) of adherent bacteria. Figures 3-4 through 3-7 show obtained using individual MAEC strains while Figure 3-3 shows the data from all strains compared with each other.

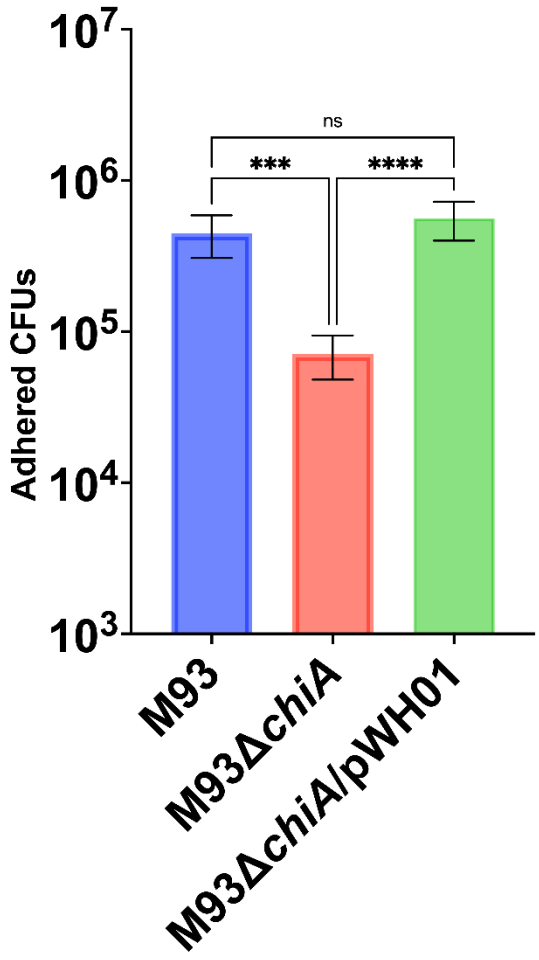


Figure 3-5: Adhered CFUs of M93 Following Incubation with MAC-T Cells for 1 hour. WT= wild type. *** p=0.001, ****p>0.0001 as determined by One-way ANOVA with Tukey's correction

M45Δ*chiA* showed an approximately 2-fold reduction compared to the wild-type ($3.1x10^5$ vs $7.2x10^5$ CFUs, respectively) as shown in Figure 3-4. This phenotype was restored upon reintroduction of *chiA* into the strain with pWH01. A one-way ANOVA showed that the reduction in adhesion was statistically significant between the wild type and the mutant (p=0.008) indicating that ChiA plays a role in adhesion to MAC-T cells.

A greater reduction was observed in M93 (>6-fold) between the wild type and mutant (Figure 3-5), which was also complemented by the pWH01 plasmid. This difference was also statistically significant when the two were compared (p=0.0001)

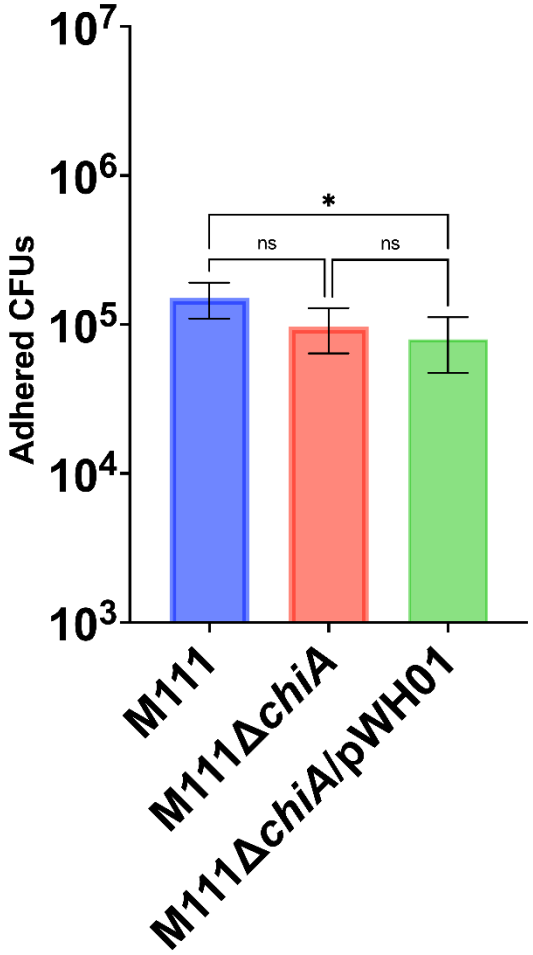


Figure 3-6: Adhered CFUs of M111 Following Incubation with MAC-T Cells for 1 hour. WT= wild type. *p<0.05 as determined by One-way ANOVA with Tukey's correction

using a one-way ANOVA. M45 and M93 were both highly competitive in a murine model of mastitis [99], and both appear to utilize ChiA in adherence to epithelial cells.

Unlike M45 and M93, M111 did not seem to rely on ChiA for adherence. There was slight reduction in adherent CFUs, when M111Δ*chiA* was compared to the wild type ($9.7x10^5$ vs $1.5x10^6$, respectively), there was no statistically significant difference (p=0.0606) (Figure 3-6). Other adhesive factors such as fimbriae play an important role in adhesion in other strains of MAEC [102]. It is possible that while the small decrease found in M111Δ*chiA* is not statistically significant, ChiA may be a secondary method of

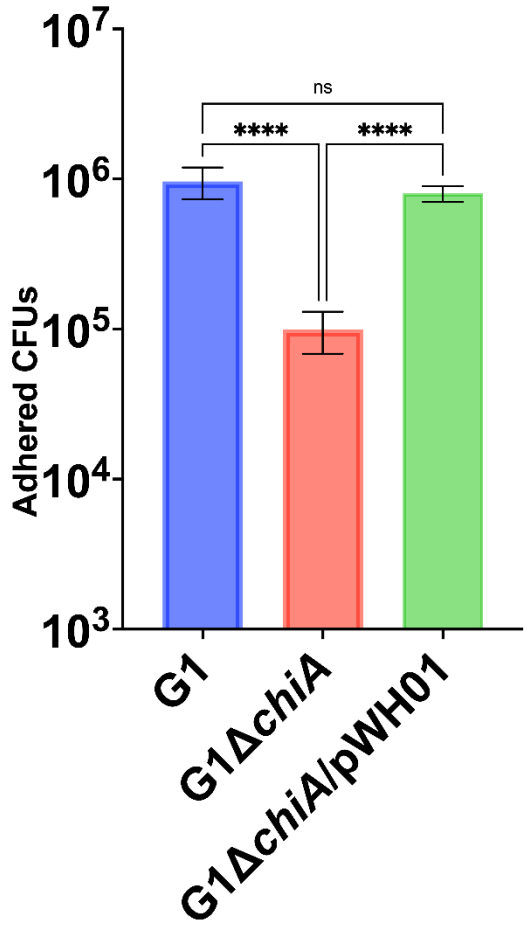


Figure 3-7: Adhered CFUs of G1 Following Incubation with MAC-T Cells for 1 hour**.** WT= wild type. ****p<0.0001 as determined by One-way ANOVA with Tukey's correction

adhesion and other factors such as pili or fimbriae play the more direct role. Future investigation into this strain should focus on other adhesins that are found in M111 and determine if they play a more significant role than ChiA.

Deletion of *chiA* in G1 caused the largest, most significant reduction in adhesion of all MAEC strains investigated. G1Δ*chiA* presented almost a 10-fold decrease in adhesion when compared to the wild type as shown in Figure 3-7. Wild type G1 resulted in an average of 9.65×10^5 adhered CFUs while G1Δ*chiA* had an average of 9.95×10^4

adhered CFUs. This difference was statistically significant ($p<0.0001$). Wild-type levels of adhesion were restored upon complementation with plasmid pWH01.

G1 was isolated from a very severe, per-acute mastitis infection that resulted in gangrene and severe tissue damage. Bacterial adhesion results in inflammation [198, 199]. An excessive amount of inflammation can result in permanent tissue damage [200, 201] with this damage being deleterious to the health of the host. In mastitis, this can result in the cow having significantly reduced milk production, repeated infections, and even early culling of the cow [9, 152, 153]. The reduction of adhesion in G1Δ*chiA* indicates ChiA is used to facilitate adhesion between strain and MAC-T cells, perhaps through binding to host proteins such as CHI3L1.

3.3 Identification of Putative Virulence Factors in Shows Insight Observed Phenotypes of 4 MAEC Strains

Many genes coding for virulence factors described in other pathogenic strains of *E. coli* are found in MAEC genomes, but very few are known to contribute to virulence or pathogenicity of MAEC [99-101, 202]. Recently, the ferric dicitrate transport system (*fecABCDE*) was proven to be vital for the growth of MAEC in raw milk [99]. This operon is highly expressed and over-represented in MAEC genomes compared to other strains [203, 204]. Swarming motility is the only phenotype that has been experimentally associated with severity of infection with strains isolated from severe mastitis exhibiting more swarming motility than those isolated from mild cases [205].

In order identify potential virulence factors, present in MAEC strains M45, M93, M111, and G1, the assembled draft genomes of each strain was analyzed using Virulence Factor Database 2.0 (Institute of Pathogen Biology, Beijing, http://www.mgc.ac.cn/VFs/) [187]. The analysis revealed 40 unique virulence factors. These were grouped based on their functions described in other strains of pathogenic *E. coli*. Groups consisted of genes coding for adhesins (n=11), autotransporters (n=4), antibiotic resistance (n=1), type 6 secretion system (n=2), complement resistance (n=3), invasins (n=2), siderophores (n=5), and toxins (n=6). Other genes were identified with unknown function (n=3) as well as SopA-like effectors of a type 3 secretion system (n=3).

Table 3-1: Genes Coding for Possible Virulence Factors found in MAEC strains M45, M93, M111, and G1

Function*	**Virulence factor**	**Strains present in**	**Reference**
Adhesin	CFA/I fimbriae	M45	[206]
	E. coli common pilus (*ecpABCDER)*	M45, M93, M111, G1	[207]
	Intimin-like adhesin (*eaeH*)	M45, M93, M111, G1	[208]
	Laminin binding fimbriae (*elfABCDG)*	M45, M111	[209]
	Hemorrhagic coli pilus	M45, M93, M111, G1	[210, 211]
	P fimbriae	M45, M93, G1	[212-214]
	Type 1 pilus (*fimABCDEFGHI*)	M45, M93, M111, G1	[215, 216]
	K88 fimbriae	M111	[217, 218]
	Long polar fimbriae	M45	[219]
	Mam7	M45, M93, M111, G1	[220, 221]
	Yad Fimbriae	M111, G1	[115, 222]
Autotransporter/Biofilm formation	Antigen 43 (*agn43*)†	M111	[223]
	ehaB†	M45, M93, M111, G1	[224]
	Vacuoloating autotransporter toxin (*vat)*	M93, G1	[225]
	upaG†	M45, G1	[226]
Complement Resistance	OmpT	M45, M93, M111, G1	[205]
	TraT	M45, M93, M111, G1	[227, 228]
	iss	M45, M93, M111, G1	[229]
Invasin	*ibeA*	M93, G1	[230, 231]
	ibeBC	M45, M93, M111, G1	
Siderophore	Aerobactin (*iucABCD/iutA*)	M45, G1	[232]

	Heme uptake (*chuASTUWXY*)	M93, G1	[233]
	Iron/Manganese transport (*sitABCD*)	M45, M93, M111, G1	[234]
	Salmochelin (*iroBCDEN*)	M45, M93	[235, 236]
	Yersiniabactin (*fyuA, irp1/2, ybtAEPQSTUX)*	M45, M93, G1	[237-239]
Type 6 Secretion System	*ace* T6SS operon	M45, M93, M111, G1	[240]
	Sci-1 T6SS	G1	[241]
Toxin	Cytolethal distending toxin (*cdtB*)	G1	[242]
	clbB	G1	[243, 244]
	Alpha-Hemolysin (*hlyABCD*)	M111, G1	[245, 246]
	Hemolysin E /Cytolysin A (*hlyE/cylA*)‡	M111	[247, 248]
	Cytotoxic necrotizing factor (*cnf1*)	G1	[249-251]
	Colicin-like usp	G1	[252]
Antibiotic Resistance	Tetracycline efflux pump (*tetA*)	G1	[253]
SopA-like T3SS effectors	*espX1, espX4, espX5*	M45, M93	[254]
Unknown Function††	*espL1*	M45, M93, M111, G1	[254]
	espL4	M45, M93	[254]
	espR1	M111	[254]

*Functions are based on previously described functions shown in other pathogenic strains of *E. coli*
†Multifunctional as adhesin
‡Also called Silent Hemolysin A (SheA) or Avian Hemolysin
††Each of these genes have been described as pseudogenes from lambdoid phage that border a pathogenicity island, but their precise function has not been shown

M111 showed the lowest level of adhesion of all 4 MAEC strains as discussed in Section 3.2 of this thesis. It was the only strain that did not demonstrate a statistically

significant reduction in adhesion to epithelial cells in the absence of *chiA*. A plausible explanation for this is the presence of alternative adhesion factors. Even though it showed the lowest level of adherence, M111 contained genes for 8/11 adhesins as wells as two autotransporters that function in both biofilm formation and adhesion. The diversity of adhesive structures encoded by M111 offers a plausible explanation for its lack of reliance on ChiA.

Interestingly, M93 and G1 only contained genes for 5/11 adhesins found in the analysis but showed the largest reduction in adhesion to epithelial cells in the absence of ChiA. Strain M111 contained genes coding for the K88 and laminin-binding fimbriae, both of which are absent in G1 and M93. These two adhesins facilitate attachment to different cell types and be important for other pathotypes of *E. coli* [209, 217, 218].

The K88 fimbriae is commonly found in enterotoxigenic *E. coli* (ETEC), specifically in strains that cause diarrhea in young pigs [218]. There are three different serological variants of K88 found in nature: K88ab, K88ac, and K88ad [217, 255, 256]. This has largely been investigated in respect to its ability to promote adhesion to porcine erythrocytes by binding to components of the extracellular matrix like fibronectin. Fibronectin is expressed by MAC-T cells and is important for adherence and invasion of *S. aureus in vitro* [257-259]. MAEC strains encode K88 pili at similar rates to other *E. coli* pathotypes [260, 261] therefore, the extent of its role in MAEC virulence has not been investigated.

The *E. coli* laminin-binding fimbriae (ELF) is highly prevalent in enteropathogenic *E. coli* (EPEC) strains and is also common in MAEC [262]; Other

mastitis pathogens such as *S. aureus* and *Streptococcus uberis* encode proteins of similar function [263-266]. The ELF operon consists of 5 genes, *elfABDCG,* with the major fimbrial subunit ElfA being the main attachment protein. It is related to G fimbriae in uropathogenic *E. coli* which binds laminin present in the extracellular matrix [209, 267]. Laminin is not produced by MAC-T cells *in vitro* but is present in the mammary gland *in vivo* [268], offering a plausible explanation for M111's apparent decreased reliance on ChiA for adhesion. MAEC is known to be able to bind to laminin present in the extracellular matrix, but ELF has not been experimentally shown to contribute to MAEC virulence.

M111 contains genes coding for both Antigen 43 (Ag43) and EhaB. Both of these proteins are classified as autotransporters which have been shown to influence cellular aggregation and biofilm formation [223, 224]. Ag43 mediates bacteria-to-bacteria interactions, auto-aggregation, and is important for survival and persistence of uropathogenic *E. coli* (UPEC) in the urinary tract [269, 270]. EhaB binds components of the extracellular matrix like laminin and collagen I in the digestive tract of cattle challenged with *E. coli* O157:H7 [224], similar to ELF. Both Ag43 and EhaB are also key factors in influencing biofilm formation in enterohemorrhagic *E. coli* [224, 269, 271]. The formation of biofilm is thought to contribute to persistent or chronic mastitis [98, 112, 244]. M111 may be able to utilize Ag43 and EhaB to facilitate adherence to epithelial cells in a different manner than the other MAEC strains tested. However, the role of these proteins in bovine mastitis remains uninvestigated.

In addition to these two fimbriae, the genome-wide association study (GWAS) conducted by Olson et al. found that strains isolated from severe cases of mastitis were

more likely to contain genes encoding the Yad fimbriae [99]. The Yad fimbriae are also found in UPEC and APEC and contribute to colonization and environmental persistence in some strains [272-274]. M111 and G1 contain genes coding for these fimbriae, however, the role of this adhesin in the context of bovine mastitis has yet to be investigated.

Interestingly, G1 contained the most genes for toxin production of all MAEC strains analyzed (n=5). The high level of adhesion exhibited by G1 in combination with the expression of these toxins might explain why it was isolated from a very severe, gangrenous case of mastitis. M111 was the only other strain that contained genes coding for toxins. Specifically, both M111 and G1 contain genes coding for an RTX toxin, alpha-hemolysin (HlyA). HlyA works by forming pores in the membrane of host cells and is one of the most extensively studied exotoxins of pathogenic *E. coli* [275]. HlyA has been proven to play an important role in the virulence of ExPEC in a mouse model [276] and is known to cause lysis of epithelial cells as well [277]. It has been found in a large variety of MAEC strains in many different areas of the world, indicating it might contribute to the damage caused by these strains in the mammary gland [263, 276, 278, 279].

In addition, G1 contains genes the cytolethal distending toxin (CDT). CDT is a heterotrimeric AB toxin that causes cell cycle arrest in mammalian cells. The active subunit, CdtB, shares a high level of homology with mammalian DNase I and is able to directly damage DNA to cause cell cycle arrest and as a result, induce apoptosis [280]. CDT has been described previously in many different MAEC strains as well, though its role or effect in virulence or severity is not yet known [119, 276, 281]. These two toxins

in addition to the other toxins genes found could help explain the high severity of mastitis G1 is able to cause.

SECTION 4: CONCLUSIONS AND FUTURE DIRECTIONS

My results demonstrate that ChiA can play an important role in the adhesion of MAEC to bovine mammary epithelial cells. A GWAS study showed that ChiA and the type-two secretion system found adjacent to it were positively associated with colonization of mouse mammary glands during a mastitis model of infection [99, 115]. Future work should include determining whether ChiA function in MAEC strains is dependent on the adjacent type two secretion system.

In these studies, I aimed to investigate environments relevant to bovine mastitis that might be able to induce expression of *chiA*. Flow cytometry showed promoter activity of *chiA* was significantly increased in hypoxic environments and in the presence of bile salts but not both. It is possible that specific bile salts influence the expression of *chiA* in a similar manner to *V. parahaemolyticus* [195, 282]. There are many other conditions relevant to mastitis that have yet to be investigated. These include growth in milk and in the presence of polymorphonuclear neutrophils. In addition, I did not confirm these results with qPCR in this thesis. This could also be investigated using RNA-sequencing or other types of reporter assays. The regulation of *chiA* is an area for further investigation. Previous research indicates it is constitutively silenced by H-NS binding to its promoter, but this has only been confirmed in the lab strain, K-12. Isogenic mutants of *hns* or conditions that repress H-NS binding such as over-expression of Lon protease or PhoP mediated displacement from DNA can be used to demonstrate this mechanism in multiple strains of MAEC.

While *chiA* is annotated as a chitinase [165] its role in bovine mastitis may not be dependent on its enzymatic activity. The chitin binding domains of ChiA, found through

homology to other chitinases, might be responsible for its role in adhesion. When *chiA* was deleted in 4 different MAEC strains of various genetic backgrounds and phenotypes, adhesion to MAC-T cells *in vitro* was significantly decreased in 3 out of 4 strains. The interaction between ChiA and host cells might be facilitated through its interaction with the chitinase-like protein, CHI3L1.

Chitinase-like proteins (CLPs) are expressed in response to pathogens that are chitinous and those that are chitin-lacking [283-286]. CLPs are also expressed during non-infectious diseases such as cancer or kidney injuries [287-289]. CHI3L1 is a CLP that is upregulated in response to mammary infection with coliform bacteria such as MAEC. Its expression in the mammary gland is controlled by caspase activity, independent of bacterial load or cytokine levels [159].

When CHI3L1 is expressed, it promotes the movement of leukocytes from the interstitial space to the alveolar lumen in mammary tissue [159, 290]. It also promotes increased proliferation of mammary epithelial cells and reduces apoptosis [291]. In the absence of CHI3L1, migration, maturation, and activation of macrophages is significantly impaired [290, 292]. Since ChiA binds to CHI3L1 [177], it is probable that MAEC expressing and secreting ChiA into the mammary gland are able to suppress these innate immune responses. This would allow them to persist longer in mammary glands, explaining the results from Olson et al. showing that strains that are more prevalent at later time points in an infection are more likely to possess *chiA* [99], though this possible interaction has not been investigated in a mammary gland setting.

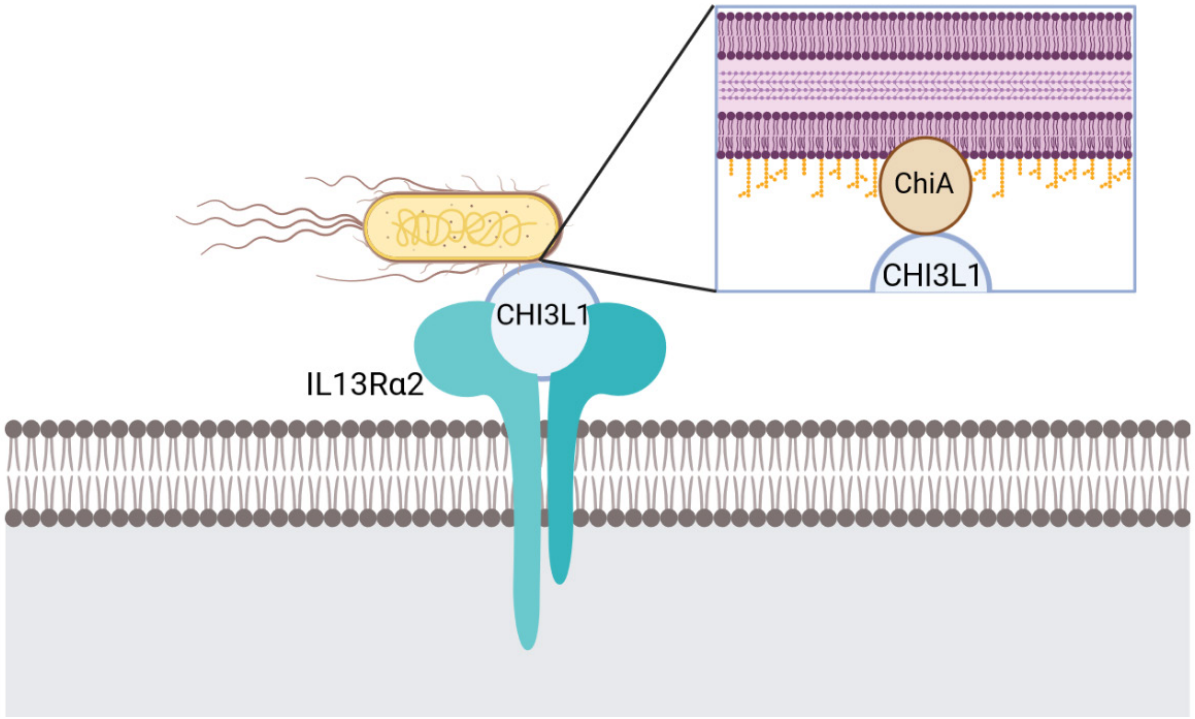


Figure 4-1: Possible Mechanism of Attachment via ChiA**.** In order for this interaction to occur as described in this thesis, IL13Rα2 must be N-glycosylated. In the absence of glycosylation, ChiA may still be able to bind CHI3L1, but this interaction will not facilitate adhesion since CHI3L1 will only bind to glycosylated IL13Rα2.

ChiA-mediated adhesion might occur when CHI3L1 is bound to interleukin-13 receptor α-2 (IL13Rα2) and IL-13 in a multimeric complex [292]. Recently, the formation of this complex was found to be heavily dependent on N-glycosylation [293]. Low et al. also showed that N-glycosylation is important for binding to be mediated between ChiA and CHI3L1 in an *in vitro* model of intestinal epithelial cells [177]. Since CHI3L1 is found in the extracellular matrix and is soluble in milk [159, 181, 293], the complex it forms with IL13Rα2 and IL-13 is likely the only time it is found tethered to the cell membrane. MAEC strains that possess *chiA* would be able to take advantage of this and bind to CHI3L1 while it is in this protein complex, bringing the bacterium in proximity to the cell, allowing colonization of the epithelium, invasion into tissue, and persistence in the mammary gland. Further investigation to confirm this possible

interaction should focus on the role that host proteins, CHI3L1, IL-13, and IL13Rα2, play. Cell lines that exhibit repressed or reduced expression of the genes for these proteins would work to confirm this interaction.

Alternatively, it is possible that ChiA does not directly bind to host proteins. Chitinases in *S. typhimurium* and *S. typhi* have been shown to modify host glycoproteins by digesting glycosyl groups present in the extracellular matrix. This results in mannose residues present on the cell surface becoming more available for attachment through type I fimbriae [174, 175]. Since ChiA shares homology with these chitinases (see Supplementary Figure 5), it is plausible it performs a similar function. ChiA might also have other functions outside of adhesion.

Chitinases in other bacterial species are known to increase survival in phagocytes by dampening the expression of host antimicrobial responses in dendritic cells and macrophages [175]. MAEC could use ChiA in a similar manner allowing for greater persistence in the mammary gland. This is a possible explanation for the results of Olson et al. showing that MAEC strains that were more prevalent in later stages of mouse and bovine models of mastitis were more likely to contain *chiA [99, 115]*.

The translocation of ChiA from the periplasm to outside of the cell is facilitated by a type-two secretion system that is found adjacent to *chiA* [165]. While the ability of chitinases found in other gram-negative bacteria to become anchored to the outer membrane has been demonstrated [176], this has not yet been demonstrated in *E. coli*. This topic should be investigated through localization studies utilizing immunoblotting and cell fractionization or confocal microscopy. For instance, the localization of ChiA in

the outer membrane could be detected by cell fractionization studies using an epitope tag such as a hemagglutinin A tag or antibodies specific to ChiA.

I also demonstrated that MAEC strain M111 possesses multiple alternative adhesive factors that offer a plausible explanation for its lack of a significant decrease in adhesion to MAC-T cells in the absence of ChiA. These include the K88 and ELF proteins that bind to glycoproteins and laminin, respectively [209, 218]. Numerous glycoproteins and possible lectins for the K88 fimbriae are present on MAC-T cells [112], however, laminin is not produced by these cells *in vitro* [268]. Creating isogenic mutants that cannot synthesize these adhesins and measuring any loss of adhesion *in vitro* is the initial step that should be taken. If a significant role for these adhesins is found *in vitro,* a mouse model of mastitis with the isogenic mutants for these adhesins would confirm any loss of virulence or ability to colonize mammary glands that may result.

I analyzed the genomes of each of the 4 MAEC strains for additional virulence factors. The annotated draft genomes of each strain were analyzed using the Virulence Factor Database. This resulted in 40 unique virulence factors being identified. G1 contained a higher number of genes for toxins than other MAEC strains analyzed. These toxins include α-hemolysin and cytolethal distending toxin among several others. The different toxins contained within G1 offer insight into the virulence and presentation of severe, gangrenous mastitis observed when it was isolated.

Better understanding MAEC virulence factors may help distinguish these bacteria from other ExPEC strains and will lead to the identification of novel targets for antimicrobials in both veterinary and human treatments. Despite the high prevalence of

genes for multiple virulence factors in many strains of MAEC, very few have been shown to play a significant role in the ability of these bacteria to cause mastitis. The impact of ChiA on adherence that I demonstrated as well as other adhesive factors I have described provide opportunities for further investigation.

REFERENCES

1. Looper, M.L. and U.o.A.C.E. Service, *Reducing Somatic Cell Count in Dairy Cattle*. 2012: Cooperative Extension Service, University of Arkansas, U.S. Department of Agriculture, and county governments cooperating.

2. Cheng, W.N. and S.G. Han, *Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments — A review*. Asian-Australas J Anim Sci, 2020. **33**(11): p. 1699-1713.

3. Döpfer, D., et al., *Recurrent Clinical Mastitis Caused by Escherichia coli in Dairy Cows*. Journal of Dairy Science, 1999. **82**(1): p. 80-85.

4. Khan, M.Z. and A. Khan. *Basic Facts of Mastitis in Dairy Animals: A Review*. 2006.

5. Shearer, J.K., *Mastitis in Dairy Goats*. 1992, University of Florida: Gainesville, Fla. p. 1-6.

6. Romero, J., E. Benavides, and C. Meza, *Assessing Financial Impacts of Subclinical Mastitis on Colombian Dairy Farms*. Front Vet Sci, 2018. **5**: p. 273.

7. Pedersen R R., K.V., Bjarnsholt T., Dahl-Pedersen K., Buhl R., Jorgensen E. , *Biofilm Research in Bovine Mastitis*. Front Vet Sci, 2021.

8. Krömker, V. and S. Leimbach, *Mastitis treatment—Reduction in antibiotic usage in dairy cows*. Reproduction in Domestic Animals, 2017. **52**(S3): p. 21-29.

9. Oliver, S.P. and L.F. Calvinho, *Influence of Inflammation on Mammary Gland Metabolism and Milk Composition*. Journal of Animal Science, 1995. **73**(suppl_2): p. 18-33.

10. Garcia, A., *Contagious vs. Environmental Mastitis*. SDSU Extension Extra Archives, 2004. **126**.

11. Zadoks, R.N., et al., *Molecular Epidemiology of Mastitis Pathogens of Dairy Cattle and Comparative Relevance to Humans*. Journal of Mammary Gland Biology and Neoplasia, 2011. **16**(4): p. 357-372.

12. Holmes, M.A. and R.N. Zadoks, *Methicillin Resistant S. aureus in Human and Bovine Mastitis*. Journal of Mammary Gland Biology and Neoplasia, 2011. **16**(4): p. 373-382.

13. Bradley, A.J., et al., *Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales*. Veterinary Record, 2007. **160**(8): p. 253-258.

14. Lee, J.H., *Methicillin (Oxacillin)-Resistant Staphylococcus aureus Strains Isolated from Major Food Animals and Their Potential Transmission to Humans*. Applied and Environmental Microbiology, 2003. **69**(11): p. 6489-6494.

15. Liu, H., et al., *Prevalence, antimicrobial susceptibility, and molecular characterization of Staphylococcus aureus isolated from dairy herds in northern China*. J Dairy Sci, 2017. **100**(11): p. 8796-8803.

16. Petrovski, K.R., et al., *The incidence and aetiology of clinical bovine mastitis on 14 farms in Northland, New Zealand*. New Zealand Veterinary Journal, 2009. **57**(2): p. 109-115.

17. Rabello, R.F., et al., *Multilocus sequence typing of Staphylococcus aureus isolates recovered from cows with mastitis in Brazilian dairy herds*. Journal of Medical Microbiology, 2007. **56**(11): p. 1505-1511.

18. Sampimon, O.C., et al., *Prevalence and herd-level risk factors for intramammary infection with coagulase-negative staphylococci in Dutch dairy herds*. Veterinary Microbiology, 2009. **134**(1): p. 37-44.

19. Steeneveld, W., et al., *Cow-specific treatment of clinical mastitis: an economic approach*. J Dairy Sci, 2011. **94**(1): p. 174-88.

20. Bradley, A.J., *Bovine Mastitis: An Evolving Disease*. The Veterinary Journal, 2002. **164**(2): p. 116-128.

21. Rollin, E., K.C. Dhuyvetter, and M.W. Overton, *The cost of clinical mastitis in the first 30 days of lactation: An economic modeling tool*. Preventive Veterinary Medicine, 2015. **122**(3): p. 257-264.

22. Bogni, C.I., et al. *War against mastitis: Current concepts on controlling bovine mastitis pathogens*. 2011.

23. Kader, M., M. Samad, and S. Saha, *Influence of host level factors on prevalence and economics of subclinical mastitis in dairy milch cows in Bangladesh*. Indian Journal of Dairy Science, 2003. **56**(4): p. 235-240.

24. OECD, Food, and A.O.o.t.U. Nations, *OECD-FAO Agricultural Outlook 2019-2028*. 2019. p. 9.

25. Commission, E., *EU agricultural outlook for markets, income and environment 2021-2031*, in *European Commission, DG Agriculture and Rural Development*, E. Commission, Editor. 2021: Brussels.

26. Pyörälä, S., *Treatment of mastitis during lactation*. Ir Vet J, 2009. **62 Suppl 4**(Suppl 4): p. S40-44.

27. Down, P.M., et al., *Current management practices and interventions prioritised as part of a nationwide mastitis control plan*. Veterinary Record, 2016. **178**(18): p. 449-449.

28. Pyörälä, S., et al., *Efficacy of Two Therapy Regimens for Treatment of Experimentally Induced Escherichia coli Mastitis in Cows*. Journal of Dairy Science, 1994. **77**(2): p. 453-461.

29. Damm, M., et al., *Differential somatic cell count-A novel method for routine mastitis screening in the frame of Dairy Herd Improvement testing programs*. J Dairy Sci, 2017. **100**(6): p. 4926-4940.

30. Schukken, Y.H., et al., *Monitoring udder health and milk quality using somatic cell counts*. Vet Res, 2003. **34**(5): p. 579-96.

31. Schalm, O.W. and D.O. Noorlander, *Experiments and observations leading to development of the California mastitis test*. J Am Vet Med Assoc, 1957. **130**(5): p. 199-204.

32. Tanni, N.S., et al., *Evaluation of sodium lauryl sulfate for the development of cow-side mastitis screening test*. Vet World, 2021. **14**(8): p. 2290-2295.

33. Viguier, C., et al., *Mastitis detection: current trends and future perspectives*. Trends in Biotechnology, 2009. **27**(8): p. 486-493.

34. Ashraf, A. and M. Imran, *Diagnosis of bovine mastitis: from laboratory to farm*. Tropical Animal Health and Production, 2018. **50**(6): p. 1193-1202.

35. Moon, J.S., et al., *Application of a New Portable Microscopic Somatic Cell Counter with Disposable Plastic Chip for Milk Analysis*. Journal of Dairy Science, 2007. **90**(5): p. 2253-2259.

36. Gunasekera, T.S., D.A. Veal, and P.V. Attfield, *Potential for broad applications of flow cytometry and fluorescence techniques in microbiological and somatic cell analyses of milk*. Int J Food Microbiol, 2003. **85**(3): p. 269-79.

37. Mansor, R., et al., *A peptidomic approach to biomarker discovery for bovine mastitis*. Journal of Proteomics, 2013. **85**: p. 89-98.

38. Bian, Y., Y. Lv, and Q. Li, *Identification of Diagnostic Protein Markers of Subclinical Mastitis in Bovine Whey Using Comparative Proteomics*. Journal of Veterinary Research, 2014. **58**(3): p. 385-392.

39. Hussein, H.A., et al., *Milk amyloid A as a biomarker for diagnosis of subclinical mastitis in cattle*. Vet World, 2018. **11**(1): p. 34-41.

40. Abdel-maged, A.D., W.A. El Sheita, and M.G. Abdelwahab, *Biochemical study of DNA markers for Bacterial infection in bovine mastitis*. Benha Veterinary Medical Journal, 2016. **31**(2): p. 93-100.

41. Patil, M.P., et al., *Somatic cell count and alkaline phosphatase activity in milk for evaluation of mastitis in buffalo*. Vet World, 2015. **8**(3): p. 363-6.

42. Kandemir, F.M., et al., *A different approach to diagnosis of subclinical mastitis: milk arginase activity*. Vet Arhiv, 2013. **83**(6): p. 603-610.

43. Addis, M.F., et al., *Evaluation of milk cathelicidin for detection of dairy sheep mastitis*. J Dairy Sci, 2016. **99**(8): p. 6446-6456.

44. Parker, A.M., et al., *Comparison of culture and a multiplex probe PCR for identifying Mycoplasma species in bovine milk, semen and swab samples*. PLoS One, 2017. **12**(3): p. e0173422.

45. Spittel, S. and M. Hoedemaker, *Mastitis diagnosis in dairy cows using PathoProof real-time polymerase chain reaction assay in comparison with conventional bacterial culture in a Northern German field study*. Berliner und Munchener tierarztliche Wochenschrift, 2012. **125**(11-12): p. 494-502.

46. Lipman, L.J., et al., *Identification of Escherichia coli strains from cows with clinical mastitis by serotyping and DNA polymorphism patterns with REP and ERIC primers*. Vet Microbiol, 1995. **43**(1): p. 13-9.

47. Charaya, G., et al., *Detection of major mastitis pathogens by multiplex polymerase chain reaction assay in buffalo milk*. Indian J. Anim. Sci, 2015. **85**(3): p. 122-125.

48. Abdelmegid, S., et al., *Identification of host defense-related proteins using label-free quantitative proteomic analysis of milk whey from cows with Staphylococcus aureus subclinical mastitis*. International journal of molecular sciences, 2017. **19**(1): p. 78.

49. Chakraborty, S., et al., *Technological interventions and advances in the diagnosis of intramammary infections in animals with emphasis on bovine population—a review*. Veterinary Quarterly, 2019. **39**(1): p. 76-94.

50. Fox, L.K., *Mycoplasma mastitis: causes, transmission, and control*. Vet Clin North Am Food Anim Pract, 2012. **28**(2): p. 225-37.

51. Makovec, J.A. and P.L. Ruegg, *Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001*. J Dairy Sci, 2003. **86**(11): p. 3466-72.

52. Barrett, D.J., et al., *Prevalence of pathogens causing subclinical mastitis in 15 dairy herds in the Republic of Ireland*. Irish Veterinary Journal, 2005. **58**(6): p. 333.

53. Koivula, M., et al., *Distribution of bacteria and seasonal and regional effects in a new database for mastitis pathogens in Finland*. Acta Agriculturae Scandinavica, Section A — Animal Science, 2007. **57**(2): p. 89-96.

54. Gildow, E.M., D.L. Fourt, and A.O. Shaw, *Sulfanilamide in the Treatment of Streptococcic Mastitis*. Journal of Dairy Science, 1938. **21**(12): p. 759-766.

55. Foley, E.J., S.W. Lee, and J.A. Epstein, *The Effect of Penicillin on Staphylococci and Streptococci Commonly Associated with Bovine Mastitis*. Journal of Milk Technology, 1945. **8**(3): p. 129-133.

56. Fleming, A., *On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to their Use in the Isolation of B. influenzæ*. Br J Exp Pathol, 1929. **10**(3): p. 226-36.

57. Mitchell, J.M., et al., *Antimicrobial Drug Residues in Milk and Meat: Causes, Concerns, Prevalence, Regulations, Tests, and Test Performance*. Journal of Food Protection, 1998. **61**(6): p. 742-756.

58. Van Boeckel, T.P., et al., *Global trends in antimicrobial use in food animals*. Proceedings of the National Academy of Sciences, 2015. **112**(18): p. 5649-5654.

59. Mia, M.T., et al., *Detection of bacterial species from clinical mastitis in dairy cows at Nilphamari district and their antibiogram studies*. Asian Journal of Medical and Biological Research, 2016. **2**(4): p. 656-663.

60. Botrel, M.-A., et al., *Distribution and Antimicrobial Resistance of Clinical and Subclinical Mastitis Pathogens in Dairy Cows in Rhône-Alpes, France*. Foodborne Pathogens and Disease, 2010. **7**(5): p. 479-487.

61. Ferri, M., et al., *Antimicrobial resistance: A global emerging threat to public health systems*. Critical Reviews in Food Science and Nutrition, 2017. **57**(13): p. 2857-2876.

62. Fang, W. and S. Pyörälä, *Mastitis-Causing Escherichia coli: Serum Sensitivity and Susceptibility to Selected Antibacterials in Milk*. Journal of Dairy Science, 1996. **79**(1): p. 76-82.

63. Louhi, M., et al., *Relevance of Sensitivity Testings (MIC) of S. aureus to Predict the Antibacterial Action in Milk*. Journal of Veterinary Medicine, Series B, 1992. **39**(1-10): p. 253-262.

64. Ziv, G., *Drug selection and use in mastitis: systemic vs local therapy*. J Am Vet Med Assoc, 1980. **176**(10 Spec No): p. 1109-15.

65. Mehta, A.C. and L.F. Muscarella, *Bronchoscope-Related "Superbug" Infections*. Chest, 2020. **157**(2): p. 454-469.

66. White, D.G. and P.F. McDermott, *Emergence and Transfer of Antibacterial Resistance*. Journal of Dairy Science, 2001. **84**: p. E151-E155.

67. Rasheed, M.U.T., Nooruddin; Ahamed, Parveez; Teklemariam, Zelalem; Jamil, Kaiser, *Antimicrobial Drug Resistance in Strains of Escherichia coli Isolated from Food Sources*. Revista do Instituto de Medicina Tropical de São Paulo, 2014. **56**(4).

68. Erskine, R., *Antimicrobial drug use in bovine mastitis*. Antimicrobial therapy in veterinary medicine, 2000: p. 712-734.

69. Barnett, B.J. and D.S. Stephens, *Urinary Tract Infection: An Overview*. The American Journal of the Medical Sciences, 1997. **314**(4): p. 245-249.

70. Bonten, M., et al., *Epidemiology of Escherichia coli Bacteremia: A Systematic Literature Review*. Clinical Infectious Diseases, 2020. **72**(7): p. 1211-1219.

71. Gaschignard, J., et al., *Neonatal Bacterial Meningitis: 444 Cases in 7 Years*. The Pediatric Infectious Disease Journal, 2011. **30**(3): p. 212-217.

72. Bonacorsi, S. and E. Bingen, *Molecular epidemiology of Escherichia coli causing neonatal meningitis*. International Journal of Medical Microbiology, 2005. **295**(6): p. 373-381.

73. Martin, G.S., et al., *The epidemiology of sepsis in the United States from 1979 through 2000*. N Engl J Med, 2003. **348**(16): p. 1546-54.

74. Patton, J.P., D.B. Nash, and E. Abrutyn, *Urinary Tract Infection: Economic Considerations*. Medical Clinics of North America, 1991. **75**(2): p. 495-513.

75. Phillips, I., et al., *EPIDEMIC MULTIRESISTANT ESCHERICHIA COLI INFECTION IN WEST LAMBETH HEALTH DISTRICT*. The Lancet, 1988. **331**(8593): p. 1038-1041.

76. Mora, A., et al., *Seropathotypes, Phylogroups, Stx Subtypes, and Intimin Types of Wildlife-Carried, Shiga Toxin-Producing Escherichia coli Strains with the Same Characteristics as Human-Pathogenic Isolates*. Applied and Environmental Microbiology, 2012. **78**(8): p. 2578-2585.

77. Pupo, G.M., et al., *Evolutionary relationships among pathogenic and nonpathogenic Escherichia coli strains inferred from multilocus enzyme electrophoresis and mdh sequence studies*. Infection and Immunity, 1997. **65**(7): p. 2685-2692.

78. Clermont, O. and S.B. Bonacorsi, Edouard, *Rapid and Simple Determination of the Escherichia coli Phylogenetic Group*. Applied and Environmental Microbiology, 2000. **66**(10): p. 4555-4558.

79. Oktay Gultekin, E., S. Tezcan Ulger, and N. Delialioğlu, *Distribution of Pathogenicity Island Markers and Virulence Factors Genes of Extraintestinal Pathogenic Escherichia coli Isolates*. Jundishapur Journal of Microbiology, 2022. **15**(4).

80. Badger-Emeka, L.I., et al., *A Three-Year Look at the Phylogenetic Profile, Antimicrobial Resistance, and Associated Virulence Genes of Uropathogenic Escherichia coli.* Pathogens, 2022. **11**(6): p. 631.

81. Xavier, R.G.C., et al., *Characterization of Escherichia coli in Dogs with Pyometra and the Influence of Diet on the Intestinal Colonization of Extraintestinal Pathogenic E. coli (ExPEC).* Veterinary Sciences, 2022. **9**(5): p. 245.

82. Radera, S., S. Srivastava, and J. Agarwal, *Virulence Genotyping and Multidrug Resistance Pattern of Escherichia coli Isolated From Community-Acquired and Hospital-Acquired Urinary Tract Infections.* Cureus, 2022. **14**(9).

83. Nascimento, J.A.S., et al., *Molecular Epidemiology and Presence of Hybrid Pathogenic Escherichia coli among Isolates from Community-Acquired Urinary Tract Infection.* Microorganisms, 2022. **10**(2): p. 302.

84. Johnson, J.R., et al., *Phylogenetic Distribution of Extraintestinal Virulence-Associated Traits in Escherichia coli.* The Journal of Infectious Diseases, 2001. **183**(1): p. 78-88.

85. Richards, V.P., et al., *Genome Based Phylogeny and Comparative Genomic Analysis of Intra-Mammary Pathogenic Escherichia coli.* PLOS ONE, 2015. **10**(3): p. e0119799.

86. Suojala, L., et al., *Phylogeny, virulence factors and antimicrobial susceptibility of Escherichia coli isolated in clinical bovine mastitis.* Veterinary Microbiology, 2011. **147**(3): p. 383-388.

87. Jouini, A., et al., *Lineages, Virulence Gene Associated and Integrons among Extended Spectrum β-Lactamase (ESBL) and CMY-2 Producing Enterobacteriaceae from Bovine Mastitis, in Tunisia.* Pathogens, 2022. **11**(8): p. 948.

88. Carlos, C., et al., *Escherichia coli phylogenetic group determination and its application in the identification of the major animal source of fecal contamination.* BMC Microbiology, 2010. **10**(1): p. 161.

89. Fernandes, J.B.C., et al., *Escherichia coli from clinical mastitis:serotypes and virulence factors.* Journal of Veterinary Diagnostic Investigation, 2011. **23**(6): p. 1146-1152.

90. Liu, Y., et al., *Phylogenetic group, virulence factors and antimicrobial resistance of Escherichia coli associated with bovine mastitis.* Research in Microbiology, 2014. **165**(4): p. 273-277.

91. Tomazi, T., et al., *Antimicrobial susceptibility patterns of Escherichia coli phylogenetic groups isolated from bovine clinical mastitis.* Journal of Dairy Science, 2018. **101**(10): p. 9406-9418.

92. Zhang, D., et al., *The phylogenetic group, antimicrobial susceptibility, and virulence genes of Escherichia coli from clinical bovine mastitis*. Journal of Dairy Science, 2018. **101**(1): p. 572-580.

93. Burvenich, C., et al., *Severity of E. coli mastitis is mainly determined by cow factors*. Vet. Res., 2003. **34**(5): p. 521-564.

94. Roussel, P., et al., *Investigating the contribution of IL-17A and IL-17F to the host response during Escherichia coli mastitis*. Veterinary Research, 2015. **46**(1): p. 56.

95. Frost, A.J., A.W. Hill, and B.E. Brooker, *Pathogenesis of experimental bovine mastitis following a small inoculum of Escherichia coli.* Research in Veterinary Science, 1982. **33**(1): p. 105-112.

96. Sulabh, S. and J. Bayan, *Bovine Immunogenetic Response to S. aureus and E. coli Mastitis: A Review*. THE INDIAN JOURNAL OF VETERINARY SCIENCES AND BIOTECHNOLOGY, 2018. **13**(03): p. 67-74.

97. Hirvonen, J., et al., *Acute Phase Response in Dairy Cows with Experimentally Induced Escherichia coli Mastitis*. Acta Veterinaria Scandinavica, 1999. **40**(1): p. 35-46.

98. Melchior, M.B., H. Vaarkamp, and J. Fink-Gremmels, *Biofilms: A role in recurrent mastitis infections?* The Veterinary Journal, 2006. **171**(3): p. 398-407.

99. Olson, M.A., et al., *Genome-Wide Identification of Fitness Factors in Mastitis-Associated Escherichia coli*. Applied and Environmental Microbiology, 2018. **84**(2): p. e02190-17.

100. Blum, S.E., et al., *Genomic and Phenomic Study of Mammary Pathogenic Escherichia coli*. PLoS One, 2015. **10**(9): p. e0136387.

101. Kempf, F., et al., *Genomic Comparative Study of Bovine Mastitis Escherichia coli*. PLOS ONE, 2016. **11**(1): p. e0147954.

102. Lehtolainen, T., et al., *Association Between Virulence Factors and Clinical Course of Escherichia coli Mastitis*. Acta Veterinaria Scandinavica, 2003. **44**(4): p. 203.

103. Ghanbarpour, R. and E. Oswald, *Phylogenetic distribution of virulence genes in Escherichia coli isolated from bovine mastitis in Iran*. Research in Veterinary Science, 2010. **88**(1): p. 6-10.

104. Jamali, H., K. Krylova, and M. Aïder, *Identification and frequency of the associated genes with virulence and antibiotic resistance of Escherichia coli isolated from cow's milk presenting mastitis pathology*. Animal Science Journal, 2018. **89**(12): p. 1701-1706.

105. Marashifard, M., et al., *Determination of antibiotic resistance pattern and virulence genes in Escherichia coli isolated from bovine with subclinical mastitis in southwest of Iran*. Tropical Animal Health and Production, 2019. **51**(3): p. 575-580.

106. Leimbach, A., et al., *Complete Genome Sequences of Escherichia coli Strains 1303 and ECC-1470 Isolated from Bovine Mastitis*. Genome Announcements, 2015. **3**(2): p. e00182-15.

107. Zhou, M., et al., *Long polar fimbriae contribute to pathogenic Escherichia coli infection to host cells*. Applied Microbiology and Biotechnology, 2019. **103**(18): p. 7317-7324.

108. Jordan, D.M., et al., *Long Polar Fimbriae Contribute to Colonization by Escherichia coli O157:H7 In Vivo*. Infection and Immunity, 2004. **72**(10): p. 6168-6171.

109. Lan, T., et al., *Antimicrobial susceptibility, phylotypes, and virulence genes of Escherichia coli from clinical bovine mastitis in five provinces of China*. Food and Agricultural Immunology, 2020. **31**(1): p. 406-423.

110. Ismail, Z.B. and S.M. Abutarbush, *Molecular characterization of antimicrobial resistance and virulence genes of Escherichia coli isolates from bovine mastitis*. Vet World, 2020. **13**(8): p. 1588-1593.

111. Cruz-Soto, A.S., et al., *Genetic relationships, biofilm formation, motility and virulence of Escherichia coli isolated from bovine mastitis*. Revista mexicana de ciencias pecuarias, 2020. **11**(1): p. 167-182.

112. Silva, V.O., et al., *Biofilm Formation on Biotic and Abiotic Surfaces in the Presence of Antimicrobials by Escherichia coli Isolates from Cases of Bovine Mastitis*. Applied and Environmental Microbiology, 2014. **80**(19): p. 6136-6145.

113. Blum, S., et al., *Identification of a bovine mastitis Escherichia coli subset*. Veterinary Microbiology, 2008. **132**(1): p. 135-148.

114. Nüesch-Inderbinen, M., et al., *Molecular types, virulence profiles and antimicrobial resistance of Escherichia coli causing bovine mastitis*. Veterinary Record Open, 2019. **6**(1): p. e000369.

115. Olson, M.A., *Identification of Genes that Determine Fitness, Virulence, and Disease Outcomes in Mastitis Associated Eschericia Coli*. 2020, Brigham Young University: Provo. p. 148.

116. Nalband, S., et al., *Characterization of Escherichia coli isolated from bovine subclinical mastitis for virulence genes, phylogenetic groups and ESBL production*. Indian Journal of Animal Research, 2020. **54**(10): p. 1265-1271.

117. Tavakoli, M. and H. Pourtaghi, *Molecular detection of virulence genes and multi-drug resistance patterns in Escherichia coli (STEC) in clinical bovine mastitis: Alborz province, Iran*. Iran J Vet Res, 2017. **18**(3): p. 208-211.

118. Zafarane, S., et al., *Characterization of virulence genes, serogroups and antimicrobial susceptibility of Shiga toxin producing Escherichia coli isolated from bovine mastitic milk in Tehran, Iran*. Tropical Biomedicine, 2017. **34**(2): p. 295-304.

119. Zaatout, N., *An overview on mastitis-associated Escherichia coli: Pathogenicity, host immunity and the use of alternative therapies*. Microbiological Research, 2022. **256**: p. 126960.

120. Raza, A., et al., *Biofilm producing Staphylococcus aureus and bovine mastitis: a review*. Molecular microbiology research, 2013. **3**(1).

121. Hall-Stoodley, L., J.W. Costerton, and P. Stoodley, *Bacterial biofilms: from the natural environment to infectious diseases*. Nat Rev Microbiol, 2004. **2**(2): p. 95-108.

122. Vlamakis, H., et al., *Sticking together: building a biofilm the Bacillus subtilis way*. Nat Rev Microbiol, 2013. **11**(3): p. 157-68.

123. Costerton, J.W., P.S. Stewart, and E.P. Greenberg, *Bacterial Biofilms: A Common Cause of Persistent Infections*. Science, 1999. **284**(5418): p. 1318-1322.

124. Sharma, G., et al., *Escherichia coli biofilm: development and therapeutic strategies*. Journal of Applied Microbiology, 2016. **121**(2): p. 309-319.

125. Ito, A., et al., *Increased Antibiotic Resistance of Escherichia coli in Mature Biofilms*. Applied and Environmental Microbiology, 2009. **75**(12): p. 4093-4100.

126. Johnson, G.M., et al., *Interference with granulocyte function by Staphylococcus epidermidis slime*. Infection and Immunity, 1986. **54**(1): p. 13-20.

127. Yasuda, H., et al., *Interaction between human polymorphonuclear leucocytes and bacteria released from in-vitro bacterial biofilm models*. Journal of Medical Microbiology, 1994. **41**(5): p. 359-367.

128. Gambino, M. and F. Cappitelli, *Mini-review: Biofilm responses to oxidative stress*. Biofouling, 2016. **32**(2): p. 167-178.

129. Beloin, C., A. Roux, and J.M. Ghigo, *Escherichia coli biofilms*. Curr Top Microbiol Immunol, 2008. **322**: p. 249-89.

130. Dunne, W.M., Jr., *Bacterial adhesion: seen any good biofilms lately?* Clin Microbiol Rev, 2002. **15**(2): p. 155-66.

131. Carpentier, B. and O. Cerf, *Biofilms and their consequences, with particular reference to hygiene in the food industry*. Journal of Applied Bacteriology, 1993. **75**(6): p. 499-511.

132. Economou, A., et al., *Secretion by numbers: Protein traffic in prokaryotes*. Mol Microbiol, 2006. **62**(2): p. 308-19.

133. Flemming, H.-C. and J. Wingender, *The biofilm matrix*. Nature Reviews Microbiology, 2010. **8**(9): p. 623-633.

134. Daniels, R., J. Vanderleyden, and J. Michiels, *Quorum sensing and swarming migration in bacteria*. FEMS Microbiology Reviews, 2004. **28**(3): p. 261-289.

135. Sturbelle, R.T., et al., *The role of quorum sensing in Escherichia coli (ETEC) virulence factors*. Veterinary Microbiology, 2015. **180**(3): p. 245-252.

136. Hensen, S.M., *Bovine Staphylococcus aureus mastitis: bacterial adhesion and invasion in relation to pathogenesis and antimicrobial sensitivity*. 2000, Utrecht University: Netherlands. p. 158.

137. Shpigel, N.Y., S. Elazar, and I. Rosenshine, *Mammary pathogenic Escherichia coli*. Current Opinion in Microbiology, 2008. **11**(1): p. 60-65.

138. Olson, M.A., et al., *Bile Salts Regulate Zinc Uptake and Capsule Synthesis in a Mastitis-Associated Extraintestinal Pathogenic Escherichia coli Strain*. Infection and Immunity, 2021. **89**(10): p. e00357-21.

139. Kalscheuer, R., et al., *The Mycobacterium tuberculosis capsule: a cell structure with key implications in pathogenesis*. Biochemical Journal, 2019. **476**(14): p. 1995-2016.

140. Minasyan, H., *Sepsis: mechanisms of bacterial injury to the patient*. Scandinavian Journal of Trauma, Resuscitation and Emergency Medicine, 2019. **27**(1): p. 19.

141. Baselga, R., I. Albizu, and B. Amorena, *Staphylococcus aureus capsule and slime as virulence factors in ruminant mastitis. A review*. Veterinary Microbiology, 1994. **39**(3): p. 195-204.

142. Whitfield, C. and I.S. Roberts, *Structure, assembly and regulation of expression of capsules in Escherichia coli*. Molecular Microbiology, 1999. **31**(5): p. 1307-1319.

143. *Complex Carbohydrate Research Center*. 10/12/22; Available from: https://ccrc.uga.edu/.

144. Kaper, J.B., J.P. Nataro, and H.L.T. Mobley, *Pathogenic Escherichia coli*. Nature Reviews Microbiology, 2004. **2**(2): p. 123-140.

145. Cassels, F.J. and M.K. Wolf, *Colonization factors of diarrheagenic E. coli and their intestinal receptors*. J Ind Microbiol, 1995. **15**(3): p. 214-26.

146. Barnich, N., et al., *Involvement of lipoprotein NlpI in the virulence of adherent invasive Escherichia coli strain LF82 isolated from a patient with Crohn's disease*. Infection and immunity, 2004. **72**(5): p. 2484-2493.

147. Teng, C.-H., et al., *NlpI Contributes to Escherichia coli K1 Strain RS218 Interaction with Human Brain Microvascular Endothelial Cells*. Infection and Immunity, 2010. **78**(7): p. 3090-3096.

148. Kaper, J.B., *Enterohemorrhagic Escherichia coli*. Current Opinion in Microbiology, 1998. **1**(1): p. 103-108.

149. Rosenshine, I., et al., *A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation*. Embo j, 1996. **15**(11): p. 2613-24.

150. Keane, O.M., *Genetic diversity, the virulence gene profile and antimicrobial resistance of clinical mastitis-associated Escherichia coli*. Research in Microbiology, 2016. **167**(8): p. 678-684.

151. Finlay, B.B. and P. Cossart, *Exploitation of Mammalian Host Cell Functions by Bacterial Pathogens*. Science, 1997. **276**(5313): p. 718-725.

152. Döpfer, D., et al., *Adhesion and invasion of Escherichia coli from single and recurrent clinical cases of bovine mastitis in vitro*. Veterinary Microbiology, 2000. **74**(4): p. 331-343.

153. Dogan, B., et al., *Adherent and invasive Escherichia coli are associated with persistent bovine mastitis*. Veterinary Microbiology, 2006. **116**(4): p. 270-282.

154. Rebers, J.E. and J.H. Willis, *A conserved domain in arthropod cuticular proteins binds chitin*. Insect Biochem Mol Biol, 2001. **31**(11): p. 1083-93.

155. Bowman, S.M. and S.J. Free, *The structure and synthesis of the fungal cell wall*. Bioessays, 2006. **28**(8): p. 799-808.

156. Hunt, S. and M. Nixon, *A comparative study of protein composition in the chitin-protein complexes of the beak, pen, sucker disc, radula and oesophageal cuticle of cephalopods*. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 1981. **68**(4): p. 535-546.

157. Durkin, C.A., T. Mock, and E.V. Armbrust, *Chitin in Diatoms and Its Association with the Cell Wall*. Eukaryotic Cell, 2009. **8**(7): p. 1038-1050.

158. Poulicek, M. and C. Jeuniaux, *Chitin biodegradation in marine environments: An experimental approach*. Biochemical Systematics and Ecology, 1991. **19**(5): p. 385-394.

159. Breyne, K., et al., *Immunomodulation of Host Chitinase 3-Like 1 During a Mammary Pathogenic Escherichia coli Infection*. Frontiers in Immunology, 2018. **9**.

160. Funkhouser, J.D. and N.N. Aronson, Jr., *Chitinase family GH18: evolutionary insights from the genomic history of a diverse protein family*. BMC Evol Biol, 2007. **7**: p. 96.

161. Monreal, J. and E.T. Reese, *The chitinase of Serratia marcescens*. Canadian Journal of Microbiology, 1969. **15**(7): p. 689-696.

162. Vaaje-Kolstad, G., et al., *The Non-catalytic Chitin-binding Protein CBP21 from Serratia marcescens Is Essential for Chitin Degradation*. Journal of Biological Chemistry, 2005. **280**(31): p. 28492-28497.

163. Bokma, E., et al., *Hevamine, a chitinase from the rubber tree Hevea brasiliensis, cleaves peptidoglycan between the C-1 of N-acetylglucosamine and C-4 of N-acetylmuramic acid and therefore is not a lysozyme*. FEBS Letters, 1997. **411**(2-3): p. 161-163.

164. Keyhani, N.O. and S. Roseman, *Wild-type Escherichia coli grows on the chitin disaccharide, N,N'-diacetylchitobiose, by expressing the cel operon*. Proceedings of the National Academy of Sciences, 1997. **94**(26): p. 14367-14371.

165. Francetic, O., et al., *Expression of the endogenous type II secretion pathway in Escherichia coli leads to chitinase secretion*. The EMBO Journal, 2000. **19**(24): p. 6697-6703.

166. Francetic˜, O., et al., *The ChiA (YheB) protein of Escherichia coli K-12 is an endochitinase whose gene is negatively controlled by the nucleoid-structuring protein H-NS*. Molecular Microbiology, 2000. **35**(6): p. 1506-1517.

167. Connell, T.D., et al., *Endochitinase is transported to the extracellular milieu by the eps-encoded general secretory pathway of Vibrio cholerae*. J Bacteriol, 1998. **180**(21): p. 5591-600.

168. Kirn, T.J., B.A. Jude, and R.K. Taylor, *A colonization factor links Vibrio cholerae environmental survival and human infection*. Nature, 2005. **438**(7069): p. 863-6.

169. Leisner, J.J., et al., *Chitin hydrolysis by Listeria spp., including L. monocytogenes*. Appl Environ Microbiol, 2008. **74**(12): p. 3823-30.

170. Chatterjee, S.S., et al., *Intracellular gene expression profile of Listeria monocytogenes*. Infect Immun, 2006. **74**(2): p. 1323-38.

171. Salunkhe, P., et al., *A Cystic Fibrosis Epidemic Strain of Pseudomonas aeruginosa Displays Enhanced Virulence and Antimicrobial Resistance*. Journal of Bacteriology, 2005. **187**(14): p. 4908-4920.

172. Fung, C., et al., *Gene expression of Pseudomonas aeruginosa in a mucin-containing synthetic growth medium mimicking cystic fibrosis lung sputum*. J Med Microbiol, 2010. **59**(Pt 9): p. 1089-1100.

173. DebRoy, S., et al., *Legionella pneumophila type II secretome reveals unique exoproteins and a chitinase that promotes bacterial persistence in the lung*. Proc Natl Acad Sci U S A, 2006. **103**(50): p. 19146-51.

174. Devlin, J.R., et al., *Salmonella enterica serovar Typhimurium chitinases modulate the intestinal glycome and promote small intestinal invasion*. PLoS Pathog, 2022. **18**(4): p. e1010167.

175. Chandra, K., et al., *GH18 family glycoside hydrolase Chitinase A of Salmonella enhances virulence by facilitating invasion and modulating host immune responses*. PLoS Pathog, 2022. **18**(4): p. e1010407.

176. Rehman, S., et al., *Structure and functional analysis of the Legionella pneumophila chitinase ChiA reveals a novel mechanism of metal-dependent mucin degradation*. PLOS Pathogens, 2020. **16**(5): p. e1008342.

177. Low, D., et al., *Chitin-binding domains of Escherichia coli ChiA mediate interactions with intestinal epithelial cells in mice with colitis*. Gastroenterology, 2013. **145**(3): p. 602-12.e9.

178. Patton, S., S.J. Gendler, and A.P. Spicer, *The epithelial mucin, MUC1, of milk, mammary gland and other tissues*. Biochim Biophys Acta, 1995. **1241**(3): p. 407-23.

179. Sando, L., et al., *Bovine Muc1 is a highly polymorphic gene encoding an extensively glycosylated mucin that binds bacteria*. J Dairy Sci, 2009. **92**(10): p. 5276-91.

180. Miquel, S., et al., *Complete Genome Sequence of Crohn's Disease-Associated Adherent-Invasive E. coli Strain LF82*. PLOS ONE, 2010. **5**(9): p. e12714.

181. Coffman, F.D., *Chitinase 3-Like-1 (CHI3L1): A Putative Disease Marker at the Interface of Proteomics and Glycomics*. Critical Reviews in Clinical Laboratory Sciences, 2008. **45**(6): p. 531-562.

182. Hanahan, D., *Studies on transformation of Escherichia coli with plasmids*. J Mol Biol, 1983. **166**(4): p. 557-80.

183. Ferrières, L., et al., *Silent mischief: bacteriophage Mu insertions contaminate products of Escherichia coli random mutagenesis performed using suicidal transposon delivery plasmids mobilized by broad-host-range RP4 conjugative machinery*. J Bacteriol, 2010. **192**(24): p. 6418-27.

184. Wiles, T.J., et al., *Modernized Tools for Streamlined Genetic Manipulation and Comparative Study of Wild and Diverse Proteobacterial Lineages*. mBio, 2018. **9**(5): p. e01877-18.

185. Calder, J.T., et al., *A Trimeric Autotransporter Enhances Biofilm Cohesiveness in Yersinia pseudotuberculosis but Not in Yersinia pestis*. Journal of Bacteriology, 2020. **202**(20): p. e00176-20.

186. Datsenko, K.A. and B.L. Wanner, *One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products*. Proc Natl Acad Sci U S A, 2000. **97**(12): p. 6640-5.

187. Liu, B., et al., *VFDB 2019: a comparative pathogenomic platform with an interactive web interface*. Nucleic Acids Research, 2018. **47**(D1): p. D687-D692.

188. Waterhouse, A.M., et al., *Jalview Version 2—a multiple sequence alignment editor and analysis workbench*. Bioinformatics, 2009. **25**(9): p. 1189-1191.

189. Mayer, S.J., et al., *Oxygen concentration in milk of healthy and mastitic cows and implications of low oxygen tension for the killing of Staphylococcus aureus by bovine neutrophils*. J Dairy Res, 1988. **55**(4): p. 513-9.

190. Feng, J., et al., *Multiple-Vessel-Based Blood Gas Profiles Analysis Revealed the Potential of Blood Oxygen in Mammary Vein as Indicator of Mammary Gland Health Risk of High-Yielding Dairy Cows*. Animals, 2022. **12**(12): p. 1484.

191. Thomas, F.C., et al., *Mastitomics, the integrated omics of bovine milk in an experimental model of Streptococcus uberis mastitis: 3. Untargeted metabolomics*. Molecular BioSystems, 2016. **12**(9): p. 2762-2769.

192. Wang, Y., et al., *Discrepancies among healthy, subclinical mastitic, and clinical mastitic cows in fecal microbiome and metabolome and serum metabolome*. Journal of Dairy Science, 2022. **105**(9): p. 7668-7688.

193. Tyagi, P., D.R. Edwards, and M.S. Coyne, *Use of Sterol and Bile Acid Biomarkers to Identify Domesticated Animal Sources of Fecal Pollution*. Water, Air, and Soil Pollution, 2008. **187**(1): p. 263-274.

194. Avican, K., et al., *RNA atlas of human bacterial pathogens uncovers stress dynamics linked to infection*. Nature Communications, 2021. **12**(1): p. 3282.

195. Li, P., et al., *Bile salt receptor complex activates a pathogenic type III secretion system*. eLife, 2016. **5**: p. e15718.

196. Padavannil, A., et al., *Structure of GrlR–GrlA complex that prevents GrlA activation of virulence genes*. Nature communications, 2013. **4**(1): p. 1-10.

197. Choi, J. and E.A. Groisman, *Salmonella expresses foreign genes during infection by degrading their silencer*. Proceedings of the National Academy of Sciences, 2020. **117**(14): p. 8074-8082.

198. Han, A., et al., *Bacterial adhesion mechanisms on dental implant surfaces and the influencing factors*. International Journal of Adhesion and Adhesives, 2016. **69**: p. 58-71.

199. Mizoguchi, E., *Chitinase 3–Like-1 Exacerbates Intestinal Inflammation by Enhancing Bacterial Adhesion and Invasion in Colonic Epithelial Cells*. Gastroenterology, 2006. **130**(2): p. 398-411.

200. Ernst, P.B., S.E. Crowe, and V.E. Reyes, *How Does Helicobacter pylori Cause Mucosal Damage? The Inflammatory Response*. Gastroenterology, 1997. **113**(6, Supplement): p. S35-S42.

201. Maes, M., et al., *In depression, bacterial translocation may drive inflammatory responses, oxidative and nitrosative stress (O&NS), and autoimmune responses directed against O&NS-damaged neoepitopes*. Acta Psychiatrica Scandinavica, 2013. **127**(5): p. 344-354.

202. Leimbach, A., et al., *No evidence for a bovine mastitis Escherichia coli pathotype*. BMC Genomics, 2017. **18**(1): p. 359.

203. Jung, D., et al., *Comparative genomic analysis of Escherichia coli isolates from cases of bovine clinical mastitis identifies nine specific pathotype marker genes*. Microb Genom, 2021. **7**(7).

204. Lin, J., J.S. Hogan, and K.L. Smith, *Antigenic homology of the inducible ferric citrate receptor (FecA) of coliform bacteria isolated from herds with naturally occurring bovine intramammary infections*. Clin Diagn Lab Immunol, 1999. **6**(6): p. 966-9.

205. Guerra, S.T., et al., *Short communication: Investigation of extra-intestinal pathogenic Escherichia coli virulence genes, bacterial motility, and multidrug resistance pattern of strains isolated from dairy cows with different severity scores of clinical mastitis*. Journal of Dairy Science, 2020. **103**(4): p. 3606-3614.

206. Jansson, L., et al., *The major subunit, CfaB, of colonization factor antigen i from enterotoxigenic Escherichia coli is a glycosphingolipid binding protein*. Infect Immun, 2006. **74**(6): p. 3488-97.

207. Rendón, M.A., et al., *Commensal and pathogenic Escherichia coli use a common pilus adherence factor for epithelial cell colonization*. Proc Natl Acad Sci U S A, 2007. **104**(25): p. 10637-42.

208. Kansal, R., et al., *Transcriptional modulation of enterotoxigenic Escherichia coli virulence genes in response to epithelial cell interactions.* Infect Immun, 2013. **81**(1): p. 259-70.

209. Samadder, P., et al., *The Escherichia coli ycbQRST operon encodes fimbriae with laminin-binding and epithelial cell adherence properties in Shiga-toxigenic E. coli O157:H7.* Environ Microbiol, 2009. **11**(7): p. 1815-26.

210. Xicohtencatl-Cortes, J., et al., *Intestinal adherence associated with type IV pili of enterohemorrhagic Escherichia coli O157:H7.* J Clin Invest, 2007. **117**(11): p. 3519-29.

211. Xicohtencatl-Cortes, J., et al., *The type 4 pili of enterohemorrhagic Escherichia coli O157:H7 are multipurpose structures with pathogenic attributes.* J Bacteriol, 2009. **191**(1): p. 411-21.

212. Roberts, J.A., et al., *The Gal(alpha 1-4)Gal-specific tip adhesin of Escherichia coli P-fimbriae is needed for pyelonephritis to occur in the normal urinary tract.* Proc Natl Acad Sci U S A, 1994. **91**(25): p. 11889-93.

213. Wullt, B., et al., *P fimbriae enhance the early establishment of Escherichia coli in the human urinary tract.* Mol Microbiol, 2000. **38**(3): p. 456-64.

214. Wullt, B., *The role of P fimbriae for Escherichia coli establishment and mucosal inflammation in the human urinary tract.* Int J Antimicrob Agents, 2003. **21**(6): p. 605-21.

215. Ofek, I., D. Mirelman, and N. Sharon, *Adherence of Escherichia coli to human mucosal cells mediated by mannose receptors.* Nature, 1977. **265**(5595): p. 623-625.

216. Mulvey, M.A., et al., *Induction and Evasion of Host Defenses by Type 1-Piliated Uropathogenic Escherichia coli.* Science, 1998. **282**(5393): p. 1494-1497.

217. Caloca, M.J., J. Soler, and S. Suárez, *Adhesion of K88ab to guinea pig erythrocytes: effect on membrane enzyme activities.* Infect Immun, 1996. **64**(8): p. 3416-8.

218. Sun, R., et al., *Inhibition of adhesion of Escherichia coli k88ac fimbria to its receptor, intestinal mucin-type glycoproteins, by a monoclonal antibody directed against a variable domain of the fimbria.* Infect Immun, 2000. **68**(6): p. 3509-15.

219. Doughty, S., et al., *Identification of a Novel Fimbrial Gene Cluster Related to Long Polar Fimbriae in Locus of Enterocyte Effacement-Negative Strains of Enterohemorrhagic Escherichia coli.* Infection and Immunity, 2002. **70**(12): p. 6761-6769.

220. Krachler, A.M. and K. Orth, *Functional Characterization of the Interaction between Bacterial Adhesin Multivalent Adhesion Molecule 7 (MAM7) Protein and Its Host Cell Ligands.* Journal of Biological Chemistry, 2011. **286**(45): p. 38939-38947.

221. Krachler, A.M., H. Ham, and K. Orth, *Outer membrane adhesion factor multivalent adhesion molecule 7 initiates host cell binding during infection by gram-negative pathogens.* Proc Natl Acad Sci U S A, 2011. **108**(28): p. 11614-9.

222. Spurbeck, R.R., et al., *Fimbrial Profiles Predict Virulence of Uropathogenic Escherichia coli Strains: Contribution of Ygi and Yad Fimbriae.* Infection and Immunity, 2011. **79**(12): p. 4753-4763.

223. Klemm, P., et al., *Structure-function analysis of the self-recognizing Antigen 43 autotransporter protein from Escherichia coli.* Molecular Microbiology, 2004. **51**(1): p. 283-296.

224. Wells, T.J., et al., *The Escherichia coli O157:H7 EhaB autotransporter protein binds to laminin and collagen I and induces a serum IgA response in O157:H7 challenged cattle.* Environmental Microbiology, 2009. **11**(7): p. 1803-1814.

225. Díaz, J.M., et al., *The Vacuolating Autotransporter Toxin (Vat) of Escherichia coli Causes Cell Cytoskeleton Changes and Produces Non-lysosomal Vacuole Formation in Bladder Epithelial Cells.* Frontiers in Cellular and Infection Microbiology, 2020. **10**.

226. Valle, J., et al., *UpaG, a New Member of the Trimeric Autotransporter Family of Adhesins in Uropathogenic Escherichia coli.* Journal of Bacteriology, 2008. **190**(12): p. 4147-4161.

227. MONTENEGRO, M.A., et al., *traT Gene Sequences, Serum Resistance and Pathogenicity-related Factors in Clinical Isolates of Escherichia coli and Other Gram-negative Bacteria.* Microbiology, 1985. **131**(6): p. 1511-1521.

228. Moll, A., P. Manning, and K. Timmis, *Plasmid-determined resistance to serum bactericidal activity: a major outer membrane protein, the traT gene product, is responsible for plasmid-specified serum resistance in Escherichia coli.* Infection and Immunity, 1980. **28**(2): p. 359-367.

229. Nolan, L.K., et al., *Resistance to Serum Complement, iss, and Virulence of Avian Escherichia coli.* Veterinary Research Communications, 2003. **27**(2): p. 101-110.

230. Huang, S.-H., et al., *Further Characterization of Escherichia coli Brain Microvascular Endothelial Cell Invasion Gene ibeA by Deletion, Complementation, and Protein Expression.* The Journal of Infectious Diseases, 2001. **183**(7): p. 1071-1078.

231. Huang, S.H., et al., *Identification and characterization of an Escherichia coli invasion gene locus, ibeB, required for penetration of brain microvascular endothelial cells.* Infection and Immunity, 1999. **67**(5): p. 2103-2109.

232. Dogan, B., et al., *Phylogroup and lpfA influence epithelial invasion by mastitis associated Escherichia coli.* Veterinary microbiology, 2012. **159**(1-2): p. 163-170.

233. Torres, A.G., et al., *TonB-Dependent Systems of Uropathogenic Escherichia coli: Aerobactin and Heme Transport and TonB Are Required for Virulence in the Mouse*. Infection and Immunity, 2001. **69**(10): p. 6179-6185.

234. Sabri, M., S. Léveillé, and C.M. Dozois, *A SitABCD homologue from an avian pathogenic Escherichia coli strain mediates transport of iron and manganese and resistance to hydrogen peroxide*. Microbiology, 2006. **152**(3): p. 745-758.

235. Hantke, K., et al., *Salmochelins, siderophores of Salmonella enterica and uropathogenic Escherichia coli strains, are recognized by the outer membrane receptor IroN*. Proceedings of the National Academy of Sciences, 2003. **100**(7): p. 3677-3682.

236. Russo, T.A., et al., *IroN Functions as a Siderophore Receptor and Is a Urovirulence Factor in an Extraintestinal Pathogenic Isolate of Escherichia coli*. Infection and Immunity, 2002. **70**(12): p. 7156-7160.

237. Koh, E.-I., et al., *Copper import in Escherichia coli by the yersiniabactin metallophore system*. Nature Chemical Biology, 2017. **13**(9): p. 1016-1021.

238. Brumbaugh, A.R., et al., *Blocking Yersiniabactin Import Attenuates Extraintestinal Pathogenic Escherichia coli in Cystitis and Pyelonephritis and Represents a Novel Target To Prevent Urinary Tract Infection*. Infection and Immunity, 2015. **83**(4): p. 1443-1450.

239. Johnson, J.R., et al., *Contribution of yersiniabactin to the virulence of an Escherichia coli sequence type 69 ("clonal group A") cystitis isolate in murine models of urinary tract infection and sepsis*. Microbial Pathogenesis, 2018. **120**: p. 128-131.

240. de Pace, F., et al., *The type VI secretion system plays a role in type 1 fimbria expression and pathogenesis of an avian pathogenic Escherichia coli strain*. Infect Immun, 2010. **78**(12): p. 4990-8.

241. Aschtgen, M.S., et al., *The SciZ protein anchors the enteroaggregative Escherichia coli Type VI secretion system to the cell wall*. Mol Microbiol, 2010. **75**(4): p. 886-99.

242. Comayras, C., et al., *Escherichia coli cytolethal distending toxin blocks the HeLa cell cycle at the G2/M transition by preventing cdc2 protein kinase dephosphorylation and activation*. Infection and Immunity, 1997. **65**(12): p. 5088-5095.

243. Johnson, J.R., et al., *Molecular epidemiology and phylogenetic distribution of the Escherichia coli pks genomic island*. J Clin Microbiol, 2008. **46**(12): p. 3906-11.

244. Ballén, V., et al., *Correlation Between Antimicrobial Resistance, Virulence Determinants and Biofilm Formation Ability Among Extraintestinal Pathogenic Escherichia coli Strains Isolated in Catalonia, Spain*. Front Microbiol, 2021. **12**: p. 803862.

245. Lally, E.T., et al., *RTX toxins recognize a beta2 integrin on the surface of human target cells*. J Biol Chem, 1997. **272**(48): p. 30463-9.

246. Soloaga, A., et al., *Insertion of Escherichia coli alpha-haemolysin in lipid bilayers as a non-transmembrane integral protein: prediction and experiment*. Mol Microbiol, 1999. **31**(4): p. 1013-24.

247. Fahie, M., et al., *A non-classical assembly pathway of Escherichia coli pore-forming toxin cytolysin A*. J Biol Chem, 2013. **288**(43): p. 31042-51.

248. Lai, X.H., et al., *Cytocidal and apoptotic effects of the ClyA protein from Escherichia coli on primary and cultured monocytes and macrophages*. Infect Immun, 2000. **68**(7): p. 4363-7.

249. Schmidt, G., et al., *Gln 63 of Rho is deamidated by Escherichia coli cytotoxic necrotizing factor-1*. Nature, 1997. **387**(6634): p. 725-9.

250. Khan, N.A., et al., *Cytotoxic necrotizing factor-1 contributes to Escherichia coli K1 invasion of the central nervous system*. J Biol Chem, 2002. **277**(18): p. 15607-12.

251. Boquet, P. and E. Lemichez, *Bacterial virulence factors targeting Rho GTPases: parasitism or symbiosis?* Trends Cell Biol, 2003. **13**(5): p. 238-46.

252. Armstrong, G.D., *Uropathogenic Escherichia coli Colicin-Like Usp and Associated Proteins: Their Evolution and Role in Pathogenesis*. The Journal of Infectious Diseases, 2013. **208**(10): p. 1539-1541.

253. McNicholas, P., I. Chopra, and D.M. Rothstein, *Genetic analysis of the tetA(C) gene on plasmid pBR322*. Journal of Bacteriology, 1992. **174**(24): p. 7926-7933.

254. Tobe, T., et al., *An extensive repertoire of type III secretion effectors in Escherichia coli O157 and the role of lambdoid phages in their dissemination*. Proceedings of the National Academy of Sciences, 2006. **103**(40): p. 14941-14946.

255. Guinée, P.A. and W.H. Jansen, *Behavior of Escherichia coli K antigens K88ab, K88ac, and K88ad in immunoelectrophoresis, double diffusion, and hemagglutination*. Infect Immun, 1979. **23**(3): p. 700-5.

256. Thiry, G., et al., *Cloning of DNA sequences encoding foreign peptides and their expression in the K88 pili*. Appl Environ Microbiol, 1989. **55**(4): p. 984-93.

257. Dziewanowska, K., et al., *Fibronectin Binding Protein and Host Cell Tyrosine Kinase Are Required for Internalization of Staphylococcus aureus by Epithelial Cells*. Infection and Immunity, 1999. **67**(9): p. 4673-4678.

258. Zhang, M., et al., *TGF-β1 promoted the infection of bovine mammary epithelial cells by Staphylococcus aureus through increasing expression of cells' fibronectin and integrin β1*. Veterinary Microbiology, 2019. **237**: p. 108420.

259. Matitashvili, E. and D.E. Bauman, *Synthesis of extracellular matrix proteins in bovine mammary epithelial cells*. In Vitro Cellular & Developmental Biology - Animal, 2001. **37**(10): p. 629-632.

260. Mörner, A.P., A. Faris, and K. Krovacek, *Virulence Determinants of Escherichia coli Isolated from the Milk of Sows with Coliform Mastitis*. Journal of Veterinary Medicine, Series B, 1998. **45**(1-10): p. 287-295.

261. Whittam, T.S. and R.A. Wilson, *Genetic relationships among pathogenic Escherichia coli of serogroup O157*. Infection and Immunity, 1988. **56**(9): p. 2467-2473.

262. Hernandes, R.T., et al., *Fimbrial Adhesins Produced by Atypical Enteropathogenic Escherichia coli Strains*. Applied and Environmental Microbiology, 2011. **77**(23): p. 8391-8399.

263. Chopra, M., et al., *Genome based phylogeny and virulence factor analysis of mastitis causing Escherichia coli isolated from Indian cattle*. The Indian Journal of Animal Sciences, 2020. **90**(12).

264. Virkola, R., et al., *Sialyloligosaccharide chains of laminin as an extracellular matrix target for S fimbriae of Escherichia coli*. Infection and Immunity, 1993. **61**(10): p. 4480-4484.

265. Kot, B., et al., *Virulence gene profiles in Staphylococcus aureus isolated from cows with subclinical mastitis in eastern Poland*. Journal of Dairy Research, 2016. **83**(2): p. 228-235.

266. Fessia, A.S. and L.M. Odierno, *Potential factors involved in the early pathogenesis of Streptococcus uberis mastitis: a review*. Folia Microbiologica, 2021. **66**(4): p. 509-523.

267. Saarela, S., et al., *The GafD protein of the G (F17) fimbrial complex confers adhesiveness of Escherichia coli to laminin*. Infect Immun, 1996. **64**(7): p. 2857-60.

268. Wang, B., et al., *Laminin-dependent integrin β1 signaling regulates milk protein synthesis via prolactin/STAT5 pathway in bovine mammary epithelial cells*. Biochemical and Biophysical Research Communications, 2020. **524**(2): p. 288-294.

269. Ulett, G.C., et al., *Functional Analysis of Antigen 43 in Uropathogenic Escherichia coli Reveals a Role in Long-Term Persistence in the Urinary Tract*. Infection and Immunity, 2007. **75**(7): p. 3233-3244.

270. Xue, Z., Y. Pang, and S. Quan, *Revisiting the functions of periplasmic chaperones in the quality control of the autotransporter Ag43 using a phenotypically homogeneous*

Escherichia coli strain. Biochemical and Biophysical Research Communications, 2022. **591**: p. 37-43.

271. Wells, T.J., et al., *EhaA is a novel autotransporter protein of enterohemorrhagic Escherichia coli O157:H7 that contributes to adhesion and biofilm formation*. Environmental Microbiology, 2008. **10**(3): p. 589-604.

272. Li, X., et al., *Compounds targeting YadC of uropathogenic Escherichia coli and its host receptor annexin A2 decrease bacterial colonization in bladder*. EBioMedicine, 2019. **50**: p. 23-33.

273. Larsonneur, F., et al., *Functional analysis of Escherichia coli Yad fimbriae reveals their potential role in environmental persistence*. Environ Microbiol, 2016. **18**(12): p. 5228-5248.

274. Verma, R., et al., *Fimbria-Encoding Gene yadC Has a Pleiotropic Effect on Several Biological Characteristics and Plays a Role in Avian Pathogenic Escherichia coli Pathogenicity*. Infect Immun, 2016. **84**(1): p. 187-93.

275. Wiles, T.J. and M.A. Mulvey, *The RTX pore-forming toxin α-hemolysin of uropathogenic Escherichia coli: progress and perspectives*. Future Microbiol, 2013. **8**(1): p. 73-84.

276. Sun, M., et al., *Insight Into the Virulence Related Secretion Systems, Fimbriae, and Toxins in O2:K1 Escherichia coli Isolated From Bovine Mastitis*. Frontiers in Veterinary Science, 2021. **8**.

277. Wiles, T.J., et al., *Inactivation of host Akt/protein kinase B signaling by bacterial pore-forming toxins*. Mol Biol Cell, 2008. **19**(4): p. 1427-38.

278. Su, Y., et al., *Fluoroquinolone-resistant and extended-spectrum β-lactamase-producing Escherichia coli from the milk of cows with clinical mastitis in Southern Taiwan*. Journal of Microbiology, Immunology and Infection, 2016. **49**(6): p. 892-901.

279. Rudenko, P., et al., *Role of microorganisms isolated from cows with mastitis in Moscow region in biofilm formation*. Veterinary World, 2021. **14**(1): p. 40.

280. Guerra, L., et al., *The biology of the cytolethal distending toxins*. Toxins (Basel), 2011. **3**(3): p. 172-90.

281. Sabry, H., A. Deutz, and M. Awad-Masalmeh, *Virulence factors, O-serogroups and antibiotic resistance of Escherichia coli isolates from cases of bovine acute mastitis in Austria*. Wiener Tierarztliche Monatsschrift, 2006. **93**(5/6): p. 136.

282. Rivera-Cancel, G. and K. Orth, *Biochemical basis for activation of virulence genes by bile salts in Vibrio parahaemolyticus*. Gut Microbes, 2017. **8**(4): p. 366-373.

283. Van Dyken, S.J., et al., *Fungal Chitin from Asthma-Associated Home Environments Induces Eosinophilic Lung Infiltration*. The Journal of Immunology, 2011. **187**(5): p. 2261-2267.

284. Wiesner, D.L., et al., *Chitin Recognition via Chitotriosidase Promotes Pathologic Type-2 Helper T Cell Responses to Cryptococcal Infection*. PLOS Pathogens, 2015. **11**(3): p. e1004701.

285. Bueter, C.L., C.A. Specht, and S.M. Levitz, *Innate Sensing of Chitin and Chitosan*. PLOS Pathogens, 2013. **9**(1): p. e1003080.

286. Lee, C.G., et al., *Role of Chitin and Chitinase/Chitinase-Like Proteins in Inflammation, Tissue Remodeling, and Injury*. Annual Review of Physiology, 2011. **73**(1): p. 479-501.

287. De Loor, J., et al., *Urinary chitinase 3-like protein 1 for early diagnosis of acute kidney injury: a prospective cohort study in adult critically ill patients*. Critical Care, 2016. **20**(1): p. 38.

288. Libreros, S., et al., *Induction of proinflammatory mediators by CHI3L1 is reduced by chitin treatment: Decreased tumor metastasis in a breast cancer model*. International Journal of Cancer, 2012. **131**(2): p. 377-386.

289. Ober, C. and G.L. Chupp, *The chitinase and chitinase-like proteins: a review of genetic and functional studies in asthma and immune-mediated diseases*. Current Opinion in Allergy and Clinical Immunology, 2009. **9**(5): p. 401-408.

290. Hughes, K., et al., *Conditional deletion of Stat3 in mammary epithelium impairs the acute phase response and modulates immune cell numbers during post-lactational regression*. The Journal of Pathology, 2012. **227**(1): p. 106-117.

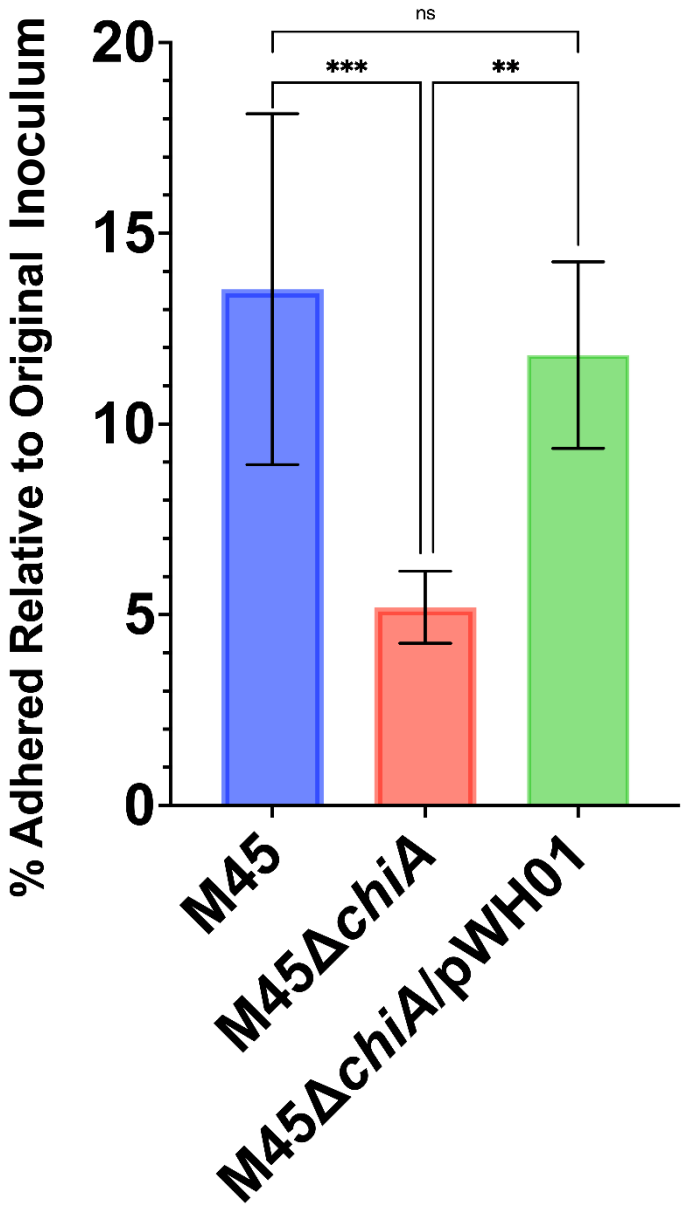
291. Anand, V., et al., *Functional characterization of Mammary Gland Protein-40, a chitinase-like glycoprotein expressed during mammary gland apoptosis*. Apoptosis, 2016. **21**(2): p. 209-224.

292. He, C.H., et al., *Chitinase 3-like 1 regulates cellular and tissue responses via IL-13 receptor α2*. Cell Rep, 2013. **4**(4): p. 830-41.

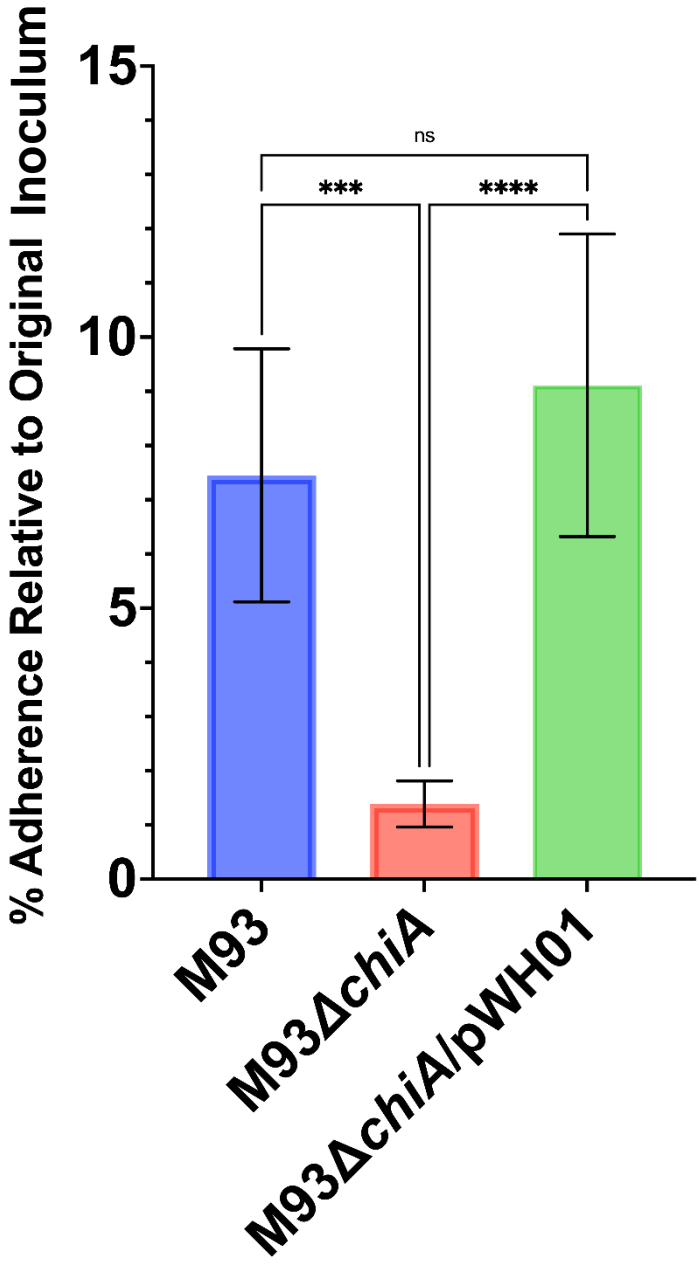
293. He, C.H., et al., *N-Glycosylation Regulates Chitinase 3-like-1 and IL-13 Ligand Binding to IL-13 Receptor α2*. Am J Respir Cell Mol Biol, 2020. **63**(3): p. 386-395.

APPENDICES

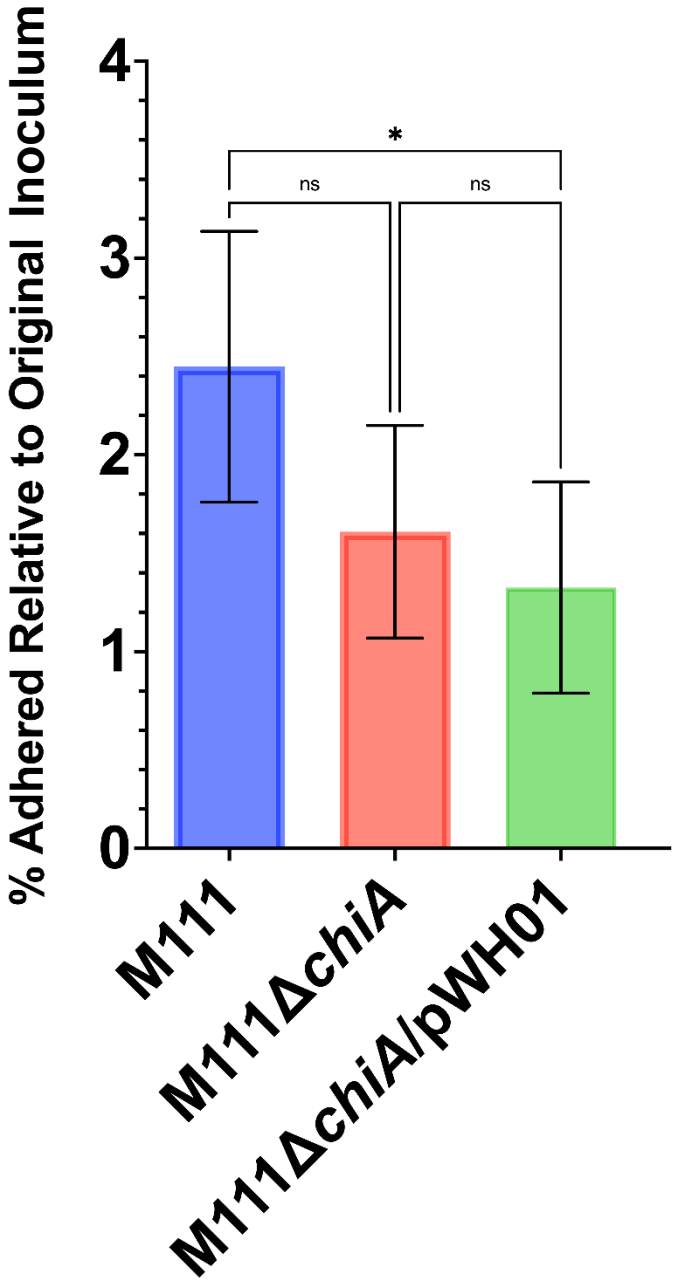
Appendix A: Supplementary figures



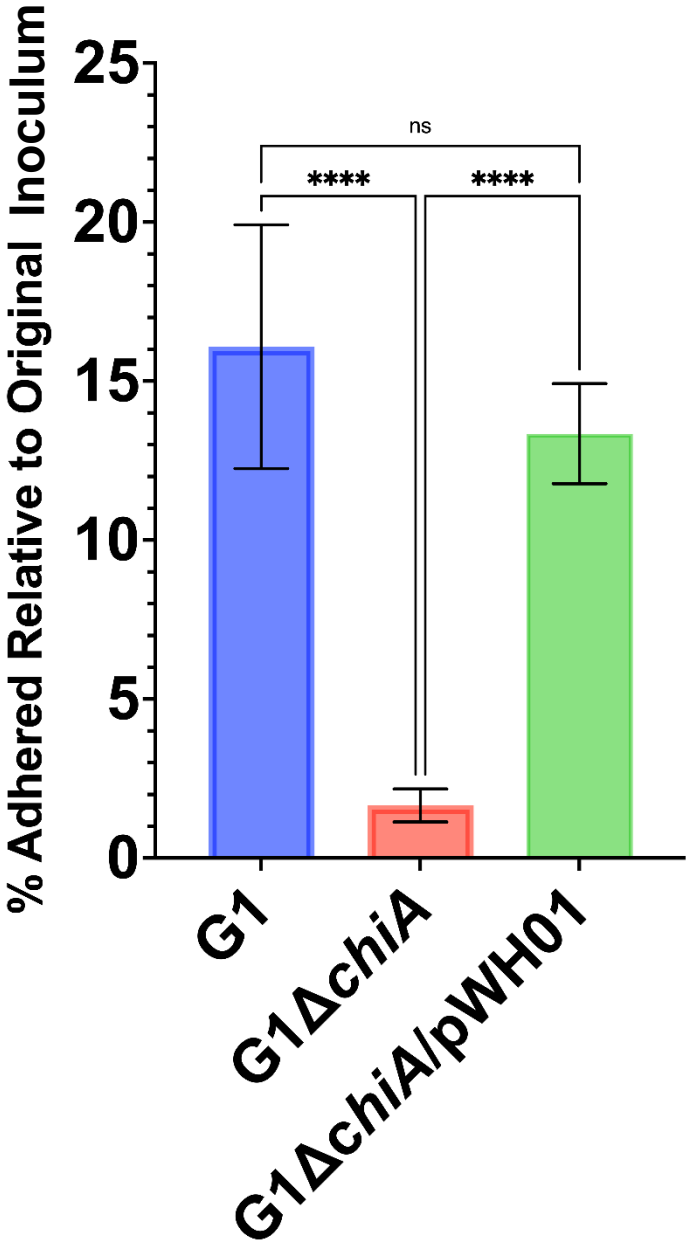
Supplementary Figure 1: Percent of Adhered CFUs Relative to Original Inoculum of M45 to MAC-T cells**.** Percentage of adhered CFUs relative to the original number of CFUs added into MAC-T cells. **p>0.01, ***p=0.001 as determined by One-Way ANOVA with Tukey's correction



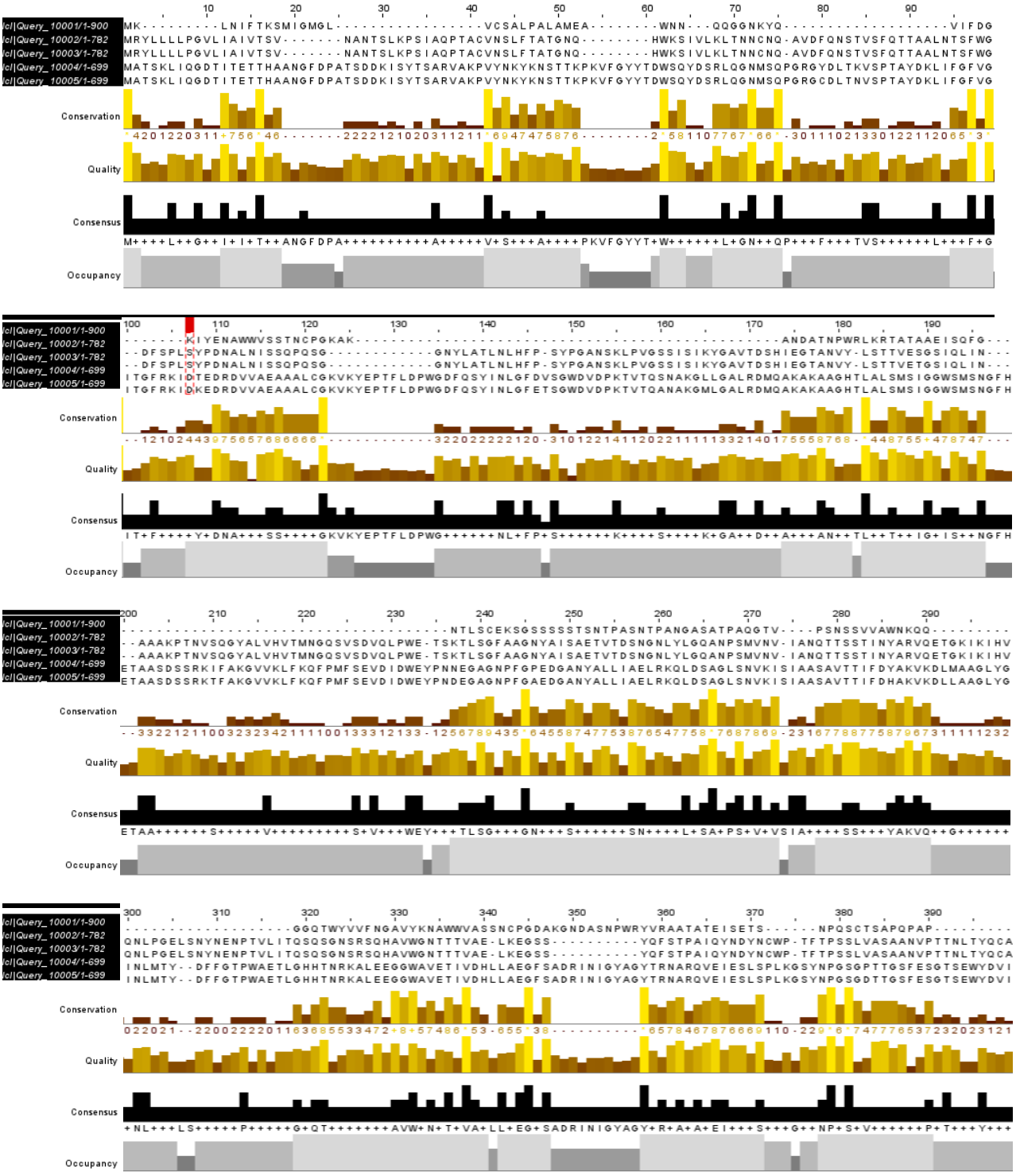
Supplementary Figure 2: Percent of Adhered CFUs Relative to Original Inoculum of M93 to MAC-T cells. Percentage of adhered CFUs relative to the original number of CFUs added into MAC-T cells. ***p=0.001 as determined by One-Way ANOVA with Tukey's correction

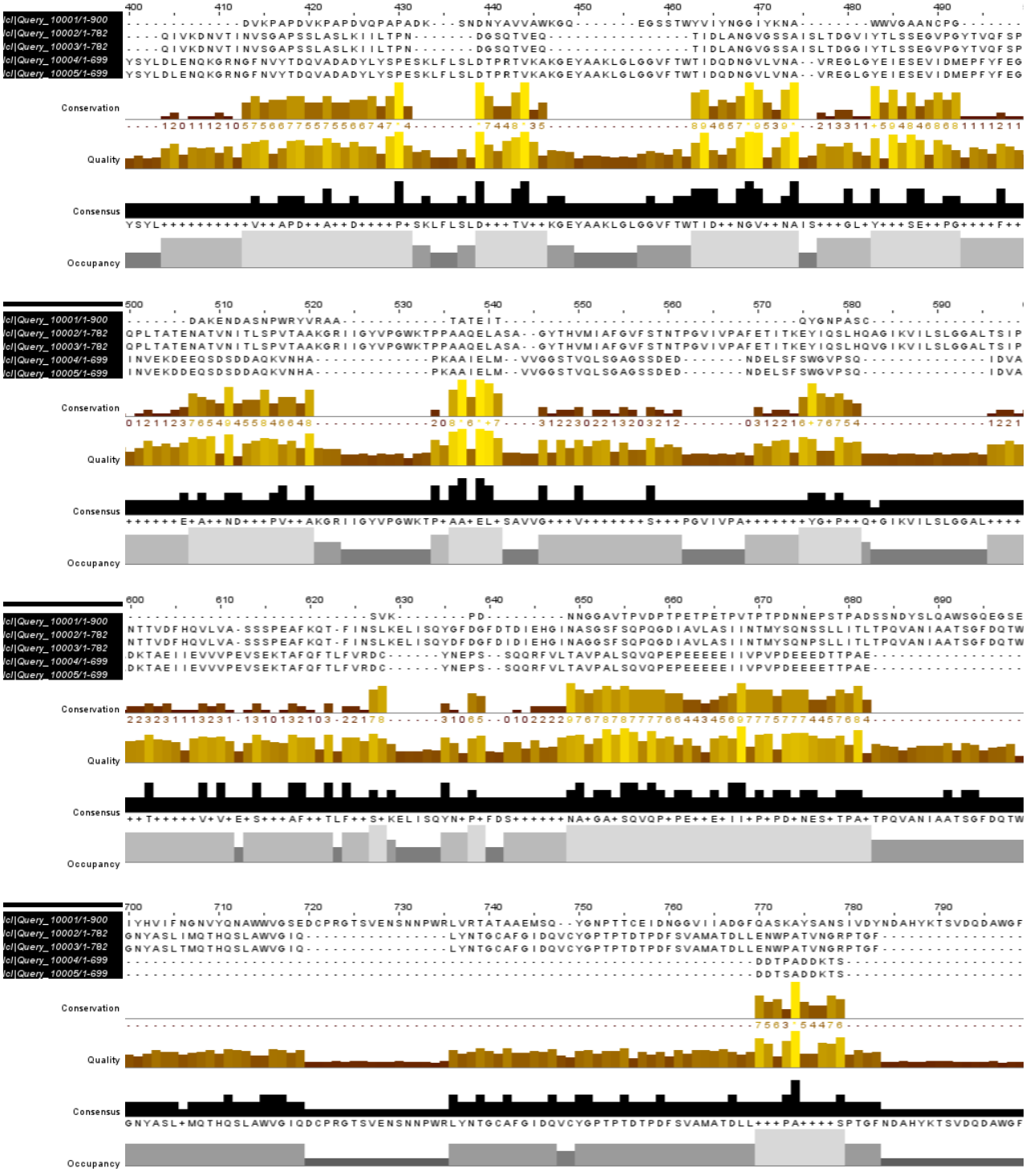


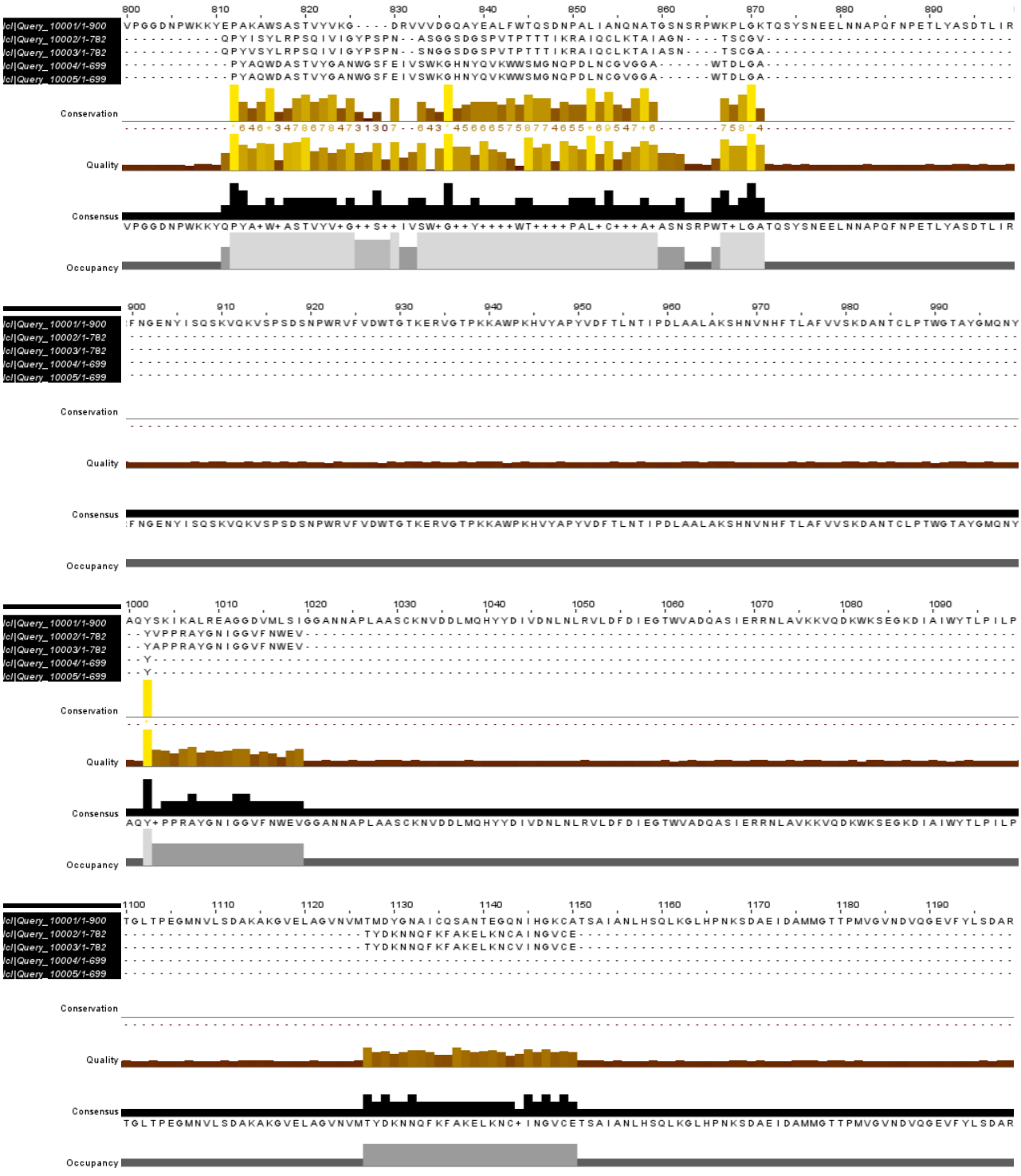
Supplementary Figure 3: Percent of Adhered CFUs Relative to Original Inoculum of M111 to MAC-T cells. Percentage of adhered CFUs relative to the original number of CFUs added into MAC-T cells. *p<0.05 as determined by One-Way ANOVA with Tukey's correction

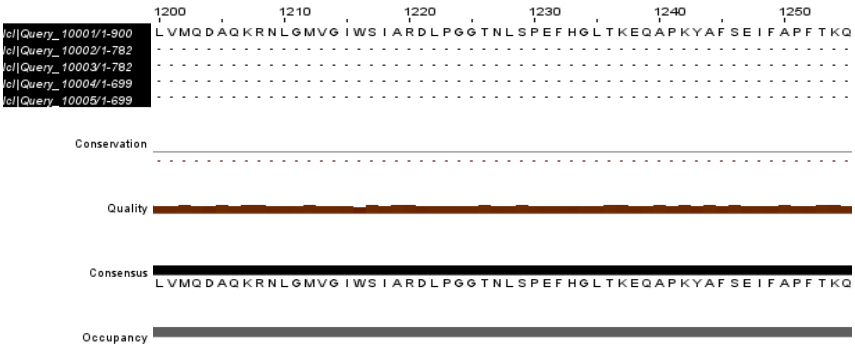


Supplementary Figure 4: Percent of Adhered CFUs Relative to Original Inoculum of M111 to MAC-T cells. Percentage of adhered CFUs relative to the original number of CFUs added into MAC-T cells. ****p<0.001 as determined by One-Way ANOVA with Tukey's correction


lcl|Query_10001/1-900
lcl|Query_10002/1-782
lcl|Query_10003/1-782
lcl|Query_10004/1-699
lcl|Query_10005/1-699
Conservation
Quality
Consensus
Occupancy


Conservation
Quality
Consensus
Occupancy


800 810 820 830 840 850 860 870 880 890
lcl|Query_10001/1-900 VPGGDNPWKKYEPAKAWSASTVYVKG----DRVVVDGQAYEALFWTQSDNPALIANQNATGSNSRPWKPLGKTQSYSNEELNNAPQFNPETLYASDTLIR
lcl|Query_10002/1-782 ----------QPYISYLRPSQIVIGYPSPN--ASGGSDGSPVTPTTTIKRAIQCLKTAIAGN----TSCGV----------
lcl|Query_10003/1-782 ----------QPYVSYLRPSQIVIGYPSPN--SNGGSDGSPVTPTTTIKRAIQCLKTAIASN----TSCGA----------
lcl|Query_10004/1-699 ----------PYAQWDASTVYGANWGSFEIVSWKGHNYQVKWWSMGNQPDLNCGVGGA------WTDLGA----------
lcl|Query_10005/1-699 ----------PYAQWDASTVYGANWGSFEIVSWKGHNYQVKWWSMGNQPDLNCGVGGA------WTDLGA----------
Conservation
Quality
Consensus
VPGGDNPWKKYQPYA+W+ASTVYV+G++S++IVSW+G++Y++++WT++++PAL+C+++A+ASNSRPWT+LGATQSYSNEELNNAPQFNPETLYASDTLIR
Occupancy
900 910 920 930 940 950 960 970 980 990
lcl|Query_10001/1-900 FNGENYISQSKVQKVSPSDSNPWRVFVDWTGTKERVGTPKKAWPKHVYAPYVDFTLNTIPDLAALAKSHNVNHFTLAFVVSKDANTCLPTWGTAYGMQNY
lcl|Query_10002/1-782 ----------
lcl|Query_10003/1-782 ----------
lcl|Query_10004/1-699 ----------
lcl|Query_10005/1-699 ----------
Conservation
Quality
Consensus
FNGENYISQSKVQKVSPSDSNPWRVFVDWTGTKERVGTPKKAWPKHVYAPYVDFTLNTIPDLAALAKSHNVNHFTLAFVVSKDANTCLPTWGTAYGMQNY
Occupancy
1000 1010 1020 1030 1040 1050 1060 1070 1080 1090
lcl|Query_10001/1-900 AQYSKIKALREAGGDVMLSIGGANNAPLAASCKNVDDLMQHYYDIVDNLNLRVLDFDIEGTWVADQASIERRNLAVKKVQDKWKSEGKDIAIWYTLPILP
lcl|Query_10002/1-782 --YVPPRAYGNIGGVFNWEV----------
lcl|Query_10003/1-782 --YAPPRAYGNIGGVFNWEV----------
lcl|Query_10004/1-699 --Y----------
lcl|Query_10005/1-699 --Y----------
Conservation
Quality
Consensus
AQY+PPRAYGNIGGVFNWEVGGANNAPLAASCKNVDDLMQHYYDIVDNLNLRVLDFDIEGTWVADQASIERRNLAVKKVQDKWKSEGKDIAIWYTLPILP
Occupancy
1100 1110 1120 1130 1140 1150 1160 1170 1180 1190
lcl|Query_10001/1-900 TGLTPEGMNVLSDAKAKGVELAGVNVMTMDYGNAICQSANTEGQNIHGKCATSAIANLHSQLKGLHPNKSDAEIDAMMGTTPMVGVNDVQGEVFYLSDAR
lcl|Query_10002/1-782 ----------TYDKNNQFKFAKELKNCAINGVCE----------
lcl|Query_10003/1-782 ----------TYDKNNQFKFAKELKNCVINGVCE----------
lcl|Query_10004/1-699 ----------
lcl|Query_10005/1-699 ----------
Conservation
Quality
Consensus
TGLTPEGMNVLSDAKAKGVELAGVNVMTYDKNNQFKFAKELKNC+INGVCETSAIANLHSQLKGLHPNKSDAEIDAMMGTTPMVGVNDVQGEVFYLSDAR
Occupancy



Supplementary Figure 5: Multisequence Alignment of 5 Unique Chitinase Protein Sequences from *S. typhimurium, S. typhi, L. pneumophilia,* and MAEC strain G1.
Genes encoding for Glycosyl Hydrolase Family 18 proteins were aligned using COBALT. Sequences are from top to bottom: MAEC strain G1, L. pneumophilia strain 130b, L. pneumophilia strain C9_S, S. enterica serovar Typhimurium strain 14028S, and S. enterica serovar Typhi strain CT18.