REGULATION OF PILUS PRODUCTION BY THE PILR/PILS TWO-COMPONENT AND AGR-LIKE QUORUM SENSING SYSTEMS IN *CLOSTRIDIUM PERFRINGENS*

BY

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ABSTRACT

Clostridium perfringens type G isolates cause necrotic enteritis (NE) in poultry. Their pilus plays a role in adhesion and is important in pathogenesis. The NE pilus in these isolates consists of three pilins (CnaA, FimA, FimB) encoded by three genes that belong to the VR-10B allele of the VR-10 locus. There are two genes downstream of the pilin genes encoding a two-component regulatory system (PilR/PilS). However, the regulatory function of PilR/PilS remains to be determined. The current study has investigated the function of PilR/PilS in the regulation of pilin production and binding to collagen by comparison of isogenic *pilR*-null and complemented strains with the parent strain CP1. In addition, the role of the Agr-like QS system in regulating pilin production and binding has been examined by comparing isogenic *agrB*-null and *virR*-null mutants and their respective complemented strains with CP1. Western blot analyses showed no detectable pilus production in mutant *pilR*, while production by its complemented strain was similar to wild-type levels. In contrast, pilus production was the same or higher in the *agrB* and *virR* mutants but reduced in their respective complements compared with CP1. Similarly, no pilus production was detected in the pilin mutant strains (*cnaA*, *fimA*, or *fimB*). These observations were supported by the binding results to collagen of mutants *pilR*, *agrB*, and *virR* and their complemented strains, with CP1 as a reference. The *pilR* mutant showed significantly less binding than CP1 ($P \leq 0.05$) to most collagen types, but its complement was similar to the parent strain in binding ($P > 0.05$). In contrast, binding of *agrB* and *virR* mutants to collagen (types I – V) showed no significant changes compared with CP1 ($P > 0.05$), whereas their complemented strains had significantly reduced binding to collagen types I – IV ($P \leq 0.05$). To confirm that the collagen binding activity of the *agrB* mutant was specifically mediated by the pilus, and not due to the expression of other

factors regulated by the Agr-like QS system, binding of CP1 and mutant *agrB* to type I and IV collagen was examined in the presence of anti-CnaA, FimA, or FimB antibodies. Antibodies against both CnaA and FimA blocked the binding of CP1 and the mutant in a dose-dependent manner. However, the antibody against FimB showed no blocking of binding. These data suggest that the PilR/PilS two-component system positively regulates pilin production, while the Agr-like QS system may negatively regulate pilin production in *C. perfringens*.

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DEDICATION

This thesis is dedicated to my parents, for their love, support and encouragement throughout my life.

FOREWORD

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LIST OF ABBREVIATIONS

Agr	Accessory gene-like regulator
AGP	Antimicrobial growth promoters
ASN	Asparagine
ASP	Aspartic Acid
CWSS	C-terminal cell wall sorting signal
Ig	Immunoglobulin
Lys	Lysine
PSA	Poultry Science Association
NE	Necrotic Enteritis
NetB	Necrotic enteritis B-like
Gly	Glycine
QS	Quorum Sensing
TCS	Two-Component Regulatory System
Thr	Threonine

TEM	Transmission electron microscopy
T4P	Type IV pili
TG	Triple glycine
VWA	von Willebrand A

CHAPTER 1 GENERAL INTRODUCTION

NE is an important poultry disease caused by *Clostridium perfringens* type G isolates. The pathogenesis of NE is complex and not fully understood but involves the pore-forming toxin NetB along with other virulence factors, including pili (Prescott et al., 2016; Lepp et al., 2021; Zhou et al., 2021).

The VR-10B (CA) locus plays an important role in the colonization of *C. perfringens* in NE development (Wade et al., 2016). Also, it has been reported previously that *netB* and pilus-encoding genes are regulated by the Agr-like quorum sensing (QS) system including the VirR/VirS two-component regulatory system (Yu et al., 2017). The function of the PilR/PilS two-component system was not known until this thesis research was completed. In addition, it was not known if this system works independently or cooperatively with the Agr-like QS system, which was also investigated in this thesis.

Therefore, the objective of the present thesis was to investigate the function of the PilR/PilS two-component system, in addition to the Agr-like QS system, in regulating the NE pilus production.

CHAPTER 2 LITERATURE REVIEW

2.1 *CLOSTRIDIUM PERFRINGENS* AND NECROTIC ENTERITIS IN POULTRY

Necrotic enteritis is an important disease in the poultry industry, resulting in extensive economical losses to the industry; an estimated $2-6 billion USD/year worldwide (Khalique et al., 2020) was reported for NE in poultry. This disease is associated with sudden death; damage to the intestinal mucosa resulting in severe necrosis; decreased digestion and absorption of nutrients and consequently reduced weight gain and decreased overall performance.

C. perfringens type G isolates cause NE in poultry, presenting a major challenge for poultry production in the post-antibiotic era. Multiple factors in *C. perfringens*, including both virulent and non-virulent, are involved in the development of the disease (Emami & Dalloul, 2021). *C. perfringens* is a diverse group of bacteria and classified into seven toxin types (Types A to G) based upon the variable production of six toxins, α-toxin (CPA), β-toxin (CPB), ε-toxin (ETX) and ι-toxin, enterotoxin (CPE) and Necrotic enteritis B-like (NetB) (Rood et al., 2018). Most of the success in colonization of this Gram-positive anaerobic bacteria are also due to the capacity of this bacteria to produce spores and bacteriocins, such as perfrin (Timbermont et al., 2014). This bacterium is commonly found in soil, sewage, litter, feces of chickens and intestines of humans and animals (Prescott et al., 2016). It is of great zoonotic importance, as it is one of the most common causes of foodborne disease in humans (Cooper & Songer, 2010). It is also an important threat to animals such as poultry and swine. For example, outbreaks of NE in poultry frequently occur between 2 and 6 weeks of age, resulting in the sudden death of the birds with mortality rates up to 50% (Lee et al., 2011). Several predisposing factors have been reported, the presence of

coccidiosis seems to be one of the most important predisposing factors (Moore, 2016; Prescott et al., 2016). Other factors such as high viscosity of digesta; GIT transit time, intestinal pH and the presence of non-digestible polysaccharides in the diet are other important predisposing factors for NE. Any factor that induces stress in chickens and disrupts the balance of intestinal microbiota and/or suppresses the immune system are triggers of NE in poultry.

In the past decades, the prophylaxis of NE has depended on the use of antibiotics at sub-therapeutic levels, which were used as antimicrobial growth promoters (AGP) to prevent birds from NE and improve their growth performance. However, the extensive use of AGPs resulted in bacteria with antibiotic resistance (e.g., *S. pneumonia*) and meat products with antibiotic residues for human consumption. These negative effects have led to the ban on the use of antibiotics as feed additives by the European Union (2006) and the Government of Canada (2018). Following this ban, a new era of research has been devoted to the development of antibiotic alternatives against *C. perfringens*. These include phage therapy (bacteriophage, lytic enzymes), probiotics, prebiotics, bacteriocins and vaccination. The reviews by Caly et al. (2015) and Khalique et al. (2020) described the advantages and developments of each of those antibiotic alternatives. A question remains though, which antibiotic alternative would be viable to use for controlling NE? The answer to this question is production system-specific and farmers choice should be based on the severity of the condition, the farm status and the individual farm management plan. According to Khalique et al. (2020), probiotics/prebiotics are the most studied and when combined are symbiotic and cost-effective. Eventually, a combination of antibiotic alternatives may be the best solution for the future of poultry production. More studies on the synergetic/additive effects and cost-effectiveness of single and/or combination of alternatives against NE are needed.

Controlling NE by any of the above antibiotic alternatives requires a clear understanding of the pathogenesis of NE. This is a complex and not fully understood topic but involves the pore-forming toxin NetB along with other virulent factors, including pili (Prescott et al., 2016; Lepp et al., 2021). Pili and in particular sortase dependent pili are important for the successful adherence, colonization and survival of Gram-positive bacteria, including *C. perfringens* pili and these surface proteins are the focus of this literature review.

2.2 PILI

Pili are hair-like appendages found on the surface of many bacteria, which are associated with bacterial adhesion and related to bacterial colonization and infection. Pili are primarily composed of protein subunits, called pilins, which are typically arranged in a helical fashion. There are different types of pili and different mechanisms of formation and roles in Gram-negative and Gram-positive bacteria. It has been well studied that pili in Gram-negative bacteria are formed by non-covalent associations of pilin subunits, and four types of pili (types I, IV and V pili, and curli pili) have been reported (Telford et al., 2006; Xu et al., 2016). In contrast, the pili of most Gram-positive bacteria are formed by covalent attachment of protein pilins to each other and to the peptidoglycan cell wall (Ton-That & Schneewind, 2004). Nevertheless, not all pili of Gram-positive bacteria show these covalent attachements. For example, Type IV pili which are found in some Gram-positive species including *C. perfringens* are dynamic filaments that are rather flexible structures that are rapidly polymerized and depolymerized from a pool of pilin subunits. This cycle of pilus extension, binding and retraction enable type IV pili to present twitching motility as well as other functions such as DNA uptake and microcolony formation (Graig et al., 2019). In Gram-

positive bacteria, the covalent associations between pilins to form the pilus and the attachment of surface proteins to cell walls are catalyzed by enzymes called sortases. Sortases are cell surface-associated or anchored cysteine transpeptidases, which are classified into six classes, A–F, based on their amino-acid sequences and biological roles (Bradshaw et al., 2015). Since the discovery of sortases, our knowledge of bacterial sortase-dependent pilus biology has progressed rapidly. In this review, we focus on pilus biogenesis and assembly in Gram-positive bacteria mediated by sortases, regulation of pilus expression and research progress of pili in *C. perfringens*.

2.3 TYPES OF PILI AND THEIR FUNCTIONS

Five types of pili have been identified in bacteria (Type I, Type IV, Curli, Type V and Sortase-dependent) and different types have distinct roles. Duguid and Campbell (1969) demonstrated that the antigen of type I pili was involved in serological agglutination in family *Salmonellae* and other *Enterobacteria*. Martinez et al. (2000) reported that an adhesin of type I pilus, FimH, plays a vital role in not only bacterial adherence but also the invasion of human bladder epithelial cells. Spaulding et al. (2018) found that the type I pilus rod is essential for its functional role in mediating *Escherichia coli* pathogenesis and FimA subunit has an important functional role in promoting colonization in the gut and infection of the bladder. Type IV pili (T4P) are widely distributed in Gram-negative and Gram-positive bacteria and have various functional roles, such as adherence, motility, and biofilm formation. Type IV pili have been widely reported to be involved in attachment to the host-cell epithelium. Freitag et al. (1995) found that the expression of type IV pili by *Neisseria gonorrhoeae* plays an essential role in the adherence to the intestinal epithelium and its persistence in the host intestine. Recent studies have shown that type IV pili are also

implicated in the motility of some bacteria, including *C. difficile* (Purcell et al., 2016) and *C. perfringens* (Varga et al., 2006). It is a type of twitching motility, which powers bacteria via the extension and retraction of the pili, as reviewed by Gordon and Wang (2019). A biofilm is an assemblage of surface-associated bacterial cells that are enclosed in an extracellular matrix (Rodrigues et al., 2019). The formation of biofilms is an important virulence factor for a number of pathogenic bacteria. Purcell et al. (2016) have reported that type IV pili are involved in biofilm formation in *C. difficile*. Curli pili are extracellular proteinaceous fibers produced by Gram-negative bacteria. Curli fibers were first discovered by Olsén et al. (1989) on *Escherichia coli* strains that caused bovine mastitis and have since been implicated in the pathogenesis of *Enterobacteriaceae*. Specifically, curli fibers are involved in adhesion to surfaces and biofilm formation (Kikuchi et al., 2005), as they serve as a major matrix component in biofilm (Hung et al., 2013). Curli pili can also mediate host cell adhesion and invasion (Tükel et al., 2009; Van Gerven et al., 2018), which suggests an important role for curli in pathogenesis. Type V pili represent a new pilus type discovered in Bacteroidia in 2016 (Xu et al., 2016). Type V fibers consist of an anchor, a stalk, an adapter and a tip pilin (Hospenthal et al., 2017). Their structure was also revealed in the bacterium *Porphromonas gingivalis*, which are composed of polymerized FimA pilin stalk subunits (Shibata et al., 2020). Their findings have revealed not only a new pilus type, but also a new mechanism of pilus formation, which will pave the way for studying the function of type V pili. Another type of pilus, present in Gram-positive bacteria are the sortase-dependent pili. *Staphylococcus aureus* sortase A (sau-SrtA), which was first identified from a mutant strain defective in cell-surface protein anchoring, is one of the best studied sortases and sortase-dependent pili (Mazmanian et al., 1999). In common with *S. aureus* and many other pathogenic Gram-positive bacteria, *C. perfringens* displays and anchors a diverse array of this

sortase-dependent pili on its cell wall with virulence functions (Khare et al., 2017). These pili are mainly involved in adherence of bacteria to the host cells or biofilm formation (adherence among bacteria) (Khare et al., 2011). Most Gram-positive bacterial species contain multiple sortase enzymes (Pallen et al., 2001). In general, at least two types of sortases are usually involved in the sortase pilus biphasic biogenesis (Khare and Narayana, 2017) in which pilus assembly by a pilus sortase is followed by pilus anchoring on the cell wall by a housekeeping sortase, as further discussed in the next section.

2.4 SORTASE-DEPENDENT PILI

2.4.1 Pilins – Basal pilins, tip pilins and backbone pilins

Bacterial pili (or fimbriae) are long, hair-like structures that protrude from the surface of many bacterial species. Despite the fact that various types of bacterial pili exist, certain features are common to all or most (Khare B and VL Narayana S, 2017; Krishnan, 2015). Firstly, the main pilus structure (sometimes known as the rod or shaft) is a polymer comprising up to a thousand or more protein subunits, known as pilins (Proft & Baker, 2009). Secondly, pili generally mediate adhesion, both intra-bacterially (during biofilm formation) and between bacterial and eukaryotic cells (during host colonization) (Proft & Baker, 2009). One characteristic unique to the pili of Gram-positive bacteria is that the pilin subunits are covalently bonded to one another (Kang & Baker, 2012). Pili in many Gram-positive pathogens consist of covalent polymers in which sortase-mediated isopeptide bonds link successive pilin subunits.

The pilins are modular proteins with frequent variant immunoglobulin folds of the CnaA and CnaB types and occasionally von Willebrand A (VWA)-like and thioester domains (Vengadesan

et al., 2011). The basal pilins are often made up of one to three CnaB like domains with or without internal isopeptide bonds. The basal pilins differ from tip and shaft pilins in their C-terminal sorting motif, often having a triple glycine (TG) motif (Krishnan, 2015).

Tip pilins are the largest in size and are made up of CnaA and CnaB domains and often with an extra domain specialized for adhesion. The CnaA/CnaB domains support the adhesive domains, connecting to the pilus shaft like a stalk and facilitating host-interaction (Krishnan, 2015).

The backbone pilins are generally made of two to four Ig-like CnaB/or CnaA domains often with an internal isopeptide bond. There is also often a lysine residue at the C-terminal end of the N-terminal domain which is involved in pilus polymerization as part of a pilin motif (YPKN) (Krishnan, 2015).

The pilin subunit proteins show wide variations in size and sequence (Kang et al., 2007). Also, the location of minor pilins on the pilus backbone has been a subject of debate; however, in most cases the minor pilins are located exclusively at the tip or base of linear, unbranched polymers (Kang et al., 2007; Nielsen et al., 2012; Khare and VL Narayana S, 2017).

2.4.2 Pilus sortases

Most Gram-positive bacteria encode multiple sortase enzymes with diverse sorting signal specificities (Khare B & VL Narayana S, 2017). According to Bradshaw et al. (2015), a scheme of six sortase classes (A-F) exist and each class recognizes a distinct sorting motif and performs mostly distinct functions. All sortases exhibit specificity towards proteins with a C-terminal cell wall sorting signal (CWSS), which consists of an LPXTG motif, or variant thereof, followed by a hydrophobic domain and short tail of positively charged residues (Boekhorst et al., 2005; Mazmanian et al., 2001). The housekeeping sortase (srtA) is a class A sortase, and is localized and

regulated independently of its LPXTG motif-containing substrates (Ton-That & Schneewind, 2004). The canonical class B enzyme of *S. aureus* recognizes a distinct NPQTN motif and srtB is expressed and regulated within the iron-regulated surface determinant (isd) locus (Kreikemeyer et al., 2011). This locus facilitates the acquisition of heme as a source of nutrient iron during infection allowing bacteria such as *S. aureus* to overcome iron restrictions during host colonization and invasive disease (Kreikemeyer et al 2011). The Cpe-SrtD sortase from *C. perfringens* and its LPQTG motif-containing substrate are clustered, meaning co-localized (its respective genes are within a few thousand base pairs of each other forming a group or gene cluster) and its cell wall-anchoring preference is not known (Suryadinata et al., 2015). Like class A, the class E sortases are not co-localized with their substrates and recognize another non-canonical LAXTG motif. Class C sortases and a few class B sortases are pilus-sortases and therefore involved in pilus assembly (Spirig et al., 2011). These pilus-sortases are enzymes primarily involved in the polymerization and incorporation of individual pilins, and may also perform secondary functions, such as anchoring pilins (Khare B & VL Narayana S, 2017). The genes that encode these enzymes and their substrates lie within a single cluster. When linking two pilins, the sortase must recognize the C-terminal sorting motif of one molecule and the appropriate lysine on the other. The covalent attachment of pili to the cell wall peptidoglycan is typically catalysed by the housekeeping sortase (Type A) (Mazmanian et al., 1999).

2.4.3 Pilus assembly

Pili are multiprotein assemblies. Pilus biogenesis in Gram-positive bacteria is a biphasic process that requires one or more sortases for assembly and a housekeeping sortase for covalent

attachment of the assembled pilus to the peptidoglycan cell wall (Kang & Baker, 2012). Based on the structural work from Ton-That and Schneewind (2004) with pili from *Corynebacterium diphtheriae*, a general model for assembly of Gram-positive pili was established. According to these authors, Gram-positive pili are built from multiple copies of a single major, or backbone, pilin to form the shaft together with one or two minor pilins, an adhesive minor pilin at the pilus tip and often a second minor pilin at the base. As the pilin subunits emerge from the cell membrane, they are linked as beads on a string by a pilus-specific sortase, which joins the C-terminus of each subunit to a lysine residue on the next by means of a covalent isopeptide (amide bond) (Ton-That & Schneewind, 2004). Moreover, a general housekeeping sortase ligates the pilus base to an amino group of the cell wall peptidoglycan, covalently anchoring the assembly. Similar assembly principles have been reported for other Gram-positive bacteria (Kang and Baker, 2012; Nielsen et al., 2012). In all cases, pilus assembly takes place on the cell membrane following Sec-dependent secretion of the components. The pilins are initially anchored to the membrane by C-terminal hydrophobic regions and subsequently incorporated into the growing pilus by a membrane-anchored cysteine transpeptidase, typically a Type C sortase (Mazmanian et al., 1999). These sortases recognize a specific sorting motif (LPXTG or other) located near the C-terminus of each pilin. Sortase cleavage between Thr and Gly is followed by ligation of the new C-terminus to the epsilon amino groups of a specific lysine on the next pilin subunit (Kang & Baker, 2012). Evidence for the pilin motif lysines in the pilins has been reported for major pilins BcpA from *Bacillus cereu*s (Hendrickx et al., 2012) and SpaA from *C. diphtheriae* (Kang et al., 2009). In *C. diphteriae*, fiber incorporation of the minor tip-associated pilus subunit SpaC required the lysine of the YPKN pilin motif in the major subunit, while the incorporation of SpaB minor subunit uses instead a glutamic acid within the enhancer box (E-box) conserved element in the major SpaA subunit (Ton-

That & Schneewind, 2004). This E-box is a DNA response element that acts as a protein binding site and has been found to regulate gene expression. Its specific DNA sequence is CANNTG (where N can be any nucleotide) and it is recognized and bound by transcription factors to initiate gene transcription (Massari and Murre, 2000).

Structural studies of the component pilins reviewed by Kang and Baker (2012) also revealed a common pattern of tandem immunoglobulin (Ig)- like domains, joined end-to-end. This assembly is further stabilized by autocatalytically generated isopeptide bond crosslinks within the domains, joining the Lys and Asn (or Asp) residues of side chains. Specialized subunits at the tip and base complete the assembly (Kang et al., 2007). Basal pilins differ from the major pilins in that their C-terminal sorting motif links to the cell wall peptidoglycan and the ligation of a basal pilin at the foot of the shaft terminates pilus growth (Mandlik et al., 2008). In major pilins containing YPKN pilin motifs, the N-domains are typically flexible and labile as in BcpA, RrgB and FimA and include the pilin motif at the C-terminal end of their last beta-strand (Krishnan, 2015). The lysine of this motif forms the sortase-mediated linkage with the preceding subunit, while the adjacent Asp has the potential to form an internal isopeptide bond within the N-domain as in other CnaB domains.

It is important to recognize that many cell wall-anchored proteins like the pili in Gram-positive bacteria are synthesized as precursor proteins equipped with two signal motifs: a) an N-terminal signal peptide for Sec-dependent secretion (Marrafini et al., 2006) and b) a C-terminal cell wall sorting signal (CWSS). The CWSS consists of a five-residue sorting motif, LPXTG, that precedes a hydrophobic and positively charged terminus. After translocation, the substrate protein is retained in the membrane via the hydrophobic and charged tail of the CWSS. A sortase enzyme recognizes the sorting motif and cleaves between the Thr and Gly residues to form an acyl enzyme

intermediate. This intermediate is then resolved through a nucleophilic attack by the amine group of the pentaglycine from the peptidoglycan precursor and releases the enzyme (Khare and Narayana, 2017). After the sortase enzyme is released, the substrate product covalently linked to the peptidoglycan precursor is incorporated into the cell wall through transglycosylation and transpeptidation reactions (Khare B & VL Narayana S, 2017).This peptidoglycan precursor is lipid II, which is a precursor molecule in the synthesis of the cell wall of bacteria. It is amphipathic and acts as a lipid anchor, embedding itself in the bacterial cell membrane (Heijenoort, 2007). Lipid II must translocate across the cell membrane to deliver and incorporate its disaccharide-pentapeptide into the peptidoglycan mesh. This lipid II is the target of several antibiotics (de Kruijff et al., 2008).

2.5 REGULATION OF PILI EXPRESSION

Pilus expression and assembly are complex processes that are often well-regulated by factors that include transcriptional regulators encoded within clusters, heterogeneous expression, two-component regulatory systems, quorum sensing systems and environmental factors, such as pH, temperature and oxygen availability (Kreikemeyer et al., 2011; Lepp et al., 2013, 2021); these mechanisms are the subject of ongoing research. Quorum sensing system is found in several Gram-positive pathogens and uses an autoinducing peptide (AIP) to signal a classical TCS (Jenul and Horswill, 2019). This system is well studied in bacteria of Staphylococci where the *agr* operon contains 4 genes including one encoding AgrD, which is the precursor for the signaling peptide AIP and another encoding AgrB which is an integral membrane endopeptidase, two encoding AgrA and AgrC, which constitute a classical TCS module (Novick & Geisinger, 2008). After the production of the AgrD precursor peptide in the cytoplasm, AgrB is thought to process this peptide to an active AIP which is then released extracellularly and acts on and induces phosphorylation of

the AgrC. The phosphate is transferred to AgrA, which activates transcription from the two *agr* promoters to subsequently modify gene expression (Novick & Geisinger, 2008; Yu et al., 2017). In *C. perfringens* as opposed to the *agr* operon in *S. aureus* that encodes AgrA/AgrC TCRS, the *agr* operon does not encode an AgrA/AgrC homolog; instead the VirS/VirR TCS is involved where the VirS membrane sensor protein is an AIP receptor for the Agr-like QS system (Gohari et al., 2021). Although regulation of pilus production in some strains of *C. perfringens* has been associated with the Agr-like quorum-sensing (QS) system (e.g., Yu et al., 2017), the clear involvement of this system on NE pilus production regulation was still not available at the time of this thesis research. This regulatory system has been shown to control NetB production, but its effect on NE pilus production was not known. The Agr-like system is an auto-regulatory system controlling gene expression in response to increasing cell density; details on the mechanism used by this system has been provided by Li and McClane (2020). Li and McClane (2020) revealed that the VirR/S TCS, which consists of the membrane sensor histidine kinase VirS and the response regulator VirR (Rood, 1998), recognizes the auto-inducing peptide (AIP) of the Agr system and subsequently modulates gene expression. There is some evidence to suggest VirS/R is involved in regulation of the NE pilus (Yu, 2017), but another TCS system located within the pilus operon, called PilRS, may also be involved. The function of the PilRS system is not known, and will be investigated on the present thesis. *C. perfringens* uses QS systems to regulate production of a number of virulence factors. The Agr-like system is the most important QS system of this bacterium for virulence.

Several Gram-positive pilus islets have been found to encode regulatory proteins that modulate their expression, though no TCSs have thus far been identified. For example, the *S. pneumoniae* PI-1 pilus islet encodes the RlrA regulator, which controls pilus production through a positive

feedback loop, resulting in a biphasic expression pattern that produces populations expressing high or low numbers of pili (Nelson et al., 2007). The PI-1 pilus was found to be preferentially expressed during early colonization stages, where adhesion is important, and reduced during later stages, presumably to evade the immune response induced against the pilus antigen (Nelson et al., 2007). It is possible that, similar to the biphasic expression of the PI-1 pilus, NE pilus production is down-regulated during later stages of infection, when it is no longer required and to evade the induced immune response, but this needs to be investigated. Another biphasic pili expression pattern was reported for the *S. pyogenes*. In this case, the FCT pilus-encoding region in this bacterial genome carries genes for two regulatory proteins, Nra/RofA and MsrR, which have contradictory effects on pilus expression, resulting in biphasic expression. In this case, the pilus is expressed at lower temperatures, corresponding to the environment of superficial skin infections, to mediate binding to keratinocytes (Abbot et al., 2007). In the case of *C. difficile*, the control of Type IV pilus expression was also not TCS associated, but rather regulated by cyclic-di-GMP (Couchman, 2016); however, the mechanism of regulation is not clearly understood.

2.6 PILI IN *C. PERFRINGENS*

2.6.1 Pilus structure (gene cluster and three pilins CnaA, FimA and FimB)

C. perfringens has been shown to produce functional Type IV pili (Varga et al., 2006). According to these authors, *C. perfringens* carries two type IV gene clusters in its genome: one cluster appears to contain all the genes necessary for type IV pilus assembly; the second cluster encodes pilB, pilC, a pilin and an unknown gene. A pilT gene is present in a third locus elsewhere on the genome (Melville & Craig, 2013; Varga et al., 2006). This type IV pilus allows *C.*

perfringens to exhibit gliding motility and it is also believed to contribute to other potentially virulence-related functions like biofilm formation and adherence (Melville & Craig, 2013). Recently, *C. perfringens* has also been shown to produce a sortase-dependent pilus, the NE pilus (Lepp et al., 2021) involved in cell adhesion, and in particular, collagen adhesion. The proposed structure and assembly are represented in Fig. 2.1 (modified from Lepp et al., 2021).

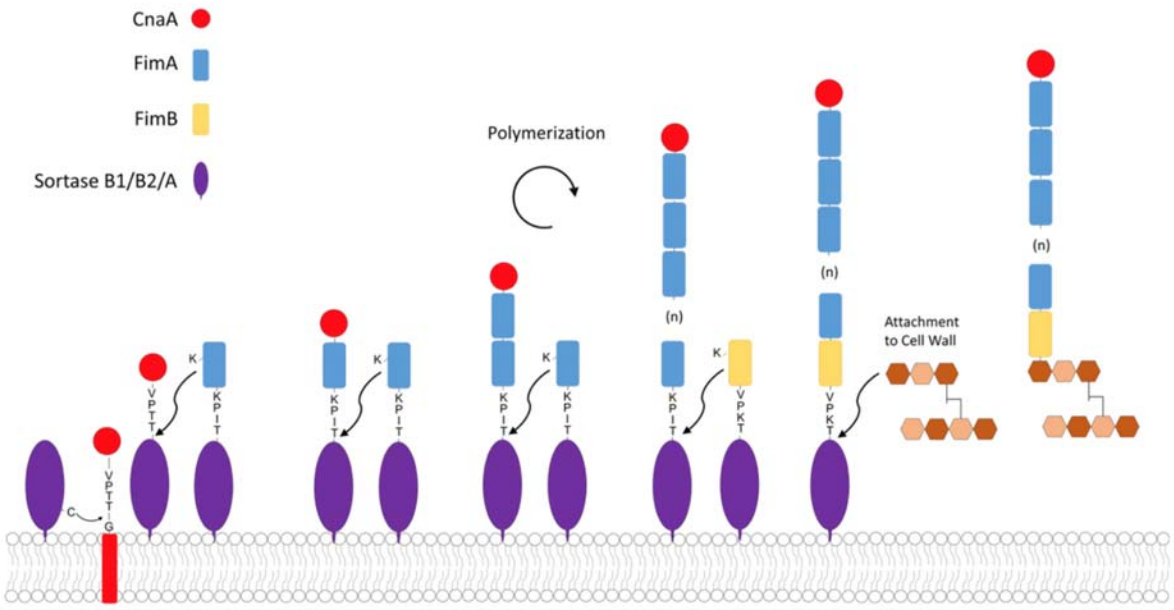

Figure 2.1. Diagram of proposed NE pilus assembly mechanism. Assembly is initiated by transfer of the CnaA tip pilin onto the sortase B1 or B2 enzyme and subsequent polymerization of FimA to form the pilus backbone. Fim B is finally linked to FimA by sortase B1, B2 or the housekeeping sortase A and complete structure is transferred onto the peptidoglycan layer. Adapted from Lepp et al (2021).

It is not clear if the anchoring of the pilus polymer to the peptidoglycan is done by a sortase type B (involved in iron uptake) or by the typical housekeeping sortase type A, as observed in

other Gram-positive bacteria. Investigation of the type of sortase used to link the NE pilus to the cell membrane and a more detailed investigation of NE pilus gene expression will aid in the development of antibiotic-independent measures to prevent NE.

2.6.2 Pilus function and their role in disease (colonization and adherence)

Both type IV pili and the NE pilus are involved in adhesion. While type IV pili seem to be involved in the adhesion of bacteria to bacteria, and therefore biofilm formation, the NE pilus is rather involved in the adherence of the bacteria to the host eukaryotic cells, in particular adherence to the host collagen (Lepp et al 2021).

Through visualization with TEM immuno-gold labelling, Lepp and colleagues (2021) were able to demonstrate that the VR-10B locus encodes the NE-pilus, which is a sortase-dependent pilus that mediates collagen-binding and is essential for NE pathogenesis (Lepp et al., 2013, 2021) . The PilRS TCS is located directly downstream from the pilus structural genes, implicating its potential in the regulation of this operon. The VR-10B locus is predicted to encode three pilin subunits (CnaA, FimA and FimB), a signal peptidase and a class B sortase (Fig. 2.1) which are expressed together in a single polycistronic transcript (Lepp et al., 2013; Wade et al., 2016). Based on the sequence similarity of the VR-10B locus operon with pilus loci from streptococcal species (Fig. 2.2) (Lepp et al., 2013), Lepp et al (2021) hypothesized that FimA and or FimB are assembled into the pilus shaft while CnaA which possesses a Cna B-type collagen binding domain, serves as the tip adhesin (Fig. 2.1). To address the hypothesis that VR-10B encodes a functional pilus, Lepp et al. (2021) used null-mutant strains for each of the pilin genes (*cnaA*, *fimA* and *fimB*) from CP1 parent strain. Immunoblotting of cell surface proteins extracted from CP1 and the isogenic mutant

strains revealed a high-molecular weight pattern indicative of a polymeric pilus that was present in CP1, but absent from the mutant strains. Each band in the ladder is consistent with an assembled pilus composed of a single subunit each of FimB and CnaA and multiple subunits of FimA. Based on the presence of a collagen-binding domain, these authors predicted that CnaA was the tip adhesin while FimA and/or FimB may serve as the base or backbone pilins. Increased intensity of the anti-FimA immunoblots in proportion to band size, suggests that multiple copies of FimA accumulate in the pilus multimer as it is assembled.

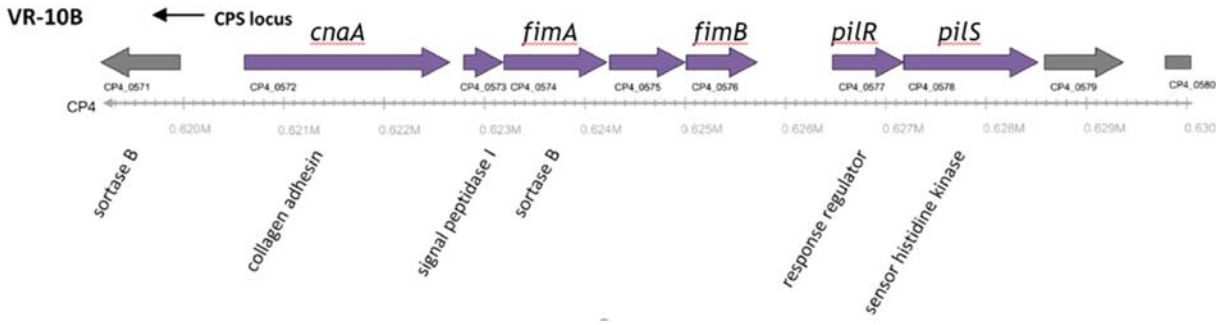

Figure 2.2. Schematic of the VR-10B locus. Adapted from Lepp et al. (2013).

Adherence of *C. perfringens* strains to extracellular matrix proteins is important in NE pathogenesis and is correlated strongly with their virulence (Prescott et al., 2016; Wade et al., 2016). It is not clear if adhesion to collagen is collagen type dependent. The presence of CnaA was found to be critical for binding of *C. perfringens* strains to collagen types IV and V and gelatin (Wade et al., 2015, 2016). Further investigations on the collagen specificity of the CP1 pilus and other strains are required to clarify if collagen adhesion is collagen-type dependent.

2.7 PILI AS VACCINE CANDIDATES

Due to their location on the bacterial cell surface, and frequent role in virulence, pili are often targeted by the host immune system. These immunogenic properties make them attractive as vaccine candidates (Rood, 2015). Pili often play a key role in adhesion, which is critical for bacterial colonization and infection. Because of this key role in virulence, targeting pilus-mediated adhesion (anti-adhesion therapy) is also seen as an alternative approach for preventing and treating bacterial infections. Wade et al. (2016) found that *cnaA*-null mutants have a reduced capacity to colonize *C. perfringens* during NE development. Furthermore, immunization of chickens with recombinant pilins CnaA and FimB partially protected against NE (Lepp et al., 2019), indicating the important role played by this pilus in NE pathogenesis. Blocking the binding of *C. perfringens* to collagen by antibodies against pilin proteins could potentially be beneficial against NE development. In fact, it was reported recently that binding of the wild-type CP1 to collagen types I and IV can be specifically blocked by rabbit antisera against pilin CnaA and FimA in a dose-dependent manner (Lepp et al., 2021), however this approach would not be feasible in practice. Nevertheless, the fact that the NE pilus is covalently linked to the bacterial cell surface, makes it susceptible to targeting by antibodies and host immune cells. Furthermore, knowing the factors that trigger evasion of these bacteria to an immune response, and whether the NE pilus is involved in this evasion, needs to be further investigated.

CHAPTER 3 HYPOTHESIS AND OBJECTIVES

3.1 HYPOTHESIS

The PilR/PilS genes located immediately downstream of the NE pilus structural genes will be involved in the regulation of NE pilus expression.

3.2 OBJECTIVES

The objective of the study was to investigate the function of the PilR/PilS TCS, in addition to the Agr-like QS system including VirSR TCS in the regulation of NE pilus expression.

CHAPTER 4 MANUSCRIPT

A NOVEL PILR/PILS TWO-COMPONENT SYSTEM REGULATES NECROTIC ENTERITIS PILUS PRODUCTION IN *CLOSTRIDIUM PERFRINGENS*

4.1 ABSTRACT

Clostridium perfringens causes necrotic enteritis (NE) in poultry. A chromosomal locus (VR-10B) was previously identified in NE-causing *C. perfringens* strains that encodes an adhesive pilus (NE pilus), along with a two-component system (TCS), designated here as PilRS. While the NE pilus is important in pathogenesis, the role of PilRS remains to be determined. The current study investigated the function of PilRS, as well as the Agr-like quorum-sensing (QS) system and VirSR TCS, in the regulation of pilin production. Isogenic *pilR*, *agrB* and *virR* null mutants were generated from parent strain CP1 by insertional inactivation using the ClosTron system, along with the respective complemented strains. Immunoblotting analyses showed no detectable pilus production in the CP1pilR mutant, while production in its complement (CP1pilR+) was greater than wild-type levels. In contrast, pilus production in the *agrB* and *virR* mutants was comparable or higher than the wild type but reduced in their respective complemented strains. When examined for collagen-binding activity, the *pilR* mutant showed significantly lower binding to most collagen types (types I – V) than CP1 ($P \leq 0.05$), whereas this activity was restored in the complemented strain ($P > 0.05$). In contrast, binding of agrB and virR mutants to collagen showed no significant differences in collagen-binding activity compared to CP1 ($P > 0.05$), whereas the complemented strains exhibited significantly reduced binding ($P \leq 0.05$). These data suggest that the PilRS TCS

positively regulates pilus production in *C. perfringens*, while the Agr-like QS system may serve as a negative regulator of this operon.

Keywords: Pilus; Binding; Two-component regulatory system; Quorum sensing; Necrotic enteritis; *Clostridium perfringens*

4.2 INTRODUCTION

Clostridium perfringens is a Gram-positive, spore-forming, anaerobic, rod-shaped bacterium that is widely distributed in the environment, particularly in soil, food, sewage and the gastrointestinal (GI) tract of both diseased and healthy animals (Hassan et al., 2015). *C. perfringens* is divided into seven toxinotypes (A through G) according to the production of six toxins, α-toxin (CPA), β-toxin (CPB), ε-toxin (ETX) and ι-toxin, enterotoxin (CPE) and Necrotic enteritis B-like (NetB) (Rood et al., 2018).

C. perfringens type G isolates, which produce NetB, cause necrotic enteritis (NE), an important disease of poultry that has been estimated to cost the poultry industry approximately $6 billion (US) in losses per year globally (Wade et al., 2015). The disease has been historically controlled by prophylactic antibiotic use; however, this application is currently being phased out due to growing concern over the development and spread of antimicrobial-resistant bacteria. The development of strategies to control NE without the use of antibiotics is now a priority to ensure the safe and cost-effective delivery of poultry products to consumers.

The host-pathogen interactions between poultry and *C. perfringens* are complex and not fully understood, involving the pore-forming toxin NetB along with other virulence factors (Keyburn et al., 2008; Lepp et al., 2013; Prescott et al., 2016). We previously identified a locus prevalent in

NE-causing *C. perfringens* isolates, designated VR-10B (Lepp et al., 2013), that encodes an adhesive sortase-dependant pilus (NE pilus), and is required for NE pathogenesis and binding to collagen (Lepp et al., 2021; Wade et al., 2016). Sortase-dependent pili play an important role in the virulence of a number of Gram-positive pathogens by mediating adhesion, host colonization, and biofilm formation (Telford et al., 2006; Danne and Dramsi, 2012; Khare et al., 2017), but haves only recently been implicated in *C. perfringens* pathogenicity. The VR-10B locus consists of seven open-reading frames (ORFs) that encode three pilin subunits (CnaA, FimA, FimB), a sortase enzyme, a signal peptidase, as well as a putative two-component regulatory system (TCS) (Lepp et al., 2013), designated here as the PilR response regulator (RR) and PilS sensor histidine kinase (SK), or PilRS. NE pilus production is abrogated in virulent *C. perfringens* strains following disruption *cnaA*, *fimA* and *fimB*, resulting in reduced collagen-binding ability and significant attenuation of virulence (Lepp et al., 2021; Wade et al., 2015). In addition, Wade et al. (2016) found that *cnaA*-null mutants have a reduced capacity to colonize chickens during NE development (Wade et al., 2016). Furthermore, immunization of chickens with recombinant pilins CnaA and FimB partially protected against NE (Lepp et al., 2019), indicating the important role played by this pilus in NE pathogenesis.

A previous report indicated that the VR-10B operon may be positively regulated by the accessory gene regulator (Agr)-like quorum sensing (QS) system (Yu et al., 2017). The Agr operon, first elucidated in *Staphylococcus aureus* (Peng et al., 1988), regulates gene expression in response to changes in local cell densities through secretion of the AgrD-encoded auto-inducing peptide (AIP), which is modified and transported across the cell membrane by AgrB, and detected in turn by the cognate AgrA/AgrC TCS (Bassler et al., 2006). *C. perfringens* carries a homologous

Agr-like operon (Ohtani et al., 2009), but unlike that of *S. aureus,* does not encode a TCS. The expression of several toxin genes, including Perfringolysin O (PFO), CPA, CPB, ETX and NetB, is dependent upon both the Agr-like system and the VirSR TCS (Yu et al., 2017; Li et al., 2011; Vidal et al., 2012; Chen and McClane, 2012; Chen et al., 2011; Cheung et al., 2010), suggesting that the VirS sensor kinase recognizes the AIP signal. VirSR is a global regulator that controls the transcription of approximately 147 genes, either through direct binding of phosphorylated VirR to upstream "VirR-box" elements, or indirectly through the regulatory RNA molecule VR-RNA (Ohtani et al., 2010). However, the relationship between the Agr-like and VirSR systems is not absolute, as the expression of ETX in Type D strains has been shown to depend on the Agr-like system, but not VirSR (Chen et al., 2011).

The PilRS genes are located immediately downstream of the NE pilus structural genes, suggesting that they may also be involved in the regulation of pilus expression. The current study objectives were therefore to investigate the function of the PilRS TCS, in addition to the Agr-like QS system and VirSR TCS, in regulating NE pilus production and function as determined by collagen-binding ability.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial strains and growth conditions

C. perfringens CP1, a field isolate from an NE case in Ontario, Canada (Thompson et al., 2006), was used as the parent strain for all of the mutants and complemented strains used in this study. The generation of *agrB* and *virR* null mutant strains CP1*agrB* and CP1*virR*, and

complemented strains CP1*agrB*+ and CP1*virR*+, has been described in Yu et al. (2017) and pilin-null mutants CP1*cnaA*, CP1*fimA* and CP1*fimB* in Lepp et al. (2021).

All *C. perfringens* strains were grown overnight at 37°C under anaerobic conditions on blood agar or Brain Heart Infusion (BHI) (Fisher) plates or in BHI broth, supplemented with 34 μg mL^{-1} chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA) and 10 μg mL^{-1} erythromycin (Thermo Fisher, Burlington, ON, Canada) as necessary. The mutant strains were supplemented with 10 μg mL^{-1} erythromycin, and the complemented strains with 10 μg mL^{-1} erythromycin and 34 μg mL^{-1} chloramphenicol. *Escherichia coli* Stellar (Takara Bio, Mountain View, CA, USA) competent cells were used for cloning in Luria-Bertani (LB) broth or agar (Difco) supplemented with 34 μg mL^{-1} chloramphenicol (Sigma-Aldrich), as required.

4.3.2 PilRS sequence analysis

The PilRS region of the VR-10B locus was amplified from CP1 and CP4 genomic DNA using primers pilRS-F and pilRS-R (Table 4-1), and Sanger sequencing was carried out on both amplicons. All predicted proteins from the CP1 genome were annotated for protein domain signatures with InterProScan v5.48-83.0. Genes were predicted to encode RRs if they contained Pfam domain PF00072, and SKs if they contained PF00512, PF07568, PF07730, PF07536, PF06580, PF01627, PF02895, PF05384, PF10090, PTHR42878, PTHR45528, PTHR43547, PTHR43711, PTHR40448. Multiple alignments of the amino acid sequences of all predicted *C. perfringens* CP1 RRs and SKs were performed separately with Clustal Omega and Neighbor-joining trees were generated. To identify putative PilR DNA-binding motifs within the VR-10B promoter, the approximately 700 bp non-coding sequence upstream of *cnaA* to the preceding gene were scanned for VirR-boxes consensus sequence “CCAnTTn (Ohtani et al., 2010) CCAnTT(Lepp

et al., 2010)" using the EMBOSS fuzzynuc tool (https://www.bioinformatics.nl/cgi-bin/emboss/), as well as the etandem, palindrome and einverted tools. To identify the VR-10B1 variant in other *C. perfringens* genomes, 200 currently available RefSeq genomes were downloaded from NCBI and local BLAST searches were performed against each using both the VR-10B and VR-10B1 sequences.

Table 4.1 Primers used in this study.

Primer	Purpose	Sequence (5'-3')
pilR-F1	Mutant verification	CGATGAGAAATTTCAATGTGC
pilR-R1	Mutant verification	TCAATTCCATTCATTTCATCCA
pilR-comp-F1	pilR complement construction	CGGTACCCGGGGATCCTAGAAGATAAAATGGTAAGTT
pilR-comp-R1	pilR complement construction	GGCCAGTGCCAAGCTTATAAATATATTCTTATTAAAC
CT_Erm-F	pilR mutant junction PCR	GATATTCACCGAACACTAGG
pJIR750-F2	pilR complement verification	GTTGGCCGATTCATTAA
pilRS-F	CP1 pilRS sequencing	TAGAAGATGAAAATGGTAAGTT
pilRS-R	CP1 pilRS sequencing	TTATAAATATATTCTTATTAAAC

4.3.3 Construction and confirmation of pilR mutant and complemented strains

The ClosTron mutagenesis system was used to insertionally inactivate *pilR* in wild-type CP1 as described by Heap et al. (2010), to generate CP1 *pilR43*::*ermB* (referred to hereafter as CP1*pilR*). The ClosTron intron-targeting region was designed to insert at 43 bp of the *pilR* open-reading frame sense-strand using the Perutka algorithm implemented at www.clostron.com. The intron-targeting region was synthesized and cloned into ClosTron plasmid pMTL007C-E2 by DNA 2.0 (Menlo Park, CA, USA) to generate pMTL007C-E2::pilR43s. The resultant plasmid was electroporated into CP1 as described previously (Yu et al., 2017; Jiraskova et al., 2005). To verify that the intron had been inserted into the expected location, PCR was performed to amplify the region spanning the insertion site with the primer pair pilR-F1/R1 (Table 4-1). Additionally, the region spanning the junction between the ClosTron insert and PilR gene was amplified using primers CT_Erm-F and pilR-F1, and verified by Sanger sequencing.

To construct the pilR complemented strain CP1 *pilR43*::*ermB* [*pilR*+] (referred to hereafter as CP1*pilR*+), *pilR* was amplified using primers pilR-comp-F1 and pilR-comp-R1 (Table 4-1) and digested with restriction enzymes BamHI and HindIII. The purified fragment was ligated into *E. coli-C. perfringens* shuttle vector pJIR750, to generate pJIR750-*pilR*, which was transformed into *E. coli* Stellar competent cells. The plasmid insert was confirmed by sequencing and electroporated into CP1*pilR* as previously described (Yu et al., 2017). Transformants were selected on BHI agar supplemented with 15 μg mL^{-1} thiamphenicol and 10 μg mL^{-1} erythromycin.

4.3.4 Isolation of cell surface protein from *C. perfringens* strains

Total cell surface proteins were extracted from *C. perfringens* cultures essentially as described by (Chang et al., 2013). Strains were grown overnight anaerobically at 37ºC on blood agar plates, and sub-cultured in BHI broth to an OD600 approximately 0.8-1.0. A 100 μl aliquot of the

subculture was spread onto a BHI plate and incubated overnight to produce a confluent lawn. The overnight culture was harvested from the surface of plates with PBS, and the suspension was centrifuged at 5,000 × g for 2 min, washed once with PBS, and adjusted to an OD600 of 1.0 in PBS. Cells (10 mL) were pelleted by centrifugation at 6,000 × g for 5 min. Bacterial cells were washed once with 1 ml SMM buffer, pH 6.8 (0.5 M sucrose, 10 mM $MgCl_2$, 10 mM maleate) and resuspended in 1 mL SMM buffer, to which 60 µL of 5 U/µL of mutanolysin (Sigma) in muramidase buffer (2 mM acetic acid, 48 mM sodium acetate) and 10 µL of 0.1M phenylmethylsulfonyl fluoride (PMSF) (Sigma) were added. After approximately 6 h incubation at 37°C with constant rotation, resultant protoplasts were centrifuged at 20,000 × g for 5 min, and the supernatant of cell wall proteins was removed. The protein content of the cell wall fraction was quantitated by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), mixed with 4× NuPAGE™ LDS Sample Buffer (Thermo Fisher Scientific) and boiled for 5 min prior to immunoblotting assays.

4.3.5 Immunoblotting assays for examination for pilus production in *C. perfringens* strains

Extracts of cell surface protein were separated on NuPAGE™ 3-8% Tris-Acetate protein gels (Thermo Fisher Scientific) by electrophoresis at 150V for 1 h. One gel was loaded with 10 µg of extracts of cell surface protein and used for staining with Biosafe Coomassie stain (BioRad), and identical gels loaded with 5 µg cell surface protein were transferred onto individual PVDF membranes (Invitrogen) by electroblotting at 30V overnight at 4°C in 1× transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, 0.1% SDS). Membranes were washed twice for 5 min in 20 ml of Milli-Q water after removal from 1× transfer buffer, then incubated in TBS blocking solution (20 mM Tris, 150 mM NaCl) containing 3% (w/v) Bovine Serum Albumin (BSA) and 0.05% (v/v)

Tween 20 for 2 h at 4°C. After washing 3 times with TBST (1 × TBS, 0.05% Tween 20) for 5 min each, the membranes were incubated with rabbit antisera (1:1000) raised against CnaA, FimA or FimB (Lepp et al., 2021) at 22°C for 1 h, washed 3 times with TBST for 5 min each and then incubated with a goat anti-rabbit IgG alkaline phosphatase (AP)-conjugated secondary antibody (1:2,000) (Cedarlane) at 22°C for 0.5 h. Membranes were washed 3 times with TBST for 5 min each and then washed 3 times with Milli-Q water for 2 min each. Alkaline phosphatase activity was detected by adding 2.5 mL of a CDP-Star® chemiluminescent substrate (Thermo Fisher Scientific) to each membrane for 5 min. Membranes were imaged on a BioRad GelDoc XR system using Image Lab software.

4.3.6 Adhesion assay

Bacterial adhesion to collagen types I through V (Sigma) was assayed as described by Xiao et al. (1998) (Xiao et al., 1998) and Wade et al. (2016) (Wade et al., 2016) with modifications. Collagen types assayed were: type I from rat tail, type II from chicken sternal cartilage, type III from human placenta, type IV from human placenta, type V from human placenta. Wells of Nunclon Delta Surface 96-well plates (Thermo Fisher) were coated with 50 µL collagen (1 mg/mL in PBS) per well overnight at 4ºC and blocked in 200 µL of PBS containing 0.5% (w/v) BSA for 2 h at 4ºC, and then rinsed 3 times with 100 µL PBS. *C. perfringens* strains were grown overnight anaerobically at 37ºC on blood agar plates, sub-cultured in BHI broth and grown to an OD600 of approximately 0.8-1.0. A 100 µL aliquot of the subculture was spread onto a BHI plate and incubated overnight to produce a confluent lawn. The overnight culture was harvested from the surface of plates in PBS and the suspension was centrifuged at 5,000 × g for 2 min, washed once with PBS, and adjusted to an OD600 of 1 in PBS. Bacterial cells were added to the wells of 96-

well plates in 50 µL aliquots and incubated at 22°C for 2.5 h with gentle shaking. Wells were rinsed 3 times with 100 µL of PBS and air-dried. Cells were stained with 0.5% (w/v) crystal violet for 5 min, rinsed 3 times with 100 µl PBS, and then air-dried. A 1:1 ethanol:acetone (v/v) solution (50 µL) was added to each well to de-stain adherent cells and absorbance was measured at 562 nm. Wells incubated with bacteria but without collagen, and wells coated with collagen but without added bacteria were used as blank and negative controls, respectively. Blank values were subtracted from all test sample absorbance values. All assays were repeated in triplicate and averaged from triplicate wells, except for assays with collagen V, which were repeated in duplicate.

4.3.7 Antibody-blocking adhesion assays

The wild type (CP1) and mutant CP1*agrB* were used to test whether their binding to collagens I and IV can be blocked by rabbit antisera against pilins CnaA, FimA or FimB. Specifically, bacterial cells (50 µL) resuspended to OD600 of 2 were incubated for 20 minutes at 22°C with three dilutions (10^{-3}, 10^{-4} and 10^{-5}) of antibodies (50 µL) against CnaA, FimA or FimB, respectively, before added to wells coated with collagen I or collagen IV. Wells incubated with bacterial cells but without collagen served as blank controls; wells coated with collagen but without added bacterial cells were used as negative controls; culture of CP1 or CP1*agrB* (50 µL) + PBS (50 µL), and 10^{-3} dilution of pre-immune rabbit serum obtained from the rabbits from which antisera against CnaA, FimA or FimB was prepared, were also used as controls. Other steps in this assay remained the same as those of regular adhesion assays described above.

4.4 RESULTS

4.4.1 Identification of novel variant VR-10B1

The PilRS TCS is located approximately 800 bp downstream of the FimB gene within the VR-10B operon in *C. perfringens* CP4, and consists of the predicted 238 amino acid (aa) response regulator PilR, and 432 aa sensor histidine kinase PilS. Amplification of the PilRS region of CP1 produced an amplicon of approximately 4 kb, instead of the expected 2.5 kb product predicted from the CP4 genome sequence (Fig. 4-1A). Sequencing of the CP1 PilRS amplicon revealed a 1,275 bp IS6 family transposase inserted 23 bp upstream of the *pilR* start codon, which accounts for the larger amplicon size in this strain (Fig. 4-1B). To determine if this variant (designated VR-10B1) is present in other *C. perfringens* strains, the VR-10B and VR-10B1 sequences encompassing *cnaA* to *pilR* were aligned against 200 available *C. perfringens* RefSeq genome sequences. Among these, 21 genomes carried an intact VR-10B locus (*i.e.*, lacking the transposase gene), whereas one strain (NCTC8081) carried an intact VR-10B1 variant (Table 4-2). Remarkably, NCTC8081 is a Type C strain isolated from the intestines of an individual that died from enteritis necroticans (EN). Additionally, two poultry isolates (JS5388 and Warren) carried the *cnaA-fimB* and *pilR* regions of VR-10B on separate contigs. In the CP1 draft genome, these two regions were also initially assembled onto separate contigs, but later determined to be misassembled as a result of the intervening repetitive transposase sequence, suggesting that JS5388 and Warren may also carry the VR-10B1 variant.

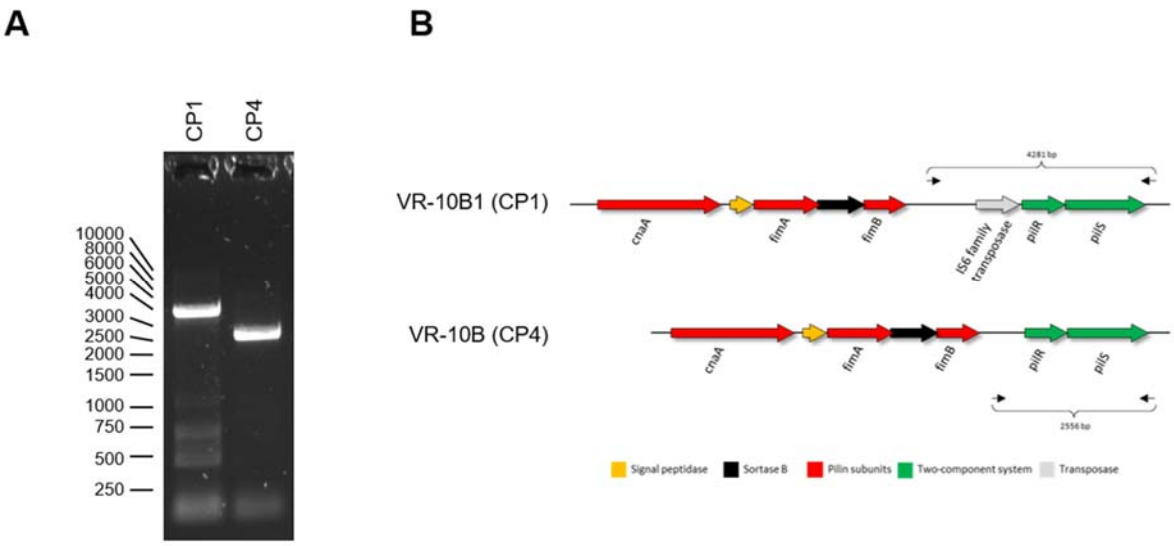

Figure 4.1 Identification of novel VR-10B1 variant. A) PCR products amplified from CP1 or CP4 genomic DNA, using primers targeting the pilR gene and upstream region. B) Schematic of the VR-10B and VR-10B1 variants, indicating primer locations (black arrows) and amplicon sizes.

Table 4.2 Summary of BLAST results for VR-10B and VR-10B1 aligned against 200 *C. perfringens* RefSeq genome sequences.

Table S2. Summary of BLAST results for VR-10B and VR-10B1 aligned against 200 *C. perfringens* RefSeq genome sequences

Strain	RefSeq Accn No	Host	Source	Collection date	Location	Query	Subject Accession	% ID	% Coverage	Region aligned
2016TE7641_69	GCF_003628405.2	turkey	intestine	2016-07	Italy: Teramo	VR-10B	NZ_RBXV01000007.1	99.985	100.02	full locus
98.78718-2	GCF_003181795.1	chicken	necrotic enteritis field case	1996	USA: Alabama	VR-10B	NZ_PJTE01000010.1	99.985	100.02	full locus
CP-14	GCF_011063105.1	NA	not applicable	1957	Yugoslavia	VR-10B	NZ_JAALMZ010000019.1	94.819	100.47	full locus
CP-15	GCF_011063045.1	chicken	not applicable	1993	China	VR-10B	NZ_JAALMY010000010.1	94.819	100.47	full locus
CP-45	GCF_011062465.1	chicken	not applicable	2018	China	VR-10B	NZ_JAALLU010000011.1	99.97	100.00	full locus
CP4	GCF_001414595.1	chicken	necrotic enteritis field case	2001	Canada;Ontario	VR-10B	NZ_LIYI01000014.1	100	100.00	full locus
Del1	GCF_002012325.1	chicken	Isolated from the intestines of sick birds in the farm	2009	USA: Millsboro, DE	VR-10B	NZ_CP019576.1	100	100.00	full locus
EHE-NE18	GCF_003203455.1	chicken	gut contents	2002-07-30	Australia:Sealake	VR-10B	NZ_CP025501.1	99.985	100.00	full locus
EUR-NE15	GCF_003181745.1	chicken	necrotic enteritis field case	22-Jul-2002	Australia: Euroa	VR-10B	NZ_PJTB01000003.1	99.985	100.00	full locus
FC2	GCF_003181735.1	chicken	necrotic enteritis field case	1995	USA: East Coast	VR-10B	NZ_PJTA01000004.1	99.985	100.00	full locus
GNP-1	GCF_003181705.1	chicken	necrotic enteritis field case	1996	USA	VR-10B	NZ_PJSZ01000006.1	99.97	100.00	full locus
ITX1105-12MP	GCF_003181695.1	chicken	necrotic enteritis field case	1996	USA	VR-10B	NZ_PJSY01000037.1	100	100.00	full locus
LLY_Tpel17	GCF_008086935.1	chicken	n/a	Oct-2018	USA: Georgia	VR-10B	NZ_VFFA01000002.1	100	100.00	full locus
NAG-NE31	GCF_003181655.1	chicken	necrotic enteritis field case	10-Sep-2004	Australia: Nagambie	VR-10B	NZ_PJSV01000016.1	99.955	100.00	full locus
Pennington	GCF_003181615.1	chicken	necrotic enteritis field case	1993	USA: Illinois	VR-10B	NZ_PJSQ01000010.1	100	100.00	full locus
SYD-NE41	GCF_003245405.1	chicken	isolate from necrotic enteritis trial	2011-06-02	Australia: Sydney	VR-10B	NZ_PJUV01000012.1	99.955	100.03	full locus
TAM-NE38	GCF_003181505.1	chicken	necrotic enteritis field case	2002	Australia: Queensland	VR-10B	NZ_PJTL01000023.1	99.97	100.00	full locus
TAM-NE40	GCF_003181475.1	chicken	necrotic enteritis field case	06-Sep-2011	Australia: Tamworth	VR-10B	NZ_PJSK01000032.1	99.955	100.02	full locus
TAM-NE46	GCF_003181405.1	chicken	necrotic enteritis field case	16-Aug-2013	Australia: Tamworth	VR-10B	NZ_PJSI01000014.1	99.955	100.00	full locus
TAMU	GCF_009259705.1	chicken	broiler chicken gut	2004	USA: Texas	VR-10B	NZ_VOVJ01000004.1	100	100.00	full locus
UDE_95-1372	GCF_003181355.1	chicken	necrotic enteritis field case	1995	USA: Delaware	VR-10B	NZ_PJTI01000007.1	99.97	100.00	full locus
JS5388	GCF_006454475.1	chicken	chicken intestine	n/a	n/a	VR-10B1	NZ_NGVU01000052.1	99.948	73.07	CnaA-pilR upstream region
						VR-10B1	NZ_NGVU01000209.1	100	11.45	pilR
Warren	GCF_003181835.1	chicken	necrotic enteritis field case	1993	USA: Illinois	VR-10B1	NZ_PJTH01000087.1	100	66.78	CnaA-pilR upstream region
						VR-10B1	NZ_PJTH01000074.1	100	13.71	pilR
NCTC8081	GCF_900461295.1	human	patient with enteritis necroticans	1946/1948	Germany: Hamburg	VR-10B1	NZ_UAWO01000002.1	93.723	100.40	full locus

To further elucidate the putative functions of PilR and PilS, amino acid sequences for each were scanned for protein domain signatures against the InterPro databases. PilR contains two domains: an N-terminal "Response regulator receiver" domain (IPR001789), responsible for accepting the phosphoryl group from the cognate SK, and a C-terminal LytTr DNA-binding domain (IPR007492), which is the effector domain activated upon phosphorylation. PilS contains a histidine kinase-like HATPase_AgrC-ComD-like ATPase domain (cd16935), also found in the *Staphylococcus aureus* AgrC and *Streptococcus pneumoniae* ComD SKs, which both recognize the Agr AIP. To evaluate how common these domains are within the CP1 genome, all predicted proteins were functionally annotated with InterProScan. Interestingly, the LytTr DNA-binding and HATPase_AgrC-ComD-like domains were detected only in VirR and VirS, respectively, aside from PilR and PilS (Fig. 4-2A). PilR and VirR share 28% identity (53% similarity), while PilS and VirS share 35% identity (57% similarity), suggesting that the VirSR and PilRS TCSs are evolutionarily related. A phylogenetic tree based on multiple sequence alignment of all predicted

SKs and RRs in the CP1 genome depicts this relationship (Fig. 4-2C). The DNA-binding activity of VirR has been determined to depend on both the FxRxHrS (Ohtani, 2016) and SKHR

(McGowan et al., 2002) motifs located in the C-terminal LytTr domain. Alignment of VirR and PilR demonstrates that while the FxRxHrS motif is conserved in PilR, the SKHR motif is not (Fig. 4-2B). Searches of the *cnaA* upstream region, which is a hypothesized target of PilR, did not reveal any putative VirR-boxes, tandem repeats or palindromic sequences that could potentially serve as a DNA-binding motif (data not shown).

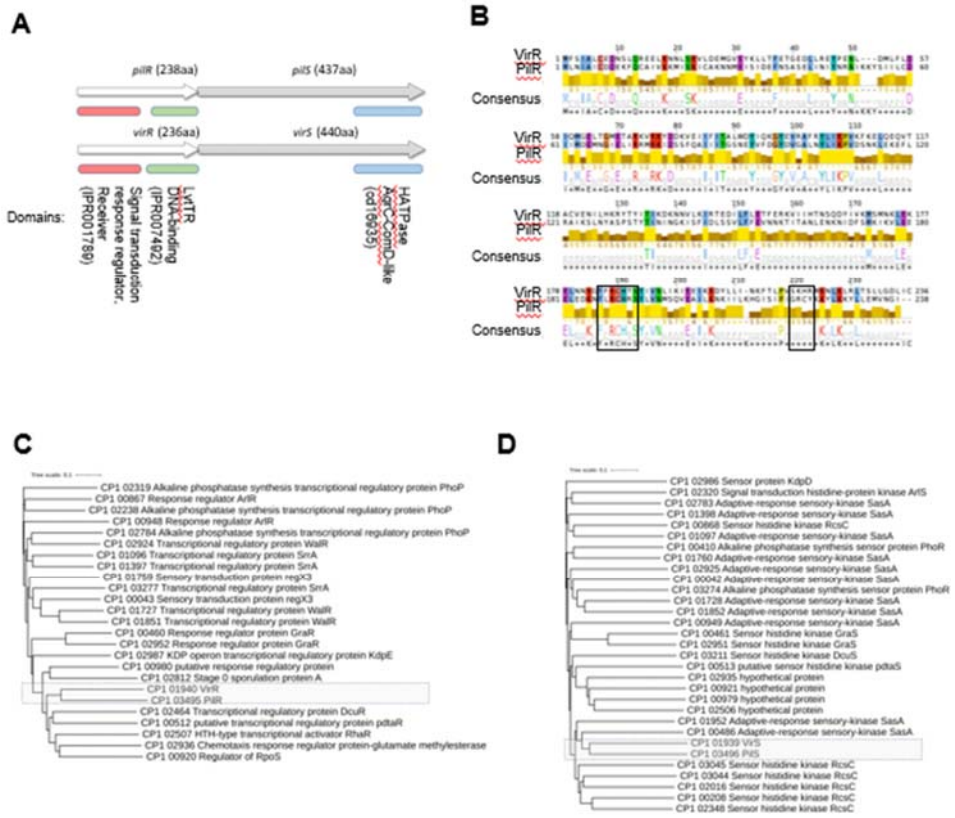

Figure 4.2 Sequence comparison of VirSR and PilRS two-component systems. A) Schematic of PilR, PilS, VirR and VirS proteins indicating shared domains. B) Amino acid sequence alignment of PilR and VirR proteins, indicating consensus sequences. The two key VirR amino acid motifs that are required for DNA-binding to VirR-box elements are outlined with boxes. C and D) Neighbour-joining phylogenetic trees built from multiple protein sequence alignment of all predicted RRs (C) or SKs (D) within the CP1 genome. The VirRS/PilRS branches are outlined with boxes.

4.4.2 Generation of pilR mutant and complement

CP1*pilR* and complemented strain CP1*pilR*+ were constructed and confirmed by PCR and sequencing. Specifically, the presence of the ClosTron insert in CP1*pilR* was verified by PCR amplification of the junction region of the insert and PilR gene, to demonstrate that the intron-targeting region had been inserted into correct location (Fig. 4-3A). The transformation of the CP1*pilR* strain with the plasmid expressing PilRS was confirmed by the presence of the plasmid DNA and *pilR* DNA (Fig. 4-3B).

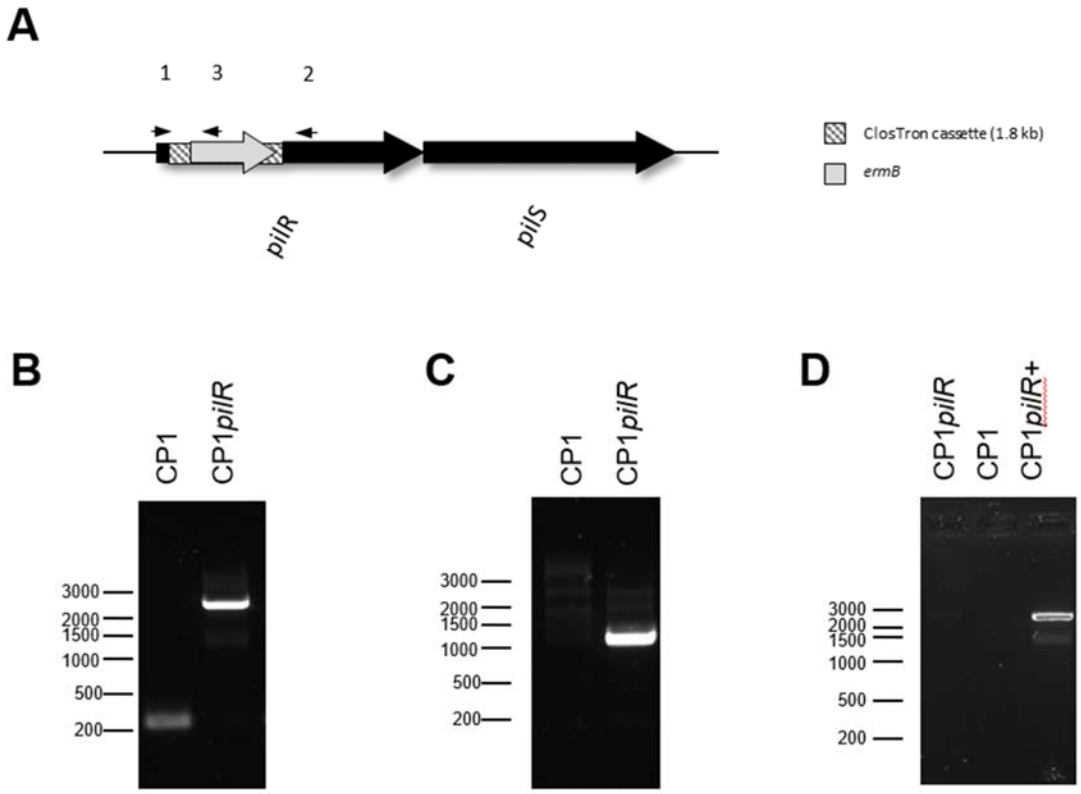

Figure 4.3 PCR confirmation of CP1 pilR-null mutant and complemented strains. A) Schematic of PilRS genes showing ClosTron insert and location of primers used for confirmation: 1) pilR-F1, 2) pilR-R1, 3) CT_erm-F. See Table 4-1 for primer details. B) PCR amplification of pilR flanking ClosTron insert using primers 1 and 2. C) PCR amplification of pilR/ClosTron junction with primers 1 and 3. D) PCR amplification of pJIR750-pilRS with primers 2 and pJIR750-F2, located on the pJIR750 shuttle vector.

4.4.3 Immunoblotting analysis of pilus production in *C. perfringens* strains

To investigate the regulatory function of the PilRS TCS and Agr-like QS system in pilus expression, an immunoblotting assay was performed using rabbit antisera raised against each of the pilin proteins (CnaA, FimA and FimB) as primary antibodies. As previously reported, proteins from the cell wall produce a ladder-like pattern, indicating the presence of pili containing variable length polymers. In CP1*pilR*, little protein was observed and no ladder-like pattern was observed (Fig. 4-4). The ladder-like banding pattern was restored in the CP1*pilR*+ strain and the intensity of the bands was greater than that of CP1. In contrast, pilus production levels were the same or higher in the CP1*virR* and CP1*agrB* mutants, but reduced in their respective complements, compared with CP1. As expected, no pilus production was observed in the three pilin mutants (CP1*cnaA,* CP1*fimA,* and CP1*fimB*).

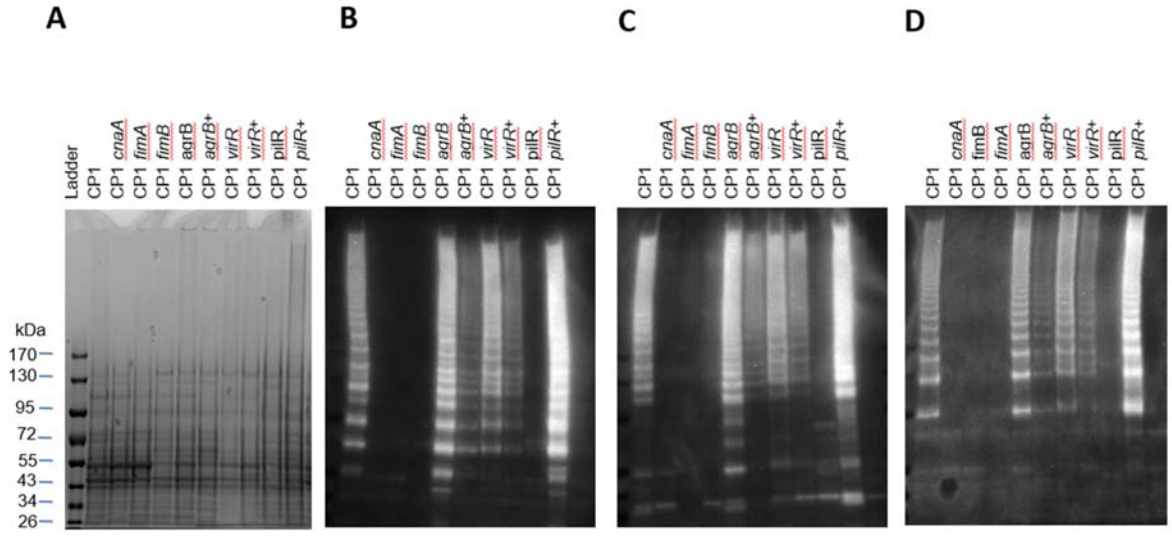

Figure 4.4 SDS-PAGE and immunoblotting analysis of cell surface fractions from *C. perfringens* CP1, isogenic null mutants and respective complemented strains. (A) Coomassie blue-stained

SDS-PAGE gel, and replicate immunoblots incubated with primary antibodies against CnaA (B), FimA (C), and FimB (D). Each lane was loaded with 5 μg of surface protein sample.

4.4.4 Collagen binding assays

To investigate the role of the proposed regulatory elements of the pili in the binding of *C. perfringens* to collagen, adhesion assays were utilized to test the binding of wild-type CP1 and isogenic CP1 mutants and complemented strains to collagen types I through V. Similar to the immunoblotting results, binding of the CP1*pilR* mutant to most collagen types was significantly lower than CP1 ($P \leq 0.05$; Tukey's), but its complement was either not significantly different or significantly higher than the parent strain ($P \leq 0.05$) (Fig. 4-5A). In contrast, binding of *agrB* and *virR* null mutants to collagen (types I – V) exhibited no significant changes compared with CP1 ($P > 0.05$), whereas the *agrB* complemented strain had significantly reduced binding to collagen types I, II and IV ($P \leq 0.05$) (Fig. 4-5B), and the *virR* complemented strain showed significantly lower binding to collagen types I, II, III and IV ($P \leq 0.05$) (Fig. 4-5C). All of the pilin mutants showed negligible binding to collagen (types I – V) than CP1 ($P \leq 0.05$) (Fig. 4-5D), as reported previously (Lepp et al., 2021).

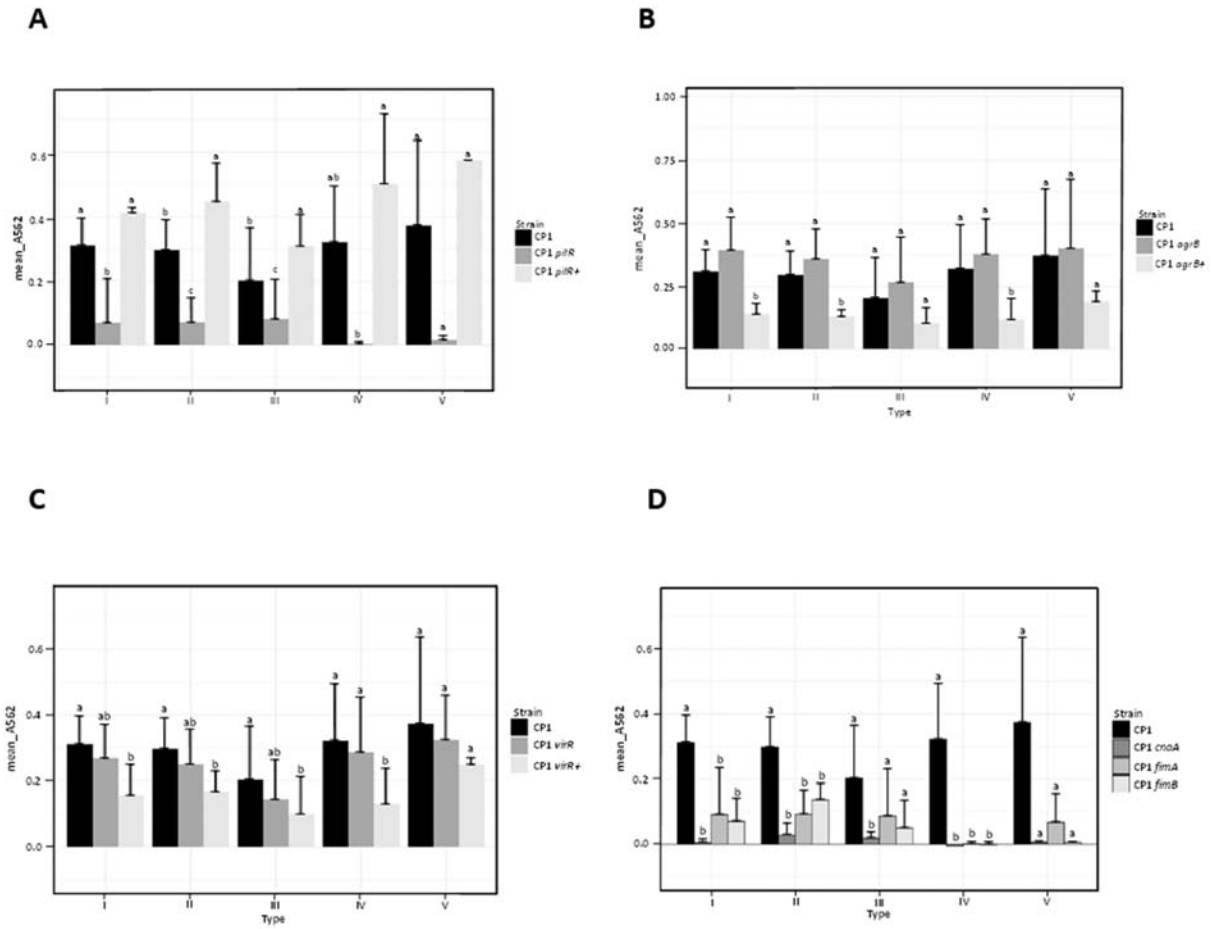

Figure 4.5 Binding of *C. perfringens* CP1, isogenic null mutants and respective complemented strains to collagen types I through V. Letters indicate significantly different groups ($P \leqslant 0.05$; Tukey's). Error bars represent standard deviations. (A) Binding of pilR-null mutant and respective complemented strains compared with CP1. (B) Binding of agrB-null mutant and complemented strains compared with CP1. (C) Binding of virR-null mutant and complemented strains compared with CP1. (D) Binding of pilin-null mutant strains compared with CP1.

4.4.5 Blocking of *C. perfringens* collagen-binding by pilin antisera

It was previously reported that binding of wild-type CP1 to collagen types I and IV is

specifically blocked by CnaA and FimA antisera in a dose-dependent manner (Lepp et al., 2021). To verify that the binding of the *agrB* null mutant was specifically due to expression of the NE pilus, and not to other unknown proteins controlled by this global regulator, the specificity of adherence to collagen types I and IV was examined in the presence of three dilutions (10^{-3}, 10^{-4}, 10^{-5}) of rabbit antisera against CnaA or FimA. The binding of CP1 and CP1*agrB* to collagen types I and IV was blocked by antisera against CnaA (Fig. 4-6A and 6B) and FimA (Fig. 4-6C and 6D). For both CnaA and FimA antiserum, as the concentration decreased, binding of CP1 and CP1*agrB* to both collagen types increased in a dose-dependent manner (Fig. 4-6). These observations suggest that the binding of mutant CP1*agrB* to collagen types I and IV is specifically mediated through the NE pilus.

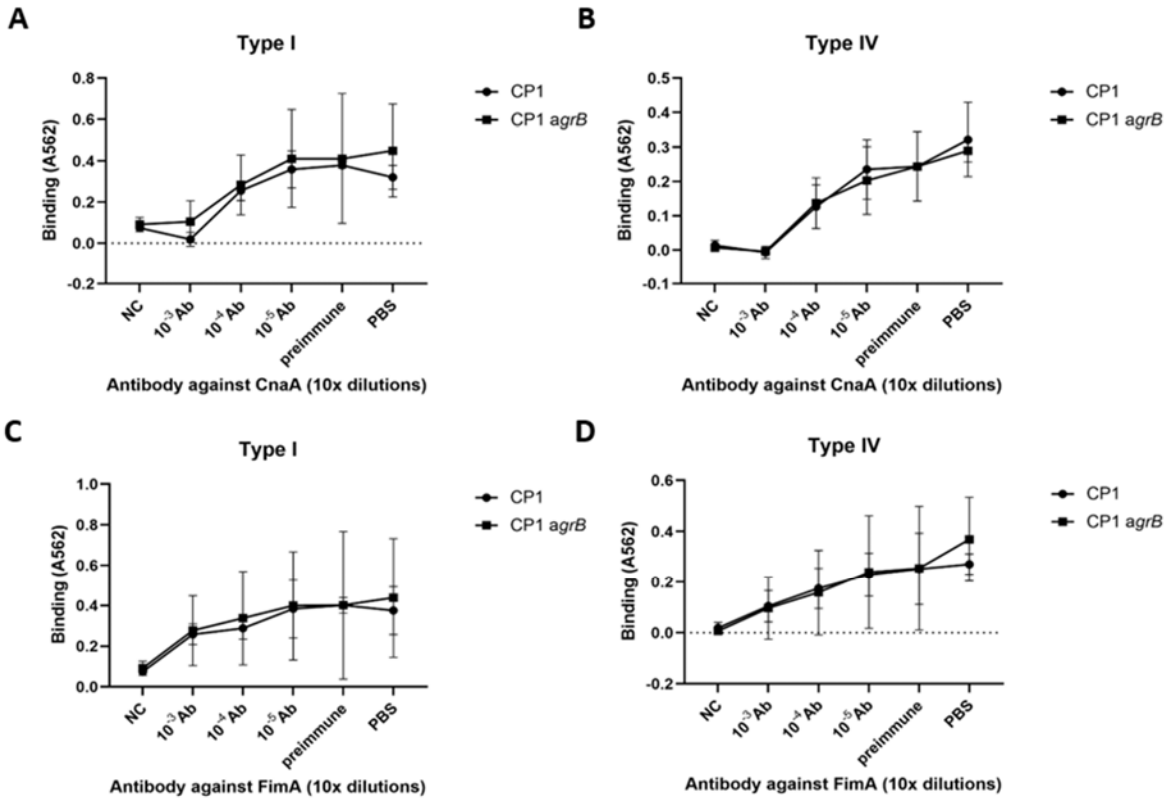

Figure 4.6 Blocking of *C. perfringens* CP1 and Agr mutant strains binding to collagen by pilin antisera. *C. perfringens* cultures were incubated with dilutions of rabbit antiserum against CnaA (A, B) or FimA (C, D) for 20 minutes before adding to wells of a 96-well plate coated with collagen types I (A, C) or IV (B, D). NC: no collagen; PBS: CP1 or CP1agrB incubated with PBS; preimmune: CP1 or CP1agrB incubated with pre-immune rabbit serum (10^{-3} dilution).

4.5 DISCUSSION

NE caused by *C. perfringens* remains a major challenge for the poultry industry, particularly in light of recently imposed restrictions on the use of preventative antibiotics, of which NE is the

primary target. A thorough understanding of the disease, including the full complement of virulence factors employed by NE-causing *C. perfringens* strains, is essential for developing novel approaches for therapy and prevention. We recently demonstrated, through visualization with TEM immuno-gold labelling, that the VR-10B locus encodes a sortase-dependent pilus (Lepp et al., 2021), which mediates collagen-binding and is essential for NE pathogenesis (Wade et al., 2016; Lepp et al., 2021). The PilRS TCS is located directly downstream from the pilus structural genes, implicating it in the regulation of this operon.

In the current study, we demonstrate that disruption of the PilR gene completely abolishes NE pilus production, while complementation restores expression to greater than wild-type levels. These results clearly show that the PilRS TCS plays an integral role as the primary positive regulator of NE pilus production. The mechanisms by which the PilR RR regulates pilus production are unknown, whether by directly controlling transcription of the VR-10B operon through binding to upstream promoter elements, or indirectly through secondary regulators. Sequence analysis of the PilRS and VirSR TCSs reveals that they are related, both in terms of sequence similarity, and shared protein domains involved in signal recognition and DNA-binding specificity. VirR activates the transcription of several genes directly through binding to upstream "VirR-boxes" (Shimizu et al., 1994; Okumura et al., 2008; Cheung and Rood, 2000), but most genes within the VirSR regulon are modulated through the secondary regulatory RNA molecule, VR-RNA (Ohtani et al., 2010). The binding of VirR to VirR-box promoter elements is dependent upon at least two amino acid motifs, FxRxHrS and SKHR (McGowan et al., 2002; McGowan et al., 2003). The fact that the SKHR motif is absent from PilR suggests that the promoter element recognized by PilR likely differs from that of VirR. Preliminary searches to identify candidate PilR-binding sequences upstream of the VR-10B operon were unsuccessful. The PilS SK, like

VirS, contains a histidine kinase-like ATPase domain similar to that of *S. aureus* AgrC, which is part of the Agr QS operon. This family also includes the *S. pneumoniae* ComD SK of the ComD-ComE TCS, involved in quorum sensing and genetic competence (Li et al., 2002). These observations warrant further investigations to better understand the respective regulons controlled by VirSR and PilRS, which may involve cross-talk with Agr-like QS, as well as define the environmental signal(s) sensed by PilS.

We previously reported that the Agr-like QS system positively regulates pilin gene transcription (Yu et al., 2017). The Agr-like QS signaling pathway typically proceeds via the VirSR TCS to affect transcription, presumably through detection of the *agrD*-encoded AIP by the VirS SK (Li et al., 2011; Vidal et al., 2012; Chen and McClane, 2012). In the current study, isogenic *virR* and *agrB* mutants did not display reduced pilus protein production as expected, but instead appeared to produce equal or greater amounts than wild-type CP1. In contrast, pilus production in both of the complemented strains was reduced, likely due to higher than wild type expression levels of the complemented gene. This suggests that the Agr-like QS system, via the VirSR TCS, is in fact a negative regulator of NE pilus production. It is unlikely that the observed difference in pilus production between strains was due to unequal protein loading, as the same amount of quantified protein was loaded for each, and similar results were obtained from several replicate experiments. Furthermore, the collagen-binding activity of the mutant and complemented strains supports the same conclusion. In general, the collagen-binding ability of both CP1*agrB* and CP1*virR* was similar to wild-type CP1, but significantly reduced in the complemented strains. This was in contrast to the PilR mutant, where the opposite trend was observed. The Agr-like QS system is a global regulator that controls a large set of genes, and it is therefore possible that other collagen-binding proteins within this regulon may have increased in expression in CP1*agrB,* and

thereby influence the collagen-binding results. The ability of CnaA and FimA antiserum to block collagen-binding of CP1*agrB* rules out this possibility and indicates that collagen binding in this mutant strain is specifically mediated by the NE pilus. The reason for the difference between the current and previous studies is unclear, although they vary in the level at which expression was examined (RNA vs protein). It is therefore possible that regulation of the NE pilus by the Agr-like QS system also occurs through post-transcriptional mechanisms, resulting in a net decrease in pilus production, despite increasing transcription. Another explanation may be differences in the bacterial growth conditions used, as the current study evaluated plate-grown cultures, while the previous study examined liquid cultures. Recently, Soncini et al. (2020) reported that *C. perfringens* could sense and respond to growth on different surfaces, leading to changes in the expression of type IV pili at both transcriptional and posttranscriptional levels between plate-grown and liquid-grown strains (Soncini et al., 2020).

It is not clear what benefit might be gained from repressing pilus production during periods of high cell density. The expression of other virulence factors, including NetB, is up-regulated by the Agr-like QS system, presumably to coordinate an effective attack on host tissues once a sufficient number of cells have accumulated (Yu et al., 2017). Several other Gram-positive pilus islets have been found to encode regulatory proteins that modulate their expression, though no TCSs have thus far been identified to the best of our knowledge. The *S. pneumoniae* PI-1 pilus islet encodes the RlrA regulator, which controls pilus production through a positive feedback loop, resulting in a biphasic expression pattern that produces populations expressing high or low numbers of pili (De Angelis et al., 2011; Hava et al., 2003). The PI-1 pilus was found to be preferentially expressed during early colonization stages, where adhesion is important, and reduced during later stages, presumably to evade the immune response induced against the pilus antigen (Pancotto et al., 2013).

Similarly, the *S. pyogenes* FCT pilus-encoding region carries genes for two regulatory proteins, Nra/RofA and MsrR, which have contradictory effects on pilus expression, resulting in bistable expression (Kreikemeyer et al., 2007; Nakata et al., 2005). In this case, the pilus is expressed at lower temperatures, corresponding to the environment of superficial skin infections, to mediate binding to keratinocytes (Abbot et al., 2007). It is therefore possible that, similar to the biphasic expression of the PI-1 pilus, NE pilus production is down-regulated during later stages of infection, when it is no longer required and to evade the induced immune response. Further studies are required to define the *in vivo* expression pattern of the NE pilus, both during various stages of infection and at different host sites.

Previous investigations of the VR-10 locus have found that VR-10B is carried exclusively by poultry isolates, of which most are associated with NE (Lepp et al., 2013; Wade et al., 2016). A recent comparative genomic analysis of 67 *C. perfringens* strains revealed that VR-10B is highly conserved, while a novel third variant (VR-10C) containing only the flanking genes was discovered in non-pathogenic strains (Lacey et al., 2018). We define here a fourth variant, designated VR-10B1, that contains a transposase gene in the region between the pilus structural genes and PilRS TCS. Transposable elements are found in a number of *Streptococci* and *Enterococci* pilus operons and point towards the likely horizontal acquisition of these islets (Cruz et al., 1995). While carriage of this variant is atypical of poultry NE strains, the transposase insertion does not appear to impact the expression of *pilR*, as demonstrated by the mutagenesis and complementation experiments shown here. In addition to CP1, VR-10B1 was found in at least one (NCTC 8081) of the other strains, which interestingly is not a poultry Type A strain, but a human Type C strain isolated in 1946 from a lethal case of Enteritis Necroticans (EN) (Zeissler et al., 1949). This represents the first known non-poultry isolate that carries the NE pilus-encoding

genes. EN is a rare but often fatal disease that was prevalent in Germany for several years following World War II. It re-emerged in the 1960s and 1970s in Papua New Guinea, where it was referred to as Pigbel (Shrestha et al., 2018). While CPB, a pore-forming toxin related to NetB (Keyburn et al., 2008), is required for the development of EN, it is not known if additional virulence factors are involved. It would be informative to survey additional EN-associated strains to determine if VR-10B1 is limited to NCTC 8081.

In summary, this work describes the previously uncharacterized PilRS TCS, which appears to be the primary positive regulator of NE pilus production. We also provide evidence that the Agr-like QS system acts as a negative regulator in this system, likely by signaling through the VirSR TCS, to which PilRS is related. These findings provide new insights into the regulation of this key NE virulence factor, as well as offer clues towards the possible evolutionary origins of this pilus islet.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSION

5.1 GENERAL DISCUSSION

C. perfringens pili contribute to adherence and virulence (Wade et al., 2015; Wade et al., 2016), in which the Agr-like QS system and VirR/VirS TCS are involved (Prescott et al., 2016, Yu et al., 2017). It was previously reported that the Agr-like QS system positively regulates the expression of two genes (CP4_0573 and CP4_0575) identified in a pilus-encoding locus VR-10B in *C. perfringens* through microarray and qPCR analysis (Yu et al., 2017). Based on similarity and synteny with streptococal pilus operons, Lepp et al (2013) predicted that the VR-10B locus, which is made up of six co-transcribed genes, would encode three structural pilin subunits (CnaA, FimA and FimB) as well as two sortases and a signal peptidase I and would be controlled by the PilR/PilS TC system. More recently, this group, through a Western blot study with serum from immunized birds provided the first evidence of the pilus production by the above locus (Lepp et al., 2019). However, these authors did not investigate the predicted regulation of the observed pilus production by the PilR/PilS TC system, which was the focus of the current study. The Agr-like QS system and VirR/VirS TC system have been shown to be involved in *C. perfringens* virulence and in particular, its key involvement on NetB toxin production (Yu et al., 2017). It was not known if this same system was involved in the production of the NE pilus at the time of this thesis research. Also, it was not known if the PilR/PilS TCS also plays a role in the pili production and adherence and if this system works cooperatively with the Agr-like QS including VirR/VirS TC system or these systems work independently. The present study investigated whether a novel PilR/PilS TCS located on the VR-10B locus could also regulate expression of pilus genes and adhesion of

structurally functional pilus product in *C. perfringens* by Western blotting analysis of pilus gene expression and pilus functional (collagen adhesion) assays.

Insertional inactivation of *pilR* resulted in reduced expression of pilus genes compared with parent strain CP1, while this expression was restored in a *pilR* complemented strain, as demonstrated in Western blotting analysis. This observation was also supported by the collagen-binding results, suggesting that not only the PilR/PilS TCS was involved in the regulation of expression of the NE pilus, but also that this pilus was functional and involved in adhesion. These findings suggest that the PilR/PilS TCS positively regulates expression of the pilus genes, although the mechanism of regulation remains to be determined. Furthermore, the pilus genes were up-regulated in the *virR* and *agrB* mutants, but down-regulated in their respective complemented strains compared with CP1, suggesting that the Agr-like QS system and/or VirR/VirS TCS repressed expression of the pilus genes. This result was contradictory to the findings by Yu et al. (2017). These authors reported that two genes (CP4_0573 and CP4_0575) involved in adhesion were downregulated in an *agrB* mutant, which suggests that the Agr-like QS system up-regulated pili production rather than repressed it. The reason for the difference between the two studies is unclear and may be due to differences in medium composition or its physical proprieties or due to differences in the growth phase of *C. perfringens* strains used. Other factors such as cell density-dependent quorum sensing responses and/or differences in the mechanisms of regulation of the gene expression of pilus-coding genes could also explain the different pilus gene expression responses.

In the present study, *C. perfringens* cultures used in Western blotting and collagen adhesion assays were grown on blood agar and BHI plates overnight (~16 h), while cultures used for

microarray and qPCR were from TPG broth medium grown to an OD600 of 0.8; therefore, *C. perfringens* cultures in the two studies were grown under different environments not only in terms of nutrient composition but also the physical characteristics of the medium used, i.e., agar vs. broth. Sunsine et al. (2020) reported changes in the expression of genes encoding type IV pili–associated proteins depending if *C. perfringens* was grown on liquid or surfaces when the same medium composition was used. These authors reported that when *C. perfringens* bacteria were placed on agar surfaces, they became elongated, flexible and had type IV pili (TFP) on their surface, traits not seen in liquid-grown cells with the same medium as the agar grown cells. These authors have also shown that the main pilin, PilA2 underwent differential post-translational modifications when grown in liquid or on plates, supporting that different growth environments could also cause differences in the expression of genes encoding pili. The same may be true for the NE pilus and requires further investigation.

Differences in growth phases of bacteria could have also influenced pilus expression and adhesion results. Growth rates of mutants and complemented strains and parent strains were not measured in the present study, and this could have been different from growth rates obtained (also not reported) by Yu et al. (2017). Not only are the growth rates of strains important to assess in the future, but it is also important to consider the timing of expression of the genes involved. Li et al. (2020) showed that *virS* was most strongly expressed early during growth while other genes, e.g., *agrD* controlled by the Agr-like QS system were mostly highly produced during late log phase -early stationary phase. Timing of expression of *pilR*, *agrB* and *virR* genes was not measured in either the present study or the study by Yu et al. (2017). This information would help us to get further insight into the dynamics of pili production and which system(s) will be more productive

at the various growth stages of the isolates and if there are timing differences in expression among isogenic strains or not.

Different growth rates and/or growth phases of the bacteria have a major effect on quorum sensing. Bacteria produce signaling molecules (autoinducers) that accumulate during specific stages of growth and the production level of autoinducers can also be influenced by the environment. Although OD 600 of 0.8 to 1.0 used for the overnight incubations of *C. perfringens* were similar in our study and the study by Yu et al. (2017), it is possible that the cultures in the two studies were grown to different population cell densities and Agr-like QS triggered by them responded to different cell densities and environment factors, resulting in separate outcomes of gene regulation and this needs further investigation for example by measuring the count (CFU/mL) changes over time. Gene regulation outcomes would not be the only variable to be affected with the cell density-dependent quorum sensing responses but also adhesion itself could have been compromised by differences in cell growth and cell densities.

The mechanism underlying the different patterns of regulation in pili expression is presently unknown and require further investigation; however, we were enlightened by one previous study which demonstrated expression of PI-1 pili in *Streptococcus pneumoniae* strains is regulated by the positive regulator RlrA, which conferred pilus expression in a biphasic expression pattern and switched pilus biogenesis from an “off” to an “on” state (De Angelis et al., 2011). For pilin expression in *C. perfringens* strains, further studies are needed to investigate whether a biphasic expression pattern is present. Using a Transwell assay, Yu et al. (2017) showed that Agr-like QS system controls NetB production through a diffusible signal. In this assay, the top and bottom chambers of transwell dishes are separated by a semipermeable membrane filter which allows

passage of proteins and/or small molecules but not bacterial cells. These authors found that when CP1 was inoculated into both the top and bottom chambers, NetB was produced whereas different productions results were obtained when other CP1 mutant strains combinations were used. Different combinations of CP1 and the *pilR, agrB,* and *virR* mutant strains could be inoculated into the top and bottom chambers of the transwell dish to assess which combinations enable pilin production and this way confirm if the PilR/PilS TCS could also be regulated by a secreted diffusible signal. It is possible that the PilR/PilS TCS could be regulated by a regulator in a biphasic expression pattern or through a diffusive signal, as the AIP signal or a third novel mechanism may be involved, and this needs further investigation.

Functional performance of *C. perfringens* pili were evaluated in collagen adhesion assays where the binding of CP1 and isogenic CP1 mutants and complemented strains to collagen types I through V were tested. Our results showed that binding of the *pilR* mutant was significantly lower than CP1, whereas binding was restored in its complemented strain. Inability of the pilR null mutant strain to bind collagen substrates suggests that the product of a gene that functions as a collagen adhesin was down-regulated by PilR/PilS TCS and therefore responsible for the inability of cells to bind collagens I to V. Wade et al. (2015) showed that the ability of *C. perfringens* cells to adhere to collagens IV and V correlated with the presence of the collagen adhesin-encoding gene cnaA located in the VR-10B locus. This hypothesis was also supported by the Western blotting results of the current study where the cnaA null strain did not show any binding to collagen. This binding may be restored in the complemented strain but was not investigated in this study and this needs confirmation.

The binding of *agrB* and *virR* mutants to collagen was similar to CP1. However, binding of the *agrB* complemented strain to collagen types I, II, and IV and binding of the *virR* complemented strain to collagen types I, II, III, and IV were significantly reduced compared to CP1. These results are consistent with the Western blotting results, showing that NE pilus production is repressed by the Agr-like QS system including VirR/VirS TCS. Not only that, but this regulatory system seems to affect adhesion to specific collagen types, in particular adhesion to collagen IV which is the most relevant collagen type in the pathogenesis of NE as collagen IV is the most abundant protein in the intestinal basement membrane of the GIT where *C. perfringens* colonization is significant (LeBleu et al., 2007).

Based on our study, there is a potential to block pilus expression by interfering with PilRS signalling. Since the pilus-null mutants are avirulent, this could prevent NE. This potential and functions of PilRS TCS should be further investigated *in vivo* at various stages of infection and different host sites, as birds' responses to NE at the cellular and immunity levels may affect the PilRS signalling. Furthermore, microbiota composition of NE affected birds may interfere with quorum sensing and therefore potentially interfere with the VirR/S TCS, which was shown in our study to likely be a negative regulator of the NE pilus production. Moreover, *in vivo* studies comparing microbiota of healthy versus NE affected birds would allow further insights on the microbiota composition predisposing to NE.

5.2 GENERAL CONCLUSION

In conclusion, our data provide the first evidence for the role of PilR/PilS TCS in the regulation of pilus expression and provide evidence for the regulatory function of Agr-like QS system in pilus

expression. In addition, the present study provides evidence for the binding of pili to five collagen types. The results of antibody blocking assays confirm this binding is mediated specifically by pili.

CHAPTER 6 FUTURE DIRECTIONS

To study the mechanism by which the PilRS TCS regulates pilus production, whether by directly controlling transcription of the VR-10B operon through binding to upstream promoter elements, or indirectly through secondary regulators.

To determine if the regulation of the NE pilus by Agr-like QS system occurs through posttranscriptional mechanisms.

To examine the difference in the regulation of NE pilus between liquid-grown and plate-grown *C. perfringens* cultures.

To define the *in vivo* expression pattern of the NE pilus, both during various stages of infection and at different host sites.

CHAPTER 7 LITERATURE CITED

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