CHARACTERIZATION OF *MANNHEIMIA HAEMOLYTICA*-SPECIFIC BACTERIOPHAGES

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A Thesis
Submitted to the School of Graduate Studies
of the University of Lethbridge
in Partial Fulfillment of the
Requirements for the Degree

MASTER OF SCIENCE
(Molecular Biology/ Microbiology)

Department of Biological Sciences
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

Abstract

Characterization of *Mannheimia haemolytica*-specific bacteriophages

Mannheimia haemolytica is the principal bacterial agent associated with bovine respiratory disease (BRD). It has a significant economic impact on the beef feedlot industry. The current methods for BRD prevention and treatment have various problems and limitations, especially with reports of increased antimicrobial resistance in *M. haemolytica.* Bacteriophage therapy presents a novel method to mitigate *M. haemolytica*. This study aimed to isolate strictly lytic *M. haemolytica*-specific bacteriophages from bovine nasopharyngeal swabs and feedlot trough water. This was accompanied by an extensive characterization of temperate bacteriophages induced from representative strains of a *M. haemolytica* collection. Phage morphology, host specificity, genomic diversity, and comparative genomics were determined. Even though temperate bacteriophages are not ideal candidates for phage therapy, they can be engineered or modified to serve this function. Genome sequences of selected temperate bacteriophages also provide a foundation for future studies on the biology of these microorganisms.

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List of Abbreviations

AMR	antimicrobial-resistant
ATCC	american type culture collection
att	attachment sites
BHIB	brain heart infusion broth
BHV-1	bovine herpes virus-1
BLAST	basic local alignment search tool
BRD	bovine respiratory disease
BRDC	bovine respiratory disease complex
BRSV	bovine respiratory syncytial virus
BVDV	bovine viral diarrhea virus
cAMP	cyclic AMP
CAS	CRISPR-associated sequence
CDS	protein coding sequence
CFU	colony-forming unit
CRISPR	clustered regularly interspaced short palindromic repeats
ETEC	enterotoxigenic *Escherichia coli*
IBR	infectious bovine rhinotracheitis virus
ICTV	international committee on taxonomy of viruses
LHT	Lwoff, Horne, and Tournier
LKT	leukotoxin
LPS	lipopolysaccharide
MH	*Mannheimia haemolytica*
MNA	modified nutrient agar
NCBI	national center for biotechnology information
ORF	open reading frame
PCNV	provisional committee on nomenclature of viruses
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulsed field gel electrophoresis
PI-3	parainfluenza type 3
RBS	ribosomal binding site
RFLP	restriction fragment length polymorphism
SPL	*Staphylococcus aureus* phage lysate
TEM	transmission electron microscope
tRNA	transfer RNA
TSA	tryptic soy agar
UGCC	University of Guelph culture collection

CHAPTER ONE

1.1 Introduction

The occurrence of bovine respiratory disease (BRD) continues to be one of the major health issues in the global cattle production industry. The morbidity and mortality associated with BRD have significant economic consequences on the feedlot industry (Duff and Galyean, 2007; Edwards, 1996). An overview of BRD is provided including various factors that contribute to its disease process and outcome, together with symptoms, pathology, and methods currently available to control and prevent BRD. *Mannheimia haemolytica* has been identified as the principal bacterial pathogen associated with BRD. It encodes several important virulence factors responsible for BRD pathogenesis and has shown increasing resistance to many antimicrobial agents (Desmolaize et al., 2011). Based on previous studies, bacteriophage therapy presents an innovative area of research for preventing bacterial infections. By understanding basic phage biology and phage characteristics during the infection and reproduction process, one can explore the possibility of using bacteriophage as a means of controlling BRD in cattle.

1.2 Economics

According to a cross-sectional survey conducted in the United States, BRD is the leading cause of morbidity and mortality in feedlots (Woolums et al., 2005), with approximately 75% of cattle morbidities and up to 70% of mortalities being linked to this disease (Edwards, 1996). Mortality from BRD and the expense of medicine and labour to

treat BRD contribute significantly to production costs. Feedlot performance and carcass traits are also negatively affected by BRD (Duff and Galyean, 2007; Gardner et al., 1999). Montgomery et al. (1984) and Roeber et al. (2001) reported that BRD negatively affected marbling scores, quality and yield grade. Calves receiving three or more medical treatments for BRD returned considerably less profit than calves that were not treated (Fulton et al., 2002). It is estimated that BRD results in losses of over $690 million annually in the United States (Badcock, 2008). When combined with expenses associated with BRD, this figure is estimated at as much as one billion dollars (Hodgins and Shewen, 2004a).

1.3 Bovine respiratory disease (BRD)

1.3.1 BRD/BRDC

Nowadays, the term bovine respiratory disease (BRD) is often being used interchangeably with bovine respiratory disease complex (BRDC) and shipping fever. Originally, BRDC was defined as three major clinical entities: 1) enzootic pneumonia of calves, 2) shipping fever complex, and 3) atypical interstitial pneumonia (Lillie, 1974). To avoid confusion in this paper, BRD refers to the clinical disease condition itself, while BRDC refers to various bacterial, viral, and environmental causes associated with this disease. The occurrences of BRD in newly-weaned or received cattle continue to be the major health problem for the US beef cattle industry (Duff and Galyean, 2007). While BRD is ultimately an infectious respiratory viral/bacterial disease in cattle, there are many potential exacerbating factors and outcomes that complicate the problem. Moreover,

the etiology of BRD is complex and multi-factored and can be categorized into three main components, bacterial, viral, and environmental/physiologic stressors.

1.4 Factors that contribute to BRD

1.4.1 Bacterial agents

The main bacterial pathogens of BRD include *M. haemolytica, Pasteurella multocida, Histophilus somni* (formerly *Haemophilus somnus*) *and Mycoplasma bovis* (Apley, 2006; Griffin et al., 2010; Pandher et al., 1998). These bacteria are ubiquitous in the cattle population as normal nasopharyngeal commensals (Panciera and Confer, 2010). When cattle encounter stress and viral or parasitic infections that impair health and suppress the host immune system, a window of opportunity is provided for secondary bacterial pathogens to colonize and reproduce in the upper respiratory tract (Griffin et al., 2010; Irsik, 2007). Regardless of the initiating events, *M. haemolytica* is considered to be the predominant bacterial pathogen associated with BRD (Duff and Galyean, 2007; Gioia et al., 2006; Griffin et al., 2010). It is the most common bacterium recovered from BRD cattle and therefore has received the most attention by researchers, even though mixed bacterial and viral infections are often associated with BRD. Pneumonia caused by these bacterial pathogens is the most significant cause of BRD associated morbidity and mortality. These pathogens may form similar pneumonic lesions even though they carry distinctive and common virulence factors (Griffin et al., 2010).

1.4.2 Environmental/Physiological agents

The current marketing and transport system used for feeder cattle in North America naturally impose stresses on animals (Duff and Galyean, 2007; Griffin et al., 2010). Various stressors are often introduced when calves are weaned and transported to a sale barn. Environmental stressors during transportation may include chilling and overheating, dehydration, starvation, and exhaustion depending on weather conditions (Rice et al., 2007). Depending on feedlot management practices, newly received calves may experience various degrees of stress caused by commingling, processing, and introduction to new feed and environments (Bowland and Shewen, 2000; Duff and Galyean, 2007). For instance, commingling of calves from various sources may destroy the social hierarchy and impose additional stress on the animals (Loerch and Fluharty, 2000). These stressors impose either psychological or physical pressure on the animals and often increase host susceptibility to BRD due to compromised immune responses (Duff and Galyean, 2007; Srikumaran et al., 2007). This is evident especially when animals are exposed to high levels of infectious agents in these situations (Jericho. 1979), and when stressors cause transient elevation of plasma cortisol levels along with a decrease in serum complement activity in calves (Rice et al., 2007; Srikumaran et al., 2007). In these environments, viral infection or inhaled irritants can lead to cellular damage in the upper respiratory tract (Irsik, 2007), and provide a window of opportunity for secondary pathogens such as *M. haemolytica* to colonize and become established. To further complicate the problem, animals are often grouped together with other cattle with unknown disease and vaccination status (Griffins et al., 2010).

1.4.3 Viral agents

Multiple viruses have been shown to be associated with BRD (Bowland and Shewen, 2000; Duff and Galyean, 2007; Fulton, 2009; Griffins et al., 2010). Co-infection with a virus and a bacterium does occur and may result in weakened clearance of infecting bacteria and lesions within the respiratory tract (Griffins et al., 2010). The major viral agents linked to BRD are bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), bovine herpes virus-1 (BHV-1), infectious bovine rhinotracheitis virus (IBR), and parainfluenza type 3 (PI-3) virus (Apley, 2006; Edwards, 2010; Griffins et al., 2010; Hodgson, 2005). Infection with BVDV leads to damage of alveolar macrophages thus inducing immunosuppression of animals (Babiuk et al., 2004). Infection with BRSV results in loss of cilia or necrosis of bronchial and bronchiolar epithelial cells, and reduces phagocytosis and opsonization by alveolar macrophages (Griffins et al. 2010). Infections of BHV-1 can spread to lower respiratory tract and cause necrosis of epithelial cells (van Vuuren, 2004). Infections with PI-3 are associated with little or no clinical symptoms in cattle, but do cause necrosis of the ciliated epithelium (Griffins et al., 2010). Most of these viruses target respiratory epithelial and ciliated cells of the animal. Viral infection in many cases promotes necrosis of epithelial cells and reduces their capacity for mucociliary clearance of agents from the respiratory tract. The accumulation of fluid and cellular debris plus formation of surface lesions in the respiratory system provide an ideal environment for secondary bacterial infection (Griffins et al., 2010).

1.5 Symptoms and Pathology of BRD

1.5.1 Host innate immunity

The respiratory epithelial surface of healthy cattle provides mechanical, chemical and microbiological barriers to prevent pathogen infection (Frank, 1984). Many bacteria including potential pathogens such as *M. haemolytica* and other members of the *Pasteurellaceae* may exist as commensals in the upper respiratory tract and nasopharynx of healthy animals (Cusack et al., 2003; Griffins et al., 2010). Mucus secretion prevents the adherence of bacteria, and the epithelial cilia provide constant upward movement of the mucus that carries potential pathogens. There are also bactericidal proteins and peptides on the epithelial surfaces of the lung. These bind to the cell surface of pathogens, promoting phagocytosis by resident macrophages and neutrophils (Griffins et al., 2010).

The lung expresses many extracellular, cell surface, endosomal, and cytoplasmic receptors that recognize conserved substances (teichoic acid, lipopolysaccharide, cytokine-phosphate-guanine DNA, etc.) produced by microbial pathogens (Ackermann et al., 2010). For instance, there are cell surface Toll-like receptors on the lung epithelia. Activation of these receptors can lead to nuclear factor kappa B activity and inflammatory reactions, or induction of interferon genes (Ackermann et al., 2010). Cytosol receptors such as nucleotide oligomerization domain-like receptors can recognize pathogens in the cytoplasm and trigger cell activation that leads to proinflammatory responses (Ackermann et al., 2010). These elements together provide the host with defence mechanisms against pathogens. To a healthy animal, some of the bacterial and viral agents associated with BRD may present little or no harm to the host. However,

under stress, the innate immune system is compromised and increased bacterial colonization may occur in stressed animals (Highlander, 2001). As BRD is typically manifested through a primary viral infection of the upper respiratory tract, this predisposes cattle to secondary bacterial infections (Edwards, 2010; Hodgson, 2005). As the disease progresses, commensal organisms may migrate into the lower respiratory tract and eventually the lungs, where bacterial infections can cause significant tissue damage. It is the combinations of stress, viral and bacterial agents that cause the clinical symptoms of BRD and even death.

1.5.2 Symptoms

As early as 7 to 10 days after calves arrive at the feedlot, clinical signs of BRD can be detected (Rice et al., 2007). Newly received or stressed animals are considered to be at high risk of developing BRD. Morbid cattle are usually identified visually, when animals display various signs of discomfort. Changes in the behaviour and appearance of individuals such as nasal/ocular discharge, depression, lethargy, anorexia, fever, increased respiratory rate, and moist cough, or combinations of above may all be indicative of BRD (Duff and Galyean, 2007; Griffins et al., 2010; Sowell et al., 1999). More severe symptoms may include respiratory distress, encrusted muzzles, excessive tear production, and dyspnea (Griffins et al., 2010). A common observation in diseased calves is a stressed stance with elbows abducted and neck extended (Duff and Galyean, 2007; Hodgins and Shewen, 2004a; Rice et al., 2007; Zecchinon at al., 2005).

1.5.3 Pathology

Regardless of the initiating event, progression of the disease often results in secondary bacterial infection of the lower respiratory tract. Eventually this may lead to severe bacterial pneumonia (Panciera and Confer, 2010), a condition that is frequently fatal. Often, calves develop pneumonia shortly after arrival at the feedlot (Gagea et al., 2006; Rice et al., 2007). The major cause of death in animals suffering from BRD is acute pleuropneumonia (Griffins et al., 2010; Rice et al., 2007), also called serofibrinous pleuropneumonia (Hodgins and Shewen, 2004a; Fulton et al., 2002), or multifactorial fibrinonecrotizing pneumonia (Highlander et al., 2000). In general, necropsy shows bilateral, cranioventrally distributed, very firm, minimally compressible lung consolidation (Griffins et al., 2010; Panciera and Confer, 2010). Consolidated lobes usually have a marbled appearance and infected areas accumulate yellow fibrin and edema (Griffins et al., 2010; Panciera and Confer, 2010). Chronic BRD infections are characterized by coagulation necrosis outlined with pale fibrous tissue and extensive adhesions formed by fibrin deposits in the pleural and pericardial cavities (Fulton et al., 2002; Griffins et al., 2010; Hodgins and Shewen, 2004a).

1.6 Management/Prevention of the BRD

1.6.1 Management

For the past three decades, efforts have been made to reduce BRD incidence through improved management strategies and early recognition of sick animals (Griffins et al., 2010). Reducing potential stressors and better management practices of highly susceptible animals seems to decrease the incidence and severity of BRD infection

(Cusack et al., 2003; Griffins et al., 2010). According to Gorden and Plummer (2010), prevention practices of BRD in dairy calves include the development and maintenance of a robust immune system, sound nutrition, proper vaccination, biosecurity, and provision of adequate ventilation. Even though these practices can be implemented to improve cattle health, they may seem impractical in the view of feedlot cattle producers and difficult to achieve in a cattle production system. An effective means of minimizing weaning stress and decreasing BRD morbidity is by pre-conditioning or pre-weaning of the cattle (Cusack et al., 2003; Duff and Galyean, 2007). These programs may include a weaning period, vaccinations, antihelminthic treatment, castration, dehorning, and acclimatization to new feed and environment (Thomson and White, 2006). However, these programs are not widely implemented as they require additional effort on behalf of the cow-calf producer that is not economically recovered (Duff and Galyean, 2007; Waggoner et al., 2005). Identifying the causative agents of BRD aids proper treatment. A nasal swab culture can be obtained easily for identifying the species responsible for causing bacterial pneumonia (De-Rosa et al., 2000). Antibiotic susceptibility of the isolated bacteria can be determined from the nasal swab as well (De-Rosa et al., 2000). However, laboratory tests for the above detections typically require additional time and cost (Duff and Galyean, 2007), which make them less useful in a therapeutic treatment program. Due to the complexity of BRD, even with a significant increase in the amount and quality of research, vaccination and therapeutic practices lack efficacy (Griffin et al., 2010).

1.6.2 Metaphylaxis/Prophylaxis

The use of metaphylactic antimicrobial therapy can be considered as prevention and treatment as cattle arriving at feedlot facility not only may be at risk of developing BRD, but also actively experiencing various stages of the disease process (Duff and Galyean, 2007; Lofgreen, 1983; Young, 1995). Prophylactic medication programs are also effective against BRD bacterial pathogens such as *M. haemolytica* (Frank and Duff, 2000; Frank et al., 2002). The metaphylactic method is based on the concept of treating the entire population at a single point in time to eliminate or minimize an expected outbreak of disease. Treatment of the population is often preferable due to the diagnostic challenges of identifying individual BRD cases in calves (Nickell and White, 2010) and metaphylaxis has proven to be more efficient and cost effective in controlling bacterial pathogens associated with BRD (Edwards, 2010). The use of metaphylaxis during relocation of cattle has been successfully used to reduce the incidence of BRD (Donkersgoed et al., 1993). Moreover, administrating long-acting antibiotics to newly arrived cattle has been shown to significantly reduce morbidity and improve rate of gain (Duff and Galyean, 2007; Rice et al., 2007; Thomson and White, 2006). Typical antimicrobial compounds administered to cattle include the injectables: ceftiofur, oxytetracycline, enrofloxacin, florfenicol, danofloxacin, tilmicosin, tulathromycin, tylosin, chlortetracycline, spectinomycin, erythromycin and penicillin (Carson et al., 2008; Fulon, 2009; Nickell and White, 2010; Thomson and White, 2006). In addition, there are several water and in-feed medications such as sulfa drugs, tetracyclines and tylosin (Fulton, 2009). These antimicrobials are listed in Table 1.1. A newer non-steroidal anti-inflammatory agent, flunixin meglumine was made available in 2009.

Table 1.1. Typical antimicrobial compounds administered to cattle to control or prevent bovine respiratory disease.

Antimicrobial	Family	Trade names	Route of Administration
ceftiofur	cephalosporin	Excede / Naxcel	Injectable
oxytetracycline	tetracycline	Terramycin / Oxy-Tet, etc	Injectable
enrofloxicin	fluoroquinolone	Baytril	Injectable
florfenicol	thiamphenicol	Nuflor	Injectable
danofloxacin	fluoroquinolone	A180	Injectable
tilmicosin	macrolide	Pulmotil / Micotil	Injectable
tulathromycin	macrolide	Draxxin	Injectable
tylosin	macrolide	Tylan	Injectable / Feed
chlortetracycline	tetracycline	Aureomycin	Injectable / Feed
spectinomycin	aminocyclitol	Trobicin / Spectam	Injectable
erythromycin	macrolide	Erythro / Gallimycin	Injectable
penicillin	beta-lactam	Crystacillin / Flo-Cillin, etc	Injectable
sulfas	sulfonamides	Tribrissen / Trivetrin	Feed
tetracyclines	tetracycline	Panmycin / Polyotic	Feed

1.6.3 Vaccination

The combination of vaccination and management strategies can provide calves an opportunity to build immunity during a time when stress and disease challenge is minimal (Edwards, 2010). However, due to the current feedlot marketing system, vaccination and background history of newly received cattle may be vague or unknown. Therefore, it becomes crucial for the feedlot producer to obtain vaccination records from the source of origin of the calves to help determine immune status (Edwards, 2010). There are concerns when administering vaccines to cattle. Immunity usually takes 2 to 3 weeks to develop, so timing is critical. Moreover, in order to elicit protective immunity, multiple doses of vaccine may be required. This adds work and expense for the cow-calf producer. While administering a vaccine provides the immune system exposure to the antigen, this does not guarantee a positive immune response from the animal (Edwards,

2010). Even though vaccination against BRD is a common practice in feedlot management, studies supporting its efficacy are limited (Bowland and Shewen, 2000; Lorenz et al., 2011; Perino and Hunsaker, 1997). Common commercial vaccination products available for BRD prevention include viral antigens for BHV-1 (IBR), BVDV, PI3, and BRSV (Table 1.2) (Bowland and Shewen, 2000; Duff and Galyean, 2007; Edwards, 2010), with some of these vaccines being integrated into preconditioning programs (Duff and Galyean, 2007). Common bacterial antigens used for control of BRD include *M. haemolytica* and *H. somni*. These antigens can be used singly or in a combined viral and bacterial vaccine, and most products can be administered subcutaneously (Edwards, 2010). Vaccination against *M. haemolytica* has produced mixed results. These vaccines are problematic as they require serological information of the infecting strain to be effective and cross-protection against other serotypes is generally not possible (Kehrenberg et al., 2001). However, other studies have shown reduced morbidity, mortality, and relapses with the use of *M. haemolytica* bacterin-toxoid vaccines (Fulton, 2009; MacGregor et al., 2003).

Table 1.2. Typical commercial vaccination products for controlling bovine respiratory-associated viruses and bacteria.

	Target agents					
Manufacture & trade names	BHV-1 (IBR)	BVDV	PI3	BRSV	*M. haemolytica*	*H. somni*
AgriLabs / Titanium® 5 [a]	√	√	√	√		
AgriPharm / Covert™10 [b]	√	√	√	√		
Aspen / TiterVac® 10 [a]	√	√	√	√		
Bayer / BRSV Vac® 4 [a]	√	√	√	√		
Boehringer Ingelheim Vetmedica / Sentry™ 4 [d]	√	√	√	√		
Durvet / Durvac™ 5 [a]	√	√	√	√		
Novartis / Vira Shield® 5 [e]	√	√	√	√		
Pharmacia & Upjohn / Herd-Vac™ 3 [c]	√	√	√			
Pfizer / Bovi-Shield® Gold 5 [a]	√	√	√	√		
Ayerst / Response® [d]					√	
Biowest / Somnu-Star Ph™ [d]					√	√
Pfizer / One Shot ™ [c]					√	
Pfizer / Somubac™ [d]						√
Pfizer / Resvac® 3 BRSV/Somubac™ [d]	√	√	√	√		√
Vetrepharm / Virabos™-4 + *H. somnus* [d]	√	√	√	√		√

Recommended dosage:

[a] 1 dose primary vaccination followed by 1 dose of BRSV vaccine 2-4 weeks later. Annual revaccination is recommended.

[b] 1 dose primary vaccination, repeat after 2-4 weeks and once annually.

[c] 1 dose primary vaccination.

[d] 2 doses primary vaccination (2-4 weeks apart). Annual revaccination is recommended.

[e] 1 dose primary vaccination and revaccinate in 4-5 weeks.

1.7 *Mannheimia haemolytica*

1.7.1 The organism

M. haemolytica is a weakly haemolytic, Gram-negative non-spore-forming coccobacillus (Griffin et al., 2010; Rice et al., 2007; Zecchinon et al., 2005). It often shows up as a complicating agent in BRD and various researchers suggest *M. haemolytica* is the principal causative agent of the disease (Duff and Galyean, 2007; Gioia et al., 2006; Griffin et al., 2010; Zecchinon et al., 2005). Among the bacterial agents associated with BRD, *M. haemolytica* is the most highly characterized as it is the bacterium most frequently recovered from cattle suffering from BRD. Beginning in the late 1970s and the early 1980s, a major BRD research emphasis was placed on *M. haemolytica* as the primary etiological agent, and challenge studies were performed (Fulton, 2009). *M. haemolytica* is characterized as being an oxidase-positive, indole-negative, fermentative, non-motile facultative anaerobe (Griffin et al., 2010; Rice et al., 2007). *M. haemolytica*, formally known as *Pasteurella haemolytica*, now belongs to the class Gamma-proteobacteria, order *Pasteurellales*, and family *Pasteurellaceae* (Griffin et al, 2010; Zecchinon et al., 2005). There are presently six species within the *Mannheimia* genus: *M. haemolytica*, *Mannheimia granulomatis*, *Mannheimia glucosida*, *Mannheimia ruminalis*, *Mannheimia varigena*, and *Mannheimia caviae* (Christensen et al., 2011; Gioia et al., 2006). There are 12 capsular serotypes in *M. haemolytica*: A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16, and A17 (Fulton, 2009; Katsuda et al., 2007; Srikumaran et al., 2007). The predominant serotype isolated from healthy cattle is serotype A2, but both serotype A1 and A2 have been found to colonize the bovine upper respiratory tract (Hodgins and Shewen, 2004b), with serotype A2 generally considered as

being non-pathogenic to cattle. However, serotype A2 does cause pneumonia in sheep (Highlander, 2001; Rice et al., 2007). When an animal's immune system becomes compromised, the commensal relationship is disrupted. *M. haemolytica* becomes pathogenic as it migrates and infects the lower respiratory tract. At this stage, A1 becomes the predominant serotype and causes characteristic bronchopneumonia (Griffin et al., 2010; Hodgins and Shewen, 2004b), and is the most frequent serotype isolated from pneumonic tissue (Gioia et al., 2006; Griffin et al., 2010; Katsuda et al., 2007; Pandher et al., 1998). There has been an increased prevalence of serotype A6 associated with BRD in the United Kingdom and the United States (Hodgins and Shewen, 2004b; Kehrenberg et al., 2001; Purdy et al., 1997). As a result, serotype A1 and A6 are now considered to be the virulent strains of *M. haemolytica* associated with BRD. Studies also show serotype A1 and A6 have extremely similar mechanisms of pathogenicity, aside from capsule differences (Davies et al., 2002; Srikumaran et al., 2007).

1.7.2 Virulence factors

Numerous papers have been published that describe the virulence and pathogenesis of *M. haemolytica*. Virulence factors allow *M. haemolytica* to evade clearance and avoid host defences while rapidly reproducing in the lower respiratory tract (Griffin et al., 2010). *M. haemolytica* has a number of virulence factors that are responsible for adherence and colonization within the respiratory tract. Virulence factors include adhesion proteins, capsular polysaccharides, lipopolysaccharide, fimbriae, sialoglycoprotease, and neuraminidase (Rice et al., 2007). Capsular polysaccharides are used for adherence and invasion, and impair the phagocytic ability of neutrophils (Apley,

2006; Rice et al., 2007). Sialoglycoprotease is believed to reduce the effectiveness of opsonizing antibodies (Lee and Shewen, 1996). Outer membrane proteins provide protective immune responses and allow *M. haemolytica* to replicate in a low-iron host-regulated environment (Apley, 2006; Highlander, 2001; Rice et al., 2007). Adhesins play a crucial role in the colonization process (Hodgins and Shewen, 2004b; Zecchinon et al., 2005). Neuraminidase also promotes colonization by reducing respiratory mucosal viscosity, thus allowing bacteria access to the cell surface (Zecchinon et al., 2005). One critical virulence factor is the lipopolysaccharide (LPS) cell wall component. It is responsible for the macrophage activation associated with endotoxin and causes haemorrhage, edema, hypoxemia, and acute inflammation in the host (Breider et al., 1990; Griffin et al., 2010). Among these virulence factors, leukotoxin (LKT), a pore-forming calcium-dependent cytotoxin belonging to the RTX family (Czuprynski et al., 2004; Gioia et al., 2006; Highlander et al., 2000), is considered as the main virulence factor in *M. haemolytica* pathogenesis (Gioia et al., 2006; Zecchinon et al., 2005). It is closely associated with pneumonic damage and it is responsible for lysis of ruminant leukocytes and platelets (Fulton, 2009; Gioia et al., 2006; Rice et al., 2007). Leukotoxin also allows the bacterium to evade destruction by the host's phagocytic cells (Rice et al., 2007), with its influence being concentration dependent. At low concentrations, LKT induces apoptosis by activating leukocytes to release various inflammatory substances including reactive oxygen intermediates, eicosanoids, and cytokines (Czuprynski et al., 2004; Hodgins and Shewen, 2004b). Higher LKT concentration impairs leukocyte function, resulting in cellular death and the formation of lung lesions and vascular damage (Czuprynski et al., 2004; Hodgins and Shewen, 2004b; Rice et al., 2007).

1.7.3 Antimicrobial resistance

The common use of antibiotics for disease prevention and growth promotion in domestic animals can potentially lead to the selection of antimicrobial-resistant (AMR) bacteria. Therefore, all bacterial pathogens associated with BRD could potentially develop resistance to antimicrobials. This discussion only describes AMR as it relates to *M. haemolytica* since it is the primary bacterial causative agent of BRD in cattle. Treatment and prevention of BRD associated with *M. haemolytica* usually entails prophylactic or metaphylactic medication with antimicrobials. Incorrect or ineffective usage of antimicrobial treatment may result in an increased occurrence of AMR in *M. haemolytica* (Hodgins and Shewen, 2004b; Watts et al., 1994). In order for treatment to be effective, antimicrobials must be administered very early in the disease process (Irsik, 2007). *M. haemolytica* has shown increasing resistance to many antimicrobial agents (Desmolaize et al., 2011). For instance, *M. haemolytica* is commonly found to be resistant to aminoglycosides, β-lactams, sulfonamides, streptomycin, macrolides, sulfamethazines, and tetracyclines (Highlander, 2001; Hodgins and Shewen, 2004b; Watts et al., 1994), and resistance to fluoroquinolones has been recently observed (Hodgins and Shewen, 2004b). In addition, due to delayed laboratory analysis, a high degree of variability is observed among isolates originating from the same animal source, and resistance rates vary over time (Hodgins and Shewen, 2004b). Most of these resistance genes seem to be associated with small or large plasmids, as well as transposons (Kehrenberg and Schwarz, 2001; Watts et al., 1994; Zecchinon et al., 2005). *M. haemolytica* is considered to be naturally competent (Gioia et al., 2006), and the resistance genes may be acquired by horizontal gene transfer (Kehrenbreg et al., 2001).

The increased incidence of AMR is a major concern in feedlot industry and monitoring of antimicrobial susceptibility in *M. haemolytica* provides insight into effective antimicrobial therapies for bovine pneumonia (Katsuda et al., 2009). Similarly, a greater emphasis must be placed on therapies that reduce or minimize the selection of resistant mutants (Griffin et al, 2010).

1.8 Introduction to Bacteriophage

1.8.1 Bacteriophage

Viruses are a complex and diverse group of microorganisms. Viruses that infect bacteria are called bacteriophages (Willey et al., 2008), or "phages" for short (Ackermann, 2003). Bacteriophages were first discovered independently by Frederick Twort and Felix d'Herelle in the early 1900s (Ackermann, 2003; Adams, 1959; Duckworth, 1976; Kutter and Sulakvelidze, 2005; Willey et al., 2008). They are probably the most abundant biomass on earth, both in terrestrial and aquatic ecosystems (Kutter and Sulakvelidze, 2005; Willey et al., 2008). The global population of tailed phages alone is estimated to be in the order of 10^{31} entities (Hendrix et al., 1999), and there can be up to 10^{8} viral particles per millilitre in various aquatic systems (Wommack and Colwell, 2000). Bacteriophages have a major influence on the structure, activity, temporal dynamics, biological diversity and evolution of microbial communities (Letarov and Kulikov, 2009). Bacteriophages contribute considerably to bacterial genetic variability. They may serve as agents of lateral gene transfer (Canchaya et al., 2003a), and carry virulence factors that confer pathogenicity to their host (Willey et al., 2008). Early research on bacteriophage was concentrated on characterizing physical, genetic,

biochemical, physiological, and morphological features, with a few model phages such as phage lambda (λ) and T-series phage that were studied extensively (Clark and March, 2006; Kutter and Sulakvelidze, 2005). Bacteriophage DNA, mRNA, and gene regulation were identified and characterized by exploring these model phage systems (Clark and March, 2006).

Shortly after the discovery of bacteriophages, they have been widely used to treat bacterial infections. Despite the discovery of antimicrobials and decreased interest in the West, phage therapy continued to be applied extensively in the former Soviet Union (Clark and March, 2006; Kutateladze and Adamia, 2010). A vast amount of therapeutic phage work has been conducted by the Georgia Eliava Institute of Bacteriophage, Microbiology and Virology in Georgia (Clark and March, 2006; Kutateladze and Adamia, 2010). In the former Soviet Union, phage preparations have been used for therapy, prophylaxis, and diagnosis of numerous bacterial infections (Kutateladze and Adamia, 2010). It was not until the 1980s that phage research started to shift towards development of new techniques that utilize phages and their enzymes (Kutter and Sulakvelidze, 2005). This included the use of phage enzymes and vectors in genetic engineering, phage display and detection technologies (Kutter and Sulakvelidze, 2005). Nevertheless, they were utilized mainly as research tools at this time in the west. Research on phage biology, including phage function, distribution within natural microbial communities, and their potential therapeutic application, was largely ignored until recently (Clark and March, 2006; Kutter and Sulakvelidze, 2005).

Phage ecology has developed rapidly with advances in molecular biology, including genomic and metagenomic studies, and methodologies applied in detection and

analysis. Recently, there has been an increased interest in potential phage applications in industry and medicine. For instance, bacteriophages have been proposed as new candidates for vaccines, a means of detecting pathogenic bacteria, a therapy against antimicrobial resistant bacteria, and a tool for various proteins, peptides, or antibodies screening applications (Clark and March, 2006).

1.8.2 Classification System

Soon after the discovery of bacteriophages, many researchers proposed various schemes of classification (Kutter and Sulakvelidze, 2005). However, most of these schemes were not accepted by the scientific community, and researchers continued to come up with revised schemes for viral classification. A classification scheme proposed in 1962 by Lwoff, Horne, and Tournier was adopted later by the Provisional Committee on Nomenclature of Viruses (PCNV). This scheme was known as the Lwoff, Horne, and Tournier (LHT) system. Classification was based on the virus' nucleic acid, capsid shape, presence of an envelope, and number of capsomers. A few years later, the PCNV became the International Committee on Taxonomy of Viruses (ICTV), which is the current organization responsible for standardizing the classification of all viruses, including bacteriophages (Kutter and Sulakvelidze, 2005; Willey et al., 2008). The committee preserved most of the taxa organized by the LHT scheme, even though it no longer uses the same classification system. The ICTV classification system now considers the nature of nuclear acids, particle structure, nucleotide or amino acid sequences, and other available information such as natural host range, pathogenicity, and mode of transmission in its classification system (Büchen-Osmond, 2003; Kutter and Sulakvelidze, 2005;

Willey et al., 2008). The current (2009) ICTV virus taxonomy contains 6 orders, 87 families, 348 genera, and 2,285 species. Due to the difficulty of establishing evolutionary relationships, there are still many viral families that are not assigned to an order, and ICTV has yet to develop complete taxonomical structures above the family level (Van Regenmortel et al., 2000; Willey et al., 2008). In addition, there are genera that cannot be grouped with other taxa due to lack of data (Kutter and Sulakvelidze, 2005). An alternative classification scheme used by many virologists is the Baltimore system. This system complements the ICTV classification as it focuses on the genome of the virus and the process of viral mRNA synthesis (Willey et al., 2008). The majority of viruses in the taxonomical system now are viruses of eukaryotes and bacteria, with few archaeal viruses been present (Willey et al., 2008).

1.8.3 Current Classification

Bacteriophages are sometimes defined as viruses of prokaryotes and they have been shown to infect more than 140 bacterial genera (Ackermann, 2001; Ackermann, 2003; Kutter and Sulakvelidze, 2005). They are extremely diverse in terms of their structural, physiological, and biological properties (Casjens, 2003; Kutter and Sulakvelidze, 2005). Currently, the bacteriophages are classified into one order, 13 families, and 34 genera (Lavigne et al., 2008 and 2009). The taxonomy of phages is still under development, as more phages are being discovered and characterized. Almost all bacteriophages contain double-stranded DNA, but phages with single-stranded DNA, single-stranded RNA, or double-stranded RNA also exist. Some phages may also possess

lipid envelopes or internal vesicles. There are four types of virions in bacteriophage: tailed, polyhedral, filamentous, and pleomorphic (Ackermann, 2003).

This review will focus on tailed phages alone as they represent the largest and most prevalent group among bacteriophages, whereas tailless (polyhedral, filamentous, and pleomorphic) phages constitute less than 4% of all phages currently recognized (Ackermann, 2001). Tailed phages have been classified to a single order, *Caudovirales* (Ackermann, 1998; Ackermann, 2003; Maniloff and Ackermann, 1998). Capsid structure is an important morphological trait utilized for bacteriophage classification. Typical tailed phage particles have icosahedral heads (capsids) and helical tails. This combination of cubic symmetry and helical tail is called binary or binal symmetry (Kutter and Sulakvelidze, 2005; Lwoff et al., 1962; Willey et al., 2008). They consist of a non-enveloped protein coat (capsid) and linear double-stranded DNA (Ackermann, 2003; Kutter and Sulakvelidze, 2005). Tailed phage DNA composition often reflects that of the host bacterium, but in rare cases can contain unusual bases such as 5-hydroxymethylcytocine (Ackermann, 2003; Kutter and Sulakvelidze, 2005). The capsids are constructed from groups of capsomers (usually 5-6 protein subunits) that can be difficult to observe (Ackermann, 2003; Kutter and Sulakvelidze, 2005) and tails are either true helices or stacked disks that often carry structures such as base plates, spikes, or fibers (Ackermann, 2003; Kutter and Sulakvelidze, 2005; Willey et al., 2008). According to ICTV virus taxonomy list (2009), there are three families and 28 genera within the order *Caudovirales*. The tailed phage families are phylogenetically related and they are determined primarily on differences in tail structure, while genera are defined by genome structure and replication criteria (Ackermann, 2003; Kutter and Sulakvelidze, 2005;

Maniloff and Ackermann, 1998). *Myoviridae*, *Siphoviridae*, and *Podoviridae* comprise the three families in the order *Caudovirales. Myoviridae* consist of 25% of tailed phages, while *Siphoviridae* and *Podoviridae* make up 61% and 14%, respectively as determined by a study of frequency of phage morphologies in various environments (Ackermann, 2001; Maniloff and Ackermann, 1998). Phages in family *Myoviridae* consist of contractile tails with two components, a sheath and a central tube (Ackermann, 2001). Phages in family *Siphoviridae* consist of long, non-contractile tails without sheath, and phages in family *Podoviridae* are similar to *Siphoviridae* except they have short, non-contractile tails. The variations in tail morphology reflect major differences in viral genome complexity, mode of infection, and in virion assembly and maturation (Maniloff and Ackermann, 1998). The current tailed phage classification into genera is an ongoing process as more phages are being discovered and many unclassified phages are expected to be grouped in the future.

1.9 Basic Phage Biology

1.9.1 General Properties of Bacteriophages

Similar to all viruses, bacteriophages are absolute parasites (Kutter and Sulakvelidze, 2005). They are able to reproduce quickly in susceptible hosts. At the same time, they are highly specific as each phage only targets a specific group of bacteria (Kutter and Sulakvelidze, 2005). Phages exist in two phases; extracellular and intracellular. They may contain a few enzymes and cannot reproduce independent of hosts in the extracellular phase, even though they possess all the required information to direct their own production (Kutter and Sulakvelidze, 2005; Willey et al., 2008). Most

phages remain infectious for a considerable period of time, even in the absence of a suitable host. During the intracellular phase, typical viruses simply induce host metabolism to synthesize viral components. Phage particles are assembled, with genome packaged inside the capsid, and released (Willey et al., 2008).

Bacteriophages differ from most viruses as some phages possess the ability to integrate into host chromosome and remain as part of the bacterial genome. Based on this particular feature, bacteriophages can be categorized into two classes: virulent and temperate phages. Virulent, or strictly lytic phages, can only replicate during a lytic cycle (Kutter and Sulakvelidze, 2005; Willey et al., 2008). After phage DNA enters the host cell, virulent phages take over host metabolism and cellular machinery to execute the production of new phages. Following accumulation of assembled phage particles, the host cell lyses and releases a group of new phages into the surrounding environment. These new phage particles disperse and infect more bacteria.

In contrast, temperate (sometimes mistakenly termed lysogenic) phages may initiate a lytic cycle or a lysogenic cycle (Clokie and Kropinski, 2009; Kutter and Sulakvelidze, 2005; Willey et al., 2008). When temperate phages enter the lysogenic cycle, the phage genome is integrated into the host genome (Freifelder and Meselson, 1970) or sometimes maintained as a plasmid within the host cell (Ikeda and Tomizowa, 1968; Inal and Karunakaran, 1996). A temperate phage in this condition is called a prophage (Clokie and Kropinski, 2009; Kutter and Sulakvelidze, 2005; Willey et al., 2008). The prophage genome is replicated during cell division in the host, thus it can constitute part of the bacterial host genome indefinitely. These infected bacteria are called lysogens or lysogenic bacteria (Casjens, 2003; Clokie and Kropinski, 2009; Kutter

and Sulakvelidze, 2005; Willey et al., 2008). Virulent phages usually encode host-lethal proteins that affect host replication, transcription, or translation (Kutter and Sulakvelidze, 2005). Host-lethal proteins are generally less common in temperate phages as restructuring of host metabolism may negatively impact long-term lysogeny (Kutter and Sulakvelidze, 2005).

1.9.2 Lysogeny

Temperate phages have several distinctive properties due to their lysogenic nature. Lysogeny is common in bacterial populations (Weinbauer, 2004), and prophages or prophage-like sequences are important contributors to bacterial diversity and bacterial evolution (Brüssow et al., 2004; Casjens, 2003; Hendrix et al., 1999). They may significantly alter host properties including modifications to the restriction system, sensitivity to antimicrobials and other environmental selective pressures (Clokie and Kropinski, 2009; Kutter and Sulakvelidze, 2005). In addition, temperate phages provide host immunity from re-infection of the same phage or other phages (Casjens, 2003; Clokie and Kropinski, 2009; Kutter and Sulakvelidze, 2005; Willey et al., 2008). The phenotype of a bacterial host can also be changed by lysogenic conversions (Casjens, 2003; Clokie and Kropinski, 2009; Willey et al., 2008). This change in cell phenotype may alter surface antigenic properties of the bacterium, or result in production of toxins due to virulence factors encoded by prophages (Canchaya et al., 2004; Casjens, 2003; Brüssow et al., 2004; Hendrix et al., 1999; Kutter and Sulakvelidze, 2005). The ability to enter the lysogenic cycle appears to be advantageous to phage in many cases. When bacteria become dormant due to nutrient deprivation, they stop synthesizing DNA or

proteins. Lysogeny allows temperate phages to enter a prophage state and survive within the host genome, whereas strictly lytic phages cannot undergo replication within a dormant host (Willey et al., 2008). In order to maintain lysogeny, temperate phages usually encode a repressor protein that blocks transcription of other phage genes such as the ones required during a lytic cycle (Clokie and Kropinski, 2009; Kutter and Sulakvelidze, 2005). Since this repressor protein may also exist in other phages, it may block lytic infection from these phages. If these phages regulate transcription with the same repressor protein, a temperate phage can provide host immunity to several different phages, all usually belonging to the same immunity group (Kutter and Sulakvelidze, 2005). The term "prophage" was first used by Lwoff and Gutmann (1950) to describe the intracellular state of phage genomes. By treating lysogenic cells with appropriate agents such as ultraviolet light or chemical mutagens that damage host DNA, prophages can be induced to initiate a lytic cycle, a process called induction (Casjens, 2003; Clokie and Kropinski, 2009; Kutter and Sulakvelidze, 2005; Willey et al., 2008). Induction ultimately leads to destruction of the bacterial host cell and production of new virions.

Even though most temperate phages have specific integration sites, there are cases where prophage DNA is excised erroneously along with adjacent fragments of bacterial host DNA (Kutter and Sulakvelidze, 2005). This may lead to specialized transduction resulting in a portion of host DNA next to the integration site being transferred to other bacterial cells, thereby altering their genomes. Generalized transduction also occurs with some phages, such as Mu, when they integrate randomly into the bacterial genome and naturally acquire host DNA during excision (Kutter and Sulakvelidze, 2005). Both temperate and virulent phages can produce generalized transduction by erroneously

packaging random fragments of bacterial host genome into capsids (Kutter and Sulakvelidze, 2005; Willey et al., 2008). Phage transduction may contribute significantly to bacterial evolution by exchanging pieces of bacterial information, or even introducing segments of genome into new organisms, either in lab or in nature (Briani et al., 2001; Kutter and Sulakvelidze, 2005). Prophage may account for genetic variability within a bacterial species through horizontal transfer of genetic information (Banks et al., 2002; Briani et al., 2001; Ohnishi et al., 2001). Prophage genomes may even carry genes that benefit the survival of bacteria in their ecological niche (Brüssow et al., 2004).

1.9.3 Lysogeny of Phage λ

The conversion from a lytic to a lysogenic phase (or vice versa) has been well studied in phage λ (Casjens, 2003; Kutter and Sulakvelidze, 2005; Willey et al., 2008). It involves the control of several regulatory proteins binding to critical promoter regions. There are two closely associated promoter regions in phage λ, one responsible for lysogeny and other for lysis. A repressor protein, CI, binds to these sites to promote lysogeny and inhibits the lytic cycle by blocking transcription of most viral genes (Willey et al., 2008). However, there is another protein, Cro, which competes with CI and promotes the lytic cycle. Both CI and Cro proteins compete for binding sites in the complex promoter/operator region which inhibit transcriptions of counterparts and promote further transcription of its own gene. (Kutter and Sulakvelidze, 2005; Willey et al., 2008).

The CII and CIII proteins are also involved in binding to critical promoter regions. They stimulate transcription of *cI* gene and transition to a lysogenic state. Therefore,

depending on the levels of CI, CII, and CIII proteins, and the levels of Cro proteins, either the lysogenic or lytic cycle may prevail (Clokie and Kropinski, 2009; Willey et al., 2008). CII proteins seem to be the key regulators during lysogeny (Kihara et al., 2001). Stability of CII protein is related to the host cell's cyclic AMP (cAMP) level. The intracellular level of cAMP is high when the host cell is deficient in energy, a condition that stabilizes CII protein and promotes lysogeny. Other nutrient-associated factors such as protease levels may influence the activity of CII protein (Willey et al., 2008). CIII protein also protects CII protein from degradation (Willey et al., 2008). This repressor-regulated system is advantageous for the phages as they can respond to different host cell conditions (Clokie and Kropinski, 2009; Kutter and Sulakvelidze, 2005; Willey et al., 2008). If a cell is low in energy, phages can enter into a prophage state to ensure survival. Conversely, host cells with sufficient energy may enable phages to successfully replicate by undergoing a lytic cycle. Integration into the host chromosome is mediated by the phage enzyme integrase (Willey et al., 2008). Once inside the host cell, the linear phage λ genome is circularized when the two cohesive ends base pair with each other. There are homologous integration regions or attachment sites (*att*) in both the host chromosome and the phage genome (Willey et al., 2008), enabling the circular phage genome to form a linear stretch of DNA within the host genome.

Induction of prophages from the bacterial genome also correlate with the stability of the repressor protein mentioned earlier. When phage DNA is damaged due to induction, the activity of the RecA protein is changed due to induced SOS response from the host (Clokie and Kropinski, 2009; Kutter and Sulakvelidze, 2005). Usually, RecA proteins are crucial in recombination and DNA repair processes (Willey et al., 2008). In

this case, RecA protein interacts with repressor CI protein resulting in its self-degradation. This eventually leads to start of lytic cycle as CI repressor protein levels continue to decrease and Cro protein levels increase. As a result, the energetic state of the host cell has significant effects on the lysogenic and lytic states of the phage infection processes (Clokie and Kropinski, 2009). Although the conversion between lysogenic and lytic states is well described for phage λ it is largely uncharacterized for other phage types.

1.10 Mechanism of phage infection/phage replication

1.10.1 Infection Process

The study of phage reproduction by a one-step growth curve was first used by Ellis and Delbrück (1939). A one-step growth experiment allows researchers to determine the details of phage-host interactions and molecular events occurring during reproduction (Kutter and Sulakvelidze, 2005; Willey et al., 2008). To carry out this experiment, bacteriophage particles are mixed with appropriate bacterial host, and a short period of time is given for phage attachment. The mixture is then diluted to avoid any new virions from binding to uninfected cells. The diluted culture is then examined by plaque assay at various time points to determine the number of infectious phage particles released from the host bacterium (Kutter and Sulakvelidze, 2005; Willey et al., 2008). Using this strategy, several parameters such as the latent period, burst size, and eclipse period can be determined. Within a common bacterial host and with the same medium and temperature conditions, these parameters are distinctive for each phage strain (Kutter and Sulakvelidze, 2005). From studying a one-step curve, the entire phage infection process can be demonstrated. This involves a series of closely programmed events including

attachment to the host cell, penetration of the host cell, taking over host metabolism, and finally phage virion assembly and release.

1.10.2 Adsorption/Penetration

Phage infection begins with virion binding to specific receptors on the host cell surface (Lindberg, 1973; Willey et al., 2008). Common receptors include cell wall lipopolysacharides and proteins, teichoic acids, flagella, and pili (Willey et al., 2008). As these receptors vary in each phage/host system, different adsorption mechanisms occur among phage-host relationships. For instance, T-even phage adsorption involves several tail structures binding to appropriate receptors on the cell surface of the host bacterium (Kutter and Sulakvelidze, 2005; Willey et al., 2008), prior to the baseplate settling on cell surface. Phage adsorption speed and efficiency are influenced by external factors and host physiological state. Many phages require divalent cations such as Ca^{2+} and Mg^{2+} as part of the binding process (Clokie and Kropinski, 2009; Kutter and Sulakvelidze, 2005; Willey et al., 2008). Mutation or alteration of receptors used by a phage can lead to bacteria developing resistance to phage, but this resistance offers no protection from other phages that recognize different cell surface receptors (Kutter and Sulakvelidze, 2005). Moreover, phages like T4 have tail fibers that recognize multiple receptors (Tetart et al., 1998), making it more difficult for bacteria to develop resistance to these phage.

The mechanisms of phage DNA transfer into the host cell are also specific for each phage (Kutter and Sulakvelidze, 2005). Usually, phage tails contain some sort of enzymatic activity that penetrates the peptidoglycan layer. In the case of T-even phages, the baseplates have lysozyme activity that help the central tube penetrate the

peptidoglycan layer (Willey et al., 2008). Conformational changes with the baseplate and sheath also result in penetration through the cell wall. Once penetration occurs, linear phage DNA is transferred through the tail tube into the host cell. However, in a linear form phage DNA is subject to degradation by host exonucleases and restriction enzymes. Consequently, phages have developed various methods to structurally protect their genomes or to disable host nucleases. Circularization of phage DNA by cohesive ends or terminal repeats (Kutter and Sulakvelidze, 2005) is one means of reducing degradation of phage DNA from exonucleases. Other mechanisms include the production of host nuclease inhibitor proteins (Belogurov et al., 1993) or the presence of unusual nucleotides such as hydroxymethyldeoxycytidine in the T4 genome to avoid digestion (Warren, 1980).

1.10.3 Phage-Directed Metabolism

In order to synthesize viral nucleic acids and proteins, phages have to take over the host metabolism and cellular machinery. Although there are few exceptions, all double-stranded DNA viruses use a similar mechanism to generate viral DNA and synthesize viral proteins (Willey et al., 2008). The initial step involves interaction of host RNA polymerase with phage promoters resulting in the transcription of early phage genes. The produced early viral mRNAs are translated to produce several protein factors (e.g., antirestriction proteins) and enzymes (e.g., ADP-ribosyltransferases) that are crucial for phage genome protection and reprogramming of the host cellular machinery to manufacture viral components (Kutter and Sulakvelidze, 2005; Willey et al., 2008). Inactivation of host proteases and restriction enzymes protect phage DNA from being

degraded. Specific viral enzymes are synthesized to terminate host biosynthesis by degrading host proteins and DNA. A set of middle genes coding for DNA polymerase proteins and hydroxymethylase directs phage DNA synthesis, followed by another set of late genes that encode structural components (e.g., head, tail fiber, tail baseplate, etc.) of phage particles (Kutter and Sulakvelidze, 2005).

1.10.4 Phage Particle Assembly/Cell Lysis

The assembly of most phages is an extremely complex process that requires specific viral proteins. These proteins include phage structural proteins, proteins that assist phage assembly, and proteins involved in host lysis (Willey et al., 2008). Phage DNA is packaged into phage proheads (procapsids) with the help of scaffolding proteins (Kutter and Sulakvelidze, 2005; Willey et al., 2008). A portal protein complex at the base of the prohead directs head assembly and DNA packaging, and serves as an attachment site for the separately assembled phage tail. For T4 phage, DNA packaging requires a set of terminase proteins that generate double-stranded ends of the concatemers produced during genome replication (Willey et al., 2008). Processed phage DNA then joins the portal protein complex for packaging into the capsid.

Two phage protein components are involved in host lysis by the tailed phages, lysin and holin (Kutter and Sulakvelidze, 2005; Willey et al., 2008). Lysin attacks the host's cell wall by cleaving bonds in the peptidoglycan layer. Holin generates pores in the cytoplasmic membrane so lysin can reach the peptidoglycan matrix during this process. The timing of lysis is tightly regulated, but also affected by growth conditions and genetics of the phage (Kutter and Sulakvelidze, 2005). Early lysis may result in too few

phages being released to the environment or production of incomplete phage particles. In contrast, delayed lysis may limit the rate at which the phage can interact with and seek new hosts (Abedon, 1990).

1.11 Phage Therapy

1.11.1 Introduction

Due to the increased emergence and spread of antimicrobial resistant bacteria, there is a renewed interest in finding alternatives to the use of antimicrobials for treating bacterial infections (Clack and March, 2006; Johnson et al., 2008; Kutateladze and Adamia, 2010; Skurnik et al., 2007). Phage therapy is considered to be one feasible approach, by selecting and utilizing bacteriophages that specifically kill targeted pathogenic bacteria (Clack and March, 2006; Skurnik et al., 2007). Not long after their discovery, phages were frequently used as therapeutics to control bacterial infections in humans, particularly in Georgia, Russia, Poland and USA (Clack and March, 2006; Kutter and Sulakvelidze, 2005). The use of phage therapy diminished following the introduction of antimicrobials and its inconsistent efficacy in early research experiments (Clack and March, 2006; Johnson et al., 2008; Kutateladze and Adamia, 2010), but a resurged interest in bacteriophage therapy is occurring due to its potential effectiveness in treating serious infections caused by antimicrobial resistant pathogens (Kutter and Sulakvelidze, 2005; Willey et al., 2008). Phage therapy can be utilized in many areas, including treating bacterial infections in humans, animals, and plants, especially when chronic infections occur (Clack and March, 2006; Skurnik et al., 2007). It also helps in eliminating pathogenic bacteria in food and water, and reduces the use of antimicrobials

in agricultural production (Kutter and Sulakvelidze, 2005; Skurnik et al., 2007). The use of antimicrobials in agriculture contributes significantly to the rapidly growing burden of antimicrobial resistant bacteria and increases the risk of their entrance into the food chain (Skurnik et al., 2007). Typically, phages with relatively broad host range are more useful for therapeutic purposes as they have the capacity to infect multiple pathogens displaying common surface receptors (Johnson et al., 2008). For instance, two novel coliphages were found to lyse multiple serotypes of pathogenic groups of *Escherichia coli*, but few non-pathogenic strains (Vicardi et al., 2008).

The diversity of phages in nature provides an abundant supply of source material, and the knowledge gained from early phage research can be utilized to develop phages for therapeutic application. Phage therapy holds several advantages over antimicrobials. Due to the mechanism of infection and the specificity of bacteriophages, they are effective against multi-drug resistant pathogenic bacteria (Skurnik et al., 2007). The specificity of bacteriophages also reduces harmful effects on beneficial bacteria or commensal flora (Clack and March, 2006; Johnson et al., 2008; Kutateladze and Adamia, 2010; Skurnik et al., 2007). Side-effects are rare since phages generally do not affect eukaryotic cells (Matsuzaki et al., 2005). The self-replicative nature of phages reduces the need for multiple doses as is often required for antimicrobials (Kutateladze and Adamia, 2010), and cost of developing a phage therapy is less than that of developing a new antimicrobial (Skurnik et al., 2007). Moreover, phage preparations can be modified in response to changes in bacterial pathogen population and susceptibility. Since the receptors on the cell wall are specific for different phages and it is almost impossible for the bacteria to mutate all receptors at once, use of a cocktail of phages can circumvent the

emergence of resistant bacteria (Kutateladze and Adamia, 2010). From early extensive use of phage therapy in humans, and from recent research studies in animals and humans, phages are generally considered safe (Johnson et al., 2008).

1.11.2 Approach

Potential candidates for phage therapy should be thoroughly characterized (Gill and Hyman, 2010). For instance, phage genome sequence and structure should be determined and subject to bioinformatic analysis. Furthermore, research trials with rigorous testing and appropriate controls for the specific application should be conducted to confirm the efficacy of therapy (Carlton et al., 2005; Skurnik et al., 2007). Virulent phages are more suitable for phage therapy as they do not undergo lysogeny (Carlton et al., 2005; Goodridge, 2010; Skurnik et al., 2007). Temperate phages may elicit undesired phenotypical changes when they integrate into the host bacterial genome. For example, they may carry genes encoding virulence factors (Johnson et al., 2008; Kutateladze and Adamia, 2010; Skurnik et al., 2007) and transfer these genes to other bacteria through transduction (Johnson et al., 2008; Kutateladze and Adamia, 2010; Kutter and Sulakvelidze, 2005). Another undesirable trait of temperate phages is that they may replicate as prophages along with the host cell, without lysing the bacterial host (Johnson et al., 2008; Kutateladze and Adamia, 2010). Virulent phages, on the other hand, always enter a lytic cycle that results in degradation of host DNA, phage replication, lysis of host cell, and release of viable phage progeny (Kutter and Sulakvelidze, 2005).

1.11.3 Animal studies

Phage therapy has been discussed in various studies to control pathogenic bacterial infections in animals. It has been extensively used in veterinary medicine and in agricultural settings (Kutter and Sulakvelidze, 2005). For example, phages were used to control enterotoxigenic *E. coli* (ETEC) infections in neonatal calves, pigs, and lambs (Smith and Huggins, 1983; Smith et al., 1987). The mortality rate of phage-treated calves was significantly lower than the untreated control groups (Smith and Huggins, 1983). Although phages did not completely eliminate pathogenic *E. coli*, numbers were reduced to a level that limited the severity of disease. Moreover, phages persisted in the animal gut as long as pathogenic *E. coli* remained present. Similar challenge studies with enterotoxigenic strains of *E. coli* also demonstrated the efficacy of phage therapy on piglets and lambs (Smith and Huggins, 1983). Phage treatment was further evaluated with calves infected with pathogenic strains of *E. coli* in subsequent studies (Smith et al., 1987). The results were promising as administration of phage quickly reduced *E. coli* to minimal levels in calves (Smith et al., 1987). Another challenge study used *Salmonella*-infected chickens to study the therapeutic value of bacteriophages (Berchieri et al., 1991), and showed that the therapy significantly reduced their mortality. A study conducted by Barrow et al. (1998) used an *E. coli*-specific lytic phage to prevent septicemia and meningitis-like infection in chickens and calves. This study demonstrated that the efficacy of phage treatment in chickens was dose-dependent. The best protection was obtained with high doses of phage, prior to bacterial challenge. Phage treatment for calves orally challenged with pathogenic *E. coli* also produced a positive therapeutic effect. Aside from calves and chickens, other studies employed different bacterial

challenges to evaluate phage therapy in various animal models, such as *Enterococcus faecium*-infected mice (Biswas et al., 2002), *Clostridium difficile*-infected hamsters (Ramesh et al., 1999), and mice infected by *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Soothill, 1992).

1.11.4 Phage Vaccines

Instead of using whole phage preparations as prophylactic or therapeutic treatments, one can also develop phage lysate-based vaccines to help reduce the use of antimicrobials in livestock animals. By reducing the incidence of bacterial infection, animal growth may be promoted, reducing the need for growth-promoting antimicrobials (Kutter and Sulakvelidze, 2005). One way to develop phage vaccines is to generate bacterial lysates using phage. Phage-induced bacterial lysates are very good immunizing agents and they elicit an immune response that is superior to conventional vaccines (Compton, 1928; Larkum, 1929; Weiss and Arnold, 1924). This is probably because conventional vaccine development usually generates antigens by inactivating pathogenic bacteria by heat, irradiation, or chemical treatment, processes which may damage relevant immunological epitopes and reduce vaccine effectiveness (Kutter and Sulakvelidze, 2005; Lauvau et al., 2001). *Staphylococcus aureus* phage lysate (SPL)® is a well known bacteriophage lysate generated using *S. aureus*-specific bacteriophages. The SPL vaccine was used in a mouse model to evaluate its efficacy in treating or preventing *S. aureus* infections (Esber et al., 1981), and the results were encouraging as SPL-treated mice had significantly higher survival rates than untreated controls. Similar to phage-generated bacterial lysate, bacterial ghost vaccines can be constructed from phage-

encoded bacteriolytic enzymes (Jalava et al., 2002; Kutter and Sulakvelidze, 2005; Mader et al., 1997; Panthel et al., 2003; Szostak et al., 1990; Szostak et al., 1996; Szostak et al., 1997; Young, 1992). This was achieved by introducing the lysis gene *e* from bacteriophage φX174 into various Gram-negative bacteria using appropriate vectors. The vectors contain regulatory genes that allow tight repression and induction of the *e* gene (Jalava et al., 2002). Lysis protein E forms pores in the bacterial cell wall, resulting in a loss of cell contents (Schon et al, 1995; Witte et al., 1990). Because bacterial ghosts retain the majority of their outer membrane and immunostimulatory LPS endotoxin structure, they elicit an immune response similar to that of regular attenuated vaccines (Jalava et al., 2002; Witte et al., 1990). Various bacterial ghosts of Gram-negative bacteria have been prepared using this method, including *E. coli* (Blasi et al., 1985), *Salmonella* species (Szostak et al., 1996), *Vibrio cholerae* (Eko et al., 2003), and *P. multocida* and *P. haemolytica* (Marchart et al., 2003). In addition to designing ghost vaccines from phage lysis gene, phage encoded enzymes can be used as antibacterial agents (Fischetti, 2005). This concept has been supported in several studies. For example, the endolysin gene of *S. aureus* bacteriophage Twort was sequenced and cloned into *E. coli* JM109 to produce purified recombinant protein (Loessner et al., 1998). Endolysin may be an effective antimicrobial against *S. aureus* infection since it was shown to rapidly cleave staphylococcal peptidoglycan. Other studies have also shown that phage-encoded lytic enzymes rapidly kill various pathogenic Gram-positive bacteria both *in vitro* and *in vivo*, while retaining host specificity (Loeffler et al., 2001; Nelson et al, 2001; Schuch et al., 2002). Moreover, these phage lysins seem to have the same degree of efficacy against antimicrobial-resistant bacteria (Loeffler et al., 2001; Nelson et al, 2001).

1.12 Bacteriophage in *M. haemolytica*

Bacteriophages were first isolated from *M. haemolytica* in the 1950s (Rifkind and Pickett, 1954; Saxena and Hoerlein, 1959). Pathogenic strains (serotype A1) of *M. haemolytica* have previously been shown to carry a prophage, which can be induced by DNA damaging elements (Richards et al., 1985). The isolated phage was named φPhaA1, and it was unable to form plaques on any of the A1 strains tested (Richards et al., 1985). As a result, the authors suspected that all tested strains harbored the same lysogenic bacteriophage (Richards et al., 1985), because temperate phage can provide host immunity against infection by other phages. Froshauer et al. (1996) also examined 14 additional strains of *M. haemolytica*, isolated from cattle with shipping fever, and found that mitomycin C could be used to induce prophage excision from all 14 strains. In all cases, the phage DNA gave the same restriction enzyme digestion pattern, corresponding to a genome of about 40 kb. They concluded that all strains examined carried the same prophage and that this phage is widely distributed among *M. haemolytica* (Froshauer et al., 1996).

The *M. haemolytica* bacteriophages investigated by Davies and Lee (2006) had relatively diverse morphologies. All of the phages possessed icosahedral heads, but three different tail morphologies were present. Based on the results of the restriction endonuclease digest, they estimated the phage genomes ranged from 22 to 45 kb in size. The identification of nine distinct restriction profiles among the 24 phages induced also supports the idea that *M haemolytica* phages are relatively diverse. Another study by Highlander et al. (2006) revealed that among 16 strains of *M. haemolytica,* six of the strains (A1, A5, A6, A7, A8, and A13) were lysed following mitomycin C treatment,

suggesting that they contain temperate phages. Strains A1 and A6 are also two of the predominant serotypes isolated from bovine respiratory disease animals (Zecchinon et al., 2005).

Based on the genome sequences of bovine *M. haemolytica* serotype A1 and A2, a considerable portion (12.3% and 11.5%) of the identified genes are bacteriophage-related (Gioia et al., 2006; Lawrence et al., 2010a). The *M. haemolytica* serotype A1 (strain BAA410) contained at least two intact prophages in the genome (Gioia et al., 2006). An intact P2-like phage, two Mu-like phages, and λ-like fragments were detected in the genome (Gioia et al., 2006). Typical P2-like phage and Mu-like phage are myoviruses, while λ is a siphovirus (Guttman et al., 2005). To date, the only *M. haemolytica* bacteriophage that has been sequenced completely is φMhaA1-PHL101. The prophage portion of this sequence was also determined and named φMhaA1-BAA410 (Highlander et al., 2006). This temperate bacteriophage was induced from *M. haemolytica* serotype A1 (strain PHL101), with a genome size of 34,525 bp (Highlander et al., 2006). It is a double-stranded DNA bacteriophage belonging to the P2 family of phages. The sequence of φMhaA1-PHL101 is very similar to the P2-like phage mentioned earlier from *M. haemolytica* strain BAA410, only 22 single-base-pair substitutions were observed between these two (Gioia et al., 2006).

The current methods for BRD prevention and treatment have various problems and limitations. Antimicrobial resistance has been reported in *M. haemolytica.* As a result, it is crucial to seek an alternative therapeutic strategy for this pathogen. Bacteriophage therapy presents a novel method to mitigate *M. haemolytica*. Several companies have already been working on therapeutic phage products to combat *E. coli*,

Salmonella typhimurium, *Listeria monocytogenes*, and *Campylobacter jejuni* in agricultural applications (Thiel, 2004). Feasibility of this approach depends on the isolation of a *M. haemolytica*-specific lytic bacteriophage from environmental sources. To date, no strictly lytic bacteriophages that are specific to *M. haemolytica* have been documented. Phages usually co-develop and share a common niche with their bacterial hosts (Brüssow and Kutter, 2005). They are highly abundant on earth (Kutter and Sulakvelidze, 2005; Willey et al., 2008). The total number is estimated at 10^{30} to 10^{32} (Brüssow and Kutter, 2005), and it is expected that all bacteria are susceptible to infection by bacteriophages (Thiel, 2004). Lytic bacteriophages have been successfully isolated and shown promising results in treating bacterial infections in ruminants, including calves challenged with various enterotoxigenic strains of *E. coli* (Smith and Huggins, 1983; Smith et al., 1987), adult beef cattle challenged with *E. coli* O157:H7 (Niu et al., 2008), and sheep challenged with *E. coli* O157:H7 (Callaway et al., 2008; Raya et al., 2006). An environmental source that contains the pathogen of interest is likely to contain phages capable of lysing that organism (Gill and Hyman, 2010). Therefore, bovine nasopharyngeal swabs were identified as the main source for isolating bacteriophages since *M. haemolytica* typically exists as a commensal organism in the upper respiratory tract in healthy ruminants (Catry et al., 2005; Highlander, 2001). Feedlot trough water is another possible phage reservoir as animals' mouths are frequently in contact with it. Based on previous studies, a lytic bacteriophage capable of lysing *Streptococcus suis* was isolated from nasal swabs of healthy Bama minipigs (Ma and Lu, 2008), and a bacteriophage isolated from the waste water of sewer treatment plants or a poultry processing plant was found to be effective in controlling *E. coli*

respiratory disease in broiler chickens (Huff et al., 2003). These studies confirmed the practicality of isolating lytic bacteriophages from nasal swabs or water samples. The efficacy of an isolated bacteriophage will be determined against field strains of *M. haemolytica* and ultimately, it was proposed that a challenge study be conducted to determine if the selected phage was effective in reducing *M. haemolytica* in the respiratory tract of feedlot cattle. Although it is relatively easy to isolate novel bacteriophages from the environment, strictly lytic phages of some species of bacteria can be rare (Goodridge, 2010), and the presence of prophages within *M. haemolytica* genome (Gioia et al., 2006; Lawrence et al., 2010a) may also provide host immunity and prevent potential lytic bacteriophage from infecting *M. haemolytica.* Other factors such as bacterial restriction-modification systems, abortive infection mechanisms, or Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) mechanisms may also affect bacteriophage infection (Gill and Hyman, 2010). As a result, we incorporate an extensive characterization of temperate bacteriophages induced from our *M. haemolytica* collection. Even though temperate bacteriophages are not the ideal candidates for phage therapy, they can be genetically engineered or modified to meet our requirement. Whole genome sequencing of selected temperate bacteriophages also provides a foundation for future phage vaccine development such as the phage-encoded lytic enzymes mentioned previously. The objectives of this study were to screen and isolate strictly-lytic or temperate bacteriophages specific to *M. haemolytica*. Isolated bacteriophages were characterized though various methods including electron microscopy, host range analysis, restriction fragment length polymorphism, and genome sequencing. The therapeutic potential of isolated bacteriophages was determined.

CHAPTER TWO

Lytic bacteriophage(s) specific to *Mannheimia haemolytica*

2.1 Introduction

Bovine respiratory disease often occurs when animals have impaired health or suppressed immune systems due to environmental or nutritional stress (Irsik, 2007). For example, viral infection or inhaled irritants can lead to cell damage in the upper respiratory tract (Irsik, 2007). This provides a window of opportunity for secondary pathogens such as *M. haemolytica* to colonize and reproduce quickly. *M. haemolytica*, a weakly haemolytic, Gram-negative coccobacillus, often shows up as a complicating agent in BRD (Zecchinon et al., 2005). Various researchers suggest *M. haemolytica* as the principle causative agent of BRD (Duff and Galyean, 2007; Gioia et al., 2006; Griffin et al., 2010; Zecchinon et al., 2005). Treatment of BRD linked to *M. haemolytica* usually entails prophylactic treatment with antimicrobials. However, to be effective, antimicrobials must be administered very early in the disease process (Irsik, 2007). Antimicrobial resistance is a major concern that needs to be monitored closely. *M. haemolytica* has shown increasing resistance to many antimicrobial agents (Watts et al., 1994). Other means of mitigation such as vaccination against *M. haemolytica* also exist, but are less efficacious (Confer et al., 2006).

The potential for resistance to antimicrobials and the lack of comprehensive preventive measures against pathogenic *M. haemolytica* have made it important to seek alternative mitigation strategies. Bacteriophage therapy presents an innovative area of research for preventing bacterial infections. Bacteriophages are a group of viruses that can infect, replicate, and cause lyses of specific bacteria (Clark and March, 2006). Phage

therapy provides a number of advantages over the use of antimicrobials. For instance, phages are effective in treating multidrug-resistant bacterial infections (Skurnik et al., 2007). Phages possess host-specificity, which means they are less likely to harm commensal bacterial flora in animals (Clack and March, 2006; Johnson et al., 2008; Kutateladze and Adamia, 2010; Skurnik et al, 2007). Moreover, phages can self replicate, reducing the need for multiple doses, as required in antimicrobial therapy (Kutateladze and Adamia, 2010; Skurnik et al., 2007).

Several studies have investigated the use of lytic phage therapy against bacterial pathogens. For example, inoculation of broiler chickens with bacteriophage significantly reduced mortality during *E. coli* challenge experiment (Huff et al, 2003). Temperate phages specific to *M. haemolytica* have been isolated and characterized (Davies and Lee, 2006; Highlander et al, 2006). Temperate phages do not lyse host cells consistently and possess a transducing capability with the host genome (Johnson et al., 2008; Kutateladze and Adamia, 2010; Kutter and Sulakvelidze, 2005; Skurnik et al., 2007). Transduction may cause undesired phenotypic changes to a recipient bacterium when temperate phages carry and integrate genes encoding virulence factors from previously infected cells during lysogenization (Adam, 1959). Usually, this type of bacteriophage is not suitable as a direct therapeutic. In this study, we attempted to isolate one or more strictly lytic phage(s) with activity against *M. haemolytica*. Strictly lytic (virulent) phages will always enter their lytic cycle and kill the bacterial host (Kutter and Sulakvelidze, 2005). Depending on the number of lytic phages isolated, desired phages will be further characterized. The activity of acquired lytic phage will be assessed against a large collection of *M. haemolytica* that have been isolated from commercial feedlot cattle. Phages that possess

strong lytic activity and broad host range will be used in further animal studies to control *M. haemolytica* infection.

2.2 Materials and methods

Bacterial Isolates

Three *M. haemolytica* reference strains and six *M. haemolytica* field strains were used to enrich lytic bacteriophage isolated from cattle. Strains of *M. haemolytica* used in this study are listed in Table 2.1. Isolates were stored at -80°C in 20% (v/v) glycerol in brain heart infusion broth (BHIB). Single colonies of *M. haemolytica* were obtained by streaking on tryptic soy agar (TSA) plates containing 5% sheep blood (v/v) (Dalynn Biologicals, Inc., Calgary, AB, Canada) and incubated overnight at 37 °C. Starter cultures were prepared by inoculating 10 mL of fresh BHIB with single colonies of each isolate. The cultures were incubated at 37 °C overnight with shaking at 120 rpm.

Table 2.1. Properties of three *M. haemolytica* reference strains and six *M. haemolytica* field strains used for phage enrichment.

Strain	Source	Serotype
BAA-410	ATCC	A1
33396	ATCC	2
33370	ATCC	6
413A	feedlot 5, arrival	2
501A	feedlot 5, arrival	1
535A	feedlot 21, arrival	1
964A	feedlot 21, arrival	6
1098A	feedlot 21, exit	2
1498A	feedlot 5, exit	2

Sample preparation

A total of 689 bovine nasopharyngeal swabs were obtained from four regional feedlots (Table 2.2). Samples were chosen randomly from *M. haemolytic*-positive and *M. haemolytic*-negative swabs received each week. Swabs were submerged and vortexed in BHI broth containing 20% glycerol. Either individual or pooled nasopharyngeal swab suspensions were filtered through 0.8/0.2-µm-pore size low protein binding syringe filters (Pall Canada Ltd., Mississauga, ON). In addition, 30 pooled water samples (500 mL) were obtained from the Lethbridge Research Centre feedlot water troughs and filtered through 0.2-µm-pore size bottle top filter (Nalgene, Fisher Scientific, Ltd. (Canada), Nepean, ON). Water filtrates (15 mL) were concentrated by Centriprep YM-30 centrifugal filter units (Millipore, Billerica, MA, USA) at 1,500 × g for 15 min at room temperature, to a final volume of 3 to 5 mL before further processing.

Table 2.2. Bovine nasopharyngeal swabs examined in this study.

Feedlot	Number of arrival samples	Number of exit samples	Total number of samples
#5	59	93	152
#21	63	79	142
#31	125	89	214
#42	97	84	181

Enrichment

The basic enrichment protocol was described by Carlson (2005). Briefly, two separate *M. haemolytica* enrichment cocktails were prepared using a mix of reference strains (BAA-410, 33396, 33370) and a mix of field isolates (413A, 501A, 535A, 964A, 1098A, 1498A). These strains were intentionally selected to represent the dominant

pathogenic and non-pathogenic serotypes of *M. haemolytica*, and broadly representative of our collection of *M. haemolytica* field strains. Cocktails of early-log *M. haemolytica* liquid culture (5 mL; approximately 5×10^8 CFU/mL) were combined with 1 mL of the nasopharyngeal swab filtrate or water filtrate, and 50 µL filter-sterilized $MgSO_4$ (1M) solution. The mixture was incubated for 18-20 h at 37° C with shaking at 150 rpm, and filtered through 0.8/0.2-µm-pore size filter. Different ratios (1:1, 5:1, 10:1) of *M. haemolytica* cocktail to sample filtrate were tested.

Detection

The double agar overlay method was used for bacteriophage detection. *M. haemolytica* strains listed in Table 2.1 were used as indicator strains. Each enriched filtrate (500 µL) was mixed with either individual or cocktails of *M. haemolytica* liquid cultures (500 µL) at early-log phase. The mixture was incubated for 15-20 min at room temperature to allow bacteriophage attachment. Molten agar (2 mL) (top agar) was added to the mixture and poured on to agar plates (bottom agar). Poured agar plates were allowed to set at room temperature for 20-30 min. Agar plates were inverted and incubated at 37 °C for 18-20 h and examined for plaque formation. Various combinations of agar, agar concentration, and divalent ion supplement were used in the double agar overlay procedure. This included modified nutrient agar (MNA), BHI agar, and modified BHI agar as bottom agar. UltraPure™ agarose (Invitrogen Canada Inc., Burlington, ON), Difco™ Agar (BD Canada, Inc., Mississauga, ON) and BHI agar were prepared with concentrations ranging from 0.4% to 0.7% to serve as top agar. Either top agar or bottom agar was supplemented with 5mM $CaCl_2$ or 10mM $MgSO_4$ divalent ions. Combinations

of the above media were first tested on identical samples wherever possible. If *M. haemolytica* cultures grew well with a particular media combination, it was adapted in bacteriophage detection. Approximately 50% of the detection assays utilized 0.7% Difco™ top agar containing 10mM $MgSO_4$ and regular BHI as bottom agar.

2.3 Results and discussion

A comprehensive screening process was undertaken in an attempt to isolate strictly lytic bacteriophage(s) specific to *M. haemolytica*. In principle, any environmental source that contains the target pathogen is likely to contain phages capable of lysing that organism (Gill and Hyman, 2010). Bovine nasopharyngeal swabs and feedlot trough water were identified as potential niches harboring specific phages. Previous studies have found bacteriophages specific to *Streptococcus suis* in nasal swabs (Ma and Lu, 2008) and to *Salmonella* Enteritidis, *E. coli*, and *Enterococcus faecalis* in water sources (Higgins et al., 2005; Huff, 2003; Uchiyama et al., 2008). Among these pathogens, *S. suis* and *E. coli* are involved in respiratory tract infections in pig and chicken, respectively. Nasopharyngeal swabs were the main focus in this study since *M. haemolytica* typically exists as a commensal organism in the upper respiratory tract in healthy ruminants (Catry et al., 2005; Highlander, 2001). Nasopharyngeal swabs were randomly selected each week from both *M. haemolytica* positive and *M. haemolytica* negative samples. Depending on the number of samples received, three to eight (usually five) swab suspensions were pooled to generate larger processing volumes and increase the probability of phage detection. The enrichment step was a method used to facilitate isolation of particularly virulent, broad-spectrum phages that may be present at low levels

in the sample (Carlson, 2005; Gill and Hyman, 2010; Jensen et al., 1998). Pooling of multiple samples allowed us to select desirable phages from a larger and potentially more diverse population of phages (Gill and Hyman, 2010). Cocktails of *M. haemolytica* were prepared by deliberately selecting reference strains and field strains that represent dominant pathogenic and non-pathogenic serotypes of *M. haemolytica*. Serotype 2 is the predominant serotype isolated from healthy cattle (Hodgins and Shewen, 2004b), whereas serotype 1 and serotype 6 are most frequently isolated from cattle with BRD (Griffin et al., 2010; Hodgins and Shewen, 2004b). The field strains were also selected to encompass the genetic diversity of *M. haemolytica* based on PFGE analysis of greater than 400 field isolates from the Lethbridge Research Centre collection (Klima et al., 2011). To increase the geographical and chronological representation of enrichment strains, isolates originating from different feedlots and separate sampling events were selected. A total of 689 bovine nasopharyngeal swabs (Table 2.2) and 30 pooled water samples were collected and screened for the presence of lytic bacteriophage(s) against *M. haemolytica*. In spite of rigorous screening effort and extensive adjustment in enrichment protocols, growth media, and phage detection assays, no lytic bacteriophage(s) specific to *M. haemolytica* were detected from either source.

This section explains several potential difficulties that may have led to the inability to isolate *M. haemolytica*-specific lytic bacteriophage(s) in this study. The number of *M. haemolytica*-specific lytic bacteriophage(s) may be too scarce in the locations tested; transportation and processing of raw samples may have further reduced phage count and viability. Even though nasopharyngeal swabs have been used as a noninvasive diagnostic method for respiratory infections in cattle harbouring *M.*

haemolytica, evaluation of this method for representative viral entities has not been conducted (Cooper and Brodersen, 2010). The process of concentrating water samples may also hinder phage carriage or survival (Gill and Hyman, 2010), possibly due to incomplete phage filtration or phage particle damage introduced by centrifugal forces. Binding to non-target particles within water of nasal samples is also a possibility. Biases introduced by enrichment and plating methods may affect desirable phage detection because enrichment could cause a single rapidly replicating phage to dominate entire culture while masking other potential phages and not all phages are efficient plaque formers under typical plating conditions (Gill and Hyman, 2010). For instance, a particularly virulent phage isolated during enrichment may be undetected in double agar overlay plaque assay, but still possesses high lytic activity *in vivo*.

Often phages require specific cofactors or divalent ions, such as Ca^{2+} or Mg^{2+}, during the infection process (Clokie and Kropinski, 2009; Kutter and Sulakvelidze, 2005; Willey et al., 2008), thus these ions were supplied in our detection method. Nevertheless, they may not fulfill specific nutrient or mineral requirements of an uncharacterized phage. In the end, the number of viable bacteriophage(s) may be too low that even after enrichment procedure they remain undetected. A more sensitive molecular screening method may be a feasible approach in this situation.

Temperate bacteriophages are widespread in bovine and ovine *M. haemolytica* isolates (Davies and Lee, 2006). During lysogeny, they provide host immunity from re-infection of the same phage or other phages (Casjens, 2003; Clokie and Kropinski, 2009; Hyman and Abedon, 2010; Kutter and Sulakvelidze, 2005). In order to persist in a bacterial genome, temperate phage usually encodes repressor proteins that block

transcription of other phage genes such as the ones required during lytic cycle (Willey et al., 2008). As a result, repressor proteins may inhibit reproduction of lytic bacteriophages specific to *M. haemolytica*.

Naturally, bacteria utilize various innate phage-resistance mechanisms to ensure survival under the pressure of phage infection. This includes mechanisms such as DNA modification, abortive infection, and phage adsorption resistance (Hyman and Abedon, 2010; Sturino and Klaenhammer, 2006). Various studies suggest clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated sequence (CAS) proteins are linked to a novel mechanism of acquired resistance against bacteriophages (Barrangou et al., 2007; Deveau et al., 2008; Hyman and Abedon, 2010; Mojica et al., 2009; Sorek et al., 2008). The CRISPR array was first discovered in *E. coli* (Ishino et al., 1987), and subsequent studies by Barrangou and colleagues confirmed its function experimentally (Barrangou et al., 2007; Deveau et al., 2008; Horvath et al., 2008). In response to phage infection, bacteria can integrate new spacers that are derived from phage genomic sequences, which results in CRISPR-mediated phage resistance (Barrangou et al., 2007). Moreover, the number and type of the spacers between CRISPR repeats are linked to phage sensitivity (Barrangou et al., 2007; Lawrence et al., 2010b). Although the exact mechanism by which CRISPR systems work is still unclear, some suggest that CRISPR functions by an RNA-silencing (RNA interference)-like mechanism (Makarova et al., 2006; Mojica et al., 2005; Sorek et al., 2008). The CRISPR system is widespread in the genomes of many bacteria (Mojica et al., 2009; Sorek et al., 2008), which implies its effectiveness in providing protection against phage attacks. Interestingly, a recent study indicates that CRISPR regions are also present in the *M.*

haemolytica genomes (Lawrence et al., 2010b). CRISPR/Cas loci were identified in all three *M. haemolytica* isolates examined (Lawrence et al., 2010b). This suggests CRISPR assays are common in *M. haemolytica* and their presence may indeed provide immunity to bacteriophages. This could have resulted in our inability to detect or isolate lytic bacteriphages specific to *M. haemolytica*.

Since our samplings were restricted to deep nasopharyngeal swabs, swabs taken from other areas of the respiratory tract or even lung tissues of morbid animals may be worth exploring. Molecular screening methods that utilize phage sequences and PCR technique may offer a more sensitive approach for identifying candidate phage, as one may design primers from sequences of closely related bacteriophages. Temperate bacteriophages of *M. haemolytica* may also have therapeutic potentials, especially with functional components such as lytic enzymes (Fischetti, 2005; Loessner et al., 1998).

CHAPTER THREE

Characterization of temperate bacteriophages induced from bovine *Mannheimia haemolytica* isolates.

3.1 Introduction

Mannheimia haemolytica, previously known as *Pasteurella haemolytica,* is a Gram-negative bacterium and a principal agent associated with bovine respiratory disease (BRD) or shipping fever (Duff and Galyean, 2007; Gioia et al., 2006; Griffin et al., 2010; Zecchinon et al., 2005). The morbidity and mortality associated with BRD have significant economic impact on the feedlot industry (Duff and Galyean, 2007; Edwards, 1996). Mortality from BRD and the expenses of preventative and prophylactic treatment contribute to its impact on the feedlot industry. Feedlot performance and carcass quality are also compromised by BRD (Duff and Galyean, 2007; Gardner et al., 1999). There are 12 capsular serotypes of *M. haemolytica* - A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16, and A17 (Fulton, 2009; Katsuda et al., 2007; Srikumaran et al., 2007). The organism normally exists as a commensal in the upper respiratory tract of healthy ruminants (Highlander, 2001), but genetically distinct subpopulations which may elicit disease also exist (Davies et al., 1997). While serotype 2 is the predominant serotype of *M. haemolytica* isolated from healthy cattle (Hodgins and Shewen, 2004b), serotypes 1 and 6 are most commonly isolated from cattle with BRD (Griffin et al., 2010; Hodgins and Shewen, 2004b; Zecchinon et al., 2005).

As agents of lateral gene transfer, bacteriophages contribute significantly to the variability and the consequential evolution of bacterial genomes (Canchaya et al., 2003a;

Davies and Lee, 2006). However, this evolutionary process also applies to genes that code for virulence factors that can confer pathogenicity to their bacterial host (Willey et al., 2008). Horizontal DNA transfer has previously been implicated in the evolution of *M. haemolytica lktA* and *ompA* genes and the emergence of new strains (Davies et al., 2001; Davies et al., 2002). Bacteriophages were first isolated from *M. haemolytica* in the 1950s (Rifkind and Pickett, 1954; Saxena and Hoerlein, 1959). Later, a phage designated φPhaA1 was isolated from *M. haemolytica* biotype A, serotype 1 (A1). As this phage was unable to form plaques on any of the A1 strains tested; it was suggested that all strains of this serotype harbored the same temperate bacteriophage (Richards et al., 1985). Froshauer et al. (1996) examined 14 strains of *M. haemolytica* isolated from cattle with shipping fever, and found all strains carried a ~40 kb prophage. A study by Highlander et al. (2006) revealed that six strains (A1, A5, A6, A7, A8, and A13) of *M. haemolytica* lysed after exposure to mitomycin C, an inducing agent that can promote the excision of prophages from the bacterial genome. Davies and Lee (2006) investigated temperate bacteriophages from 15 bovine and 17 ovine *M. haemolytica* isolates, with genome sizes ranging from of 22 to 45 kb.

Froshauer et al. (1996) demonstrated that danofloxacin, a fluoroquinolone with broad spectrum antibacterial activity, causes DNA damage in *M. haemolytica*. This damage induces prophage in *M. haemolytica* to undergo the lytic cycle and leads to cell lysis. It was discovered that the degree of prophage induction is related to total exposure of bacteria to the antimicrobial compound. The authors concluded that the presence of an inducible prophage increases the sensitivity of *M. haemolytica* to danofloxacin (Froshauer et al., 1996).

The objective of this study was to extend previous research on *M. haemolytica* phage by screening a diverse collection of bovine *M. haemolytica* isolates for temperate bacteriophages. The *M. haemolytica* isolates have been characterized extensively in terms of genetic diversity and antimicrobial susceptibility (Klima et al., 2011). Based upon previous reports in the literature, I hypothesized that temperate bacteriophages would be prevalent and diverse in our *M. haemolytica* culture collection. Mitomycin C treatment was used to induce prophages in the *M. haemolytica* isolates and the diversity of isolated bacteriophage particles was determined by comparing virion morphology, host specificity, restriction fragment length polymorphisms and genomic sequences. This study also determined that enrofloxacin, a typical fluoroquinolone antimicrobial administered to cattle, did not provide similar effect to that of danofloxacin, where the presence of an inducible prophage in *M. haemolytica* correlated positively to its antimicrobial resistance. These results suggest that *M. haemolytica* isolates with higher enrofloxacin susceptibility are not more likely to contain inducible phages.

3.2 Materials and methods

Bacterial strains

A total of 82 *M. haemolytica* isolates were examined in this study (Table 3.1). Ten reference strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and University of Guelph culture collection (UGCC, Guelph, ON, Canada). Field strains of *M. haemolytica* were isolated from bovine nasopharyngeal swabs collected from feedlot cattle in southern Alberta, Canada. Identification of *M. haemolytica* isolates was confirmed by multiplex polymerase chain reaction (Alexander

et al., 2008). Isolates were stored at -80°C in brain heart infusion broth (BHIB) containing 20% (v/v) glycerol. A total of 72 *M. haemolytica* field strains, representing each of the three serotypes detected (serotypes 1, 2, and 6), were selected from isolates collected from cattle at either arrival or > 60 days on feed at four regional feedlots. Single colonies of *M. haemolytica* isolates were obtained by streaking cells on tryptic soy agar (TSA) plates containing 5% sheep blood (v/v) (Dalynn Biologicals, Inc., Calgary, AB, Canada). The inoculated plates were incubated overnight at 37 °C. Starter cultures were prepared by inoculating 10 mL of fresh BHIB with single colonies of each isolate and incubated overnight at 37 °C with shaking at 120 rpm. A preliminary *M. haemolytica* growth curve analysis was carried out to determine inoculation and incubation conditions necessary to obtain early-log cultures for phage induction and plating procedures.

Table 3.1. Properties of 10 *M. haemolytica* reference strains and 72 *M. haemolytica* field isolates.

Reference Strains

Isolate	Serotype	Source	ENRO ZD (mm)[1]	Phage morphology	Bacterial PFGE Type[2]	Phage RFLP Type[3]
BAA410	1	ATCC	N/A	*Myoviridae/ Siphoviridae*[4]	N/A[5]	N/A
G1	1	Geulph	N/A	Undetected	N/A	N/A
G2	2	Geulph	N/A	Undetected	N/A	N/A
G5	5	Geulph	N/A	Undetected	N/A	N/A
33370	6	ATCC	N/A	Undetected	N/A	N/A
29698	7	ATCC	N/A	Undetected	N/A	N/A
G8	8	Guelph	N/A	*Myoviridae/ Siphoviridae*	N/A	N/A
29700	9	ATCC	N/A	Undetected	N/A	N/A
G12	12	Guelph	N/A	Undetected	N/A	N/A
G14	14	Guelph	N/A	Undetected	N/A	N/A

Field Strains

Isolate	Serotype	Feedlot information		ENRO ZD (mm)	Phage morphology	Bacterial PFGE Type	Phage RFLP Type
25A	N/A	21	Arrival	36	*Myoviridae*	N/A	11
140A	2	21	Arrival	35	*Siphoviridae*	B	9
143A	2	21	Arrival	26	*Siphoviridae*	A	5
251B	2	21	Arrival	37	*Siphoviridae*	A	8
254A	2	21	Arrival	36	*Siphoviridae*	A	8
309A	2	21	Arrival	26	*Siphoviridae*	A	6
369A	2	5	Arrival	25	Undetected	N/A	N/A
394A	2	5	Arrival	25	Undetected	N/A	N/A
407A	2	21	Arrival	26	*Siphoviridae*	C	8
410A	2	5	Arrival	25	*Siphoviridae*	A	14
413A	2	5	Arrival	34	*Myoviridae*	A	N/A
501A	1	5	Arrival	32	*Siphoviridae*	F	7
535A	1	21	Arrival	25	*Siphoviridae*	G	7
587A	2	21	Exit	38	*Myoviridae*	A	5
611A	1	5	Exit	26	*Siphoviridae*	H	12
613A	2	21	Exit	36	*Myoviridae/ Siphoviridae*	A	N/A
622A	2	21	Exit	36	*Myoviridae/ Siphoviridae*	C	N/A
755A	2	5	Arrival	36	*Myoviridae*	A	1
813A	2	21	Exit	35	Undetected	N/A	N/A
842A	2	21	Arrival	35	*Myoviridae*	A	13
863A	2	21	Arrival	36	Undetected	N/A	N/A
882A	2	21	Exit	26	Undetected	N/A	N/A
925A	2	21	Exit	35	Undetected	N/A	N/A
940A	2	5	Exit	38	Undetected	N/A	N/A

946A	2	5	Exit	34	*Siphoviridae*	B	N/A
964A	6	21	Arrival	32	*Myoviridae*	H	N/A
1040A	2	21	Exit	36	*Myoviridae*	A	5
1059A	2	5	Exit	25	*Myoviridae*	A	5
1098A	2	21	Exit	30	*Siphoviridae*	C	N/A
1127A	2	21	Exit	25	*Myoviridae*	B	3
1152A	6	21	Exit	25	*Myoviridae*	H	7
1312A	2	5	Exit	25	*Myoviridae*	A	10
1316A	2	5	Exit	25	Undetected	N/A	N/A
1391A	2	5	Exit	26	Undetected	N/A	N/A
1492A	2	5	Exit	35	*Myoviridae*	A	5
1498A	2	5	Exit	31	*Myoviridae*	B	5
1719A	2	21	Exit	25	*Myoviridae*	A	N/A
1731A	1	21	Exit	25	Undetected	N/A	N/A
1745A	2	5	Exit	26	*Myoviridae*	A	5
1795A	2	21	Exit	26	*Myoviridae/ Siphoviridae*	A	N/A
1829A	2	5	Exit	26	*Siphoviridae*	A	8
2024A	2	21	Exit	26	*Myoviridae/ Siphoviridae*	E	N/A
2237A	1	5	Arrival	25	*Myoviridae*	H	7
2256A	1	5	Arrival	25	*Siphoviridae*	H	7
2259A	1	5	Arrival	25	Undetected	N/A	N/A
2318A	2	21	Arrival	25	*Myoviridae*	H	7
2657A	2	5	Arrival	35	*Siphoviridae*	I	N/A
2673B	2	5	Arrival	39	*Siphoviridae*	I	N/A
2677A	2	5	Arrival	35	*Myoviridae*	A	N/A
2679A	2	5	Arrival	35	*Myoviridae*	I	N/A
2707A	2	5	Arrival	45	*Myoviridae*	A	N/A
2713A	N/A	5	Arrival	25	*Siphoviridae*	F	N/A
2791A	1	21	Exit	25	*Myoviridae*	H	5
3083A	6	5	Exit	25	*Myoviridae*	F	N/A
3089A	6	5	Exit	26	*Siphoviridae*	H	N/A
3672A	1	21	Exit	26	Undetected	N/A	N/A
3674A	1	21	Exit	25	Undetected	N/A	N/A
3721A	2	21	Exit	35	*Myoviridae*	I	5
3724A	2	21	Exit	35	*Myoviridae*	A	8
3728A	2	21	Exit	35	Undetected	N/A	N/A
3911A	2	5	Exit	35	*Myoviridae*	D	5
3927A	6	5	Exit	24	*Myoviridae*	D	4
3931A	2	5	Exit	26	Undetected	N/A	N/A
4031A	2	21	Exit	37	Undetected	N/A	N/A
4386A	2	21	Exit	36	*Siphoviridae*	C	5
4387A	2	21	Exit	35	*Myoviridae*	C	5
4645A	2	21	Exit	35	*Siphoviridae*	C	6
4653A	2	21	Exit	35	*Siphoviridae*	I	N/A
4661A	2	21	Exit	36	Undetected	N/A	N/A
4664A	2	21	Exit	37	*Myoviridae*	A	2
5039A	2	5	Exit	35	*Myoviridae*	A	N/A
5042A	2	5	Exit	36	Undetected	N/A	N/A

[1] ENRO ZD: Enrofloxacin inhibition zone diameter in millimeter (mm).
[2] Bacterial PFGE Type: Type A - Type I based on ≥65% similarity cut-off (relatedness).
[3] Phage RFLP Type: Type 1 - Type 14 based on ≥70% similarity cut-off (relatedness).
[4] *Myoviridae/Siphoviridae*: Both *Myoviridae* and *Siphoviridae* morphologies were detected.
[5] N/A: Not applicable.

Bacteriophage isolation

Bacteriophages were induced from *M. haemolytica* isolates using a modification of the procedure by Davies and Lee (2006). Briefly, fresh 30 mL of BHIB was inoculated with an appropriate volume (0.3-1.0 mL) of overnight liquid starter culture of each isolate and incubated at 37°C with shaking at 120 rpm. Each culture was incubated for 3-6 h to achieve early-log phase (approximately 5×10^8 CFU/mL). Phage induction was initiated by adding freshly prepared mitomycin C (Sigma-Aldrich Canada Ltd., Oakville, ON) solution to each culture to a final concentration of 0.2 μg/mL. Optical density (OD_{660nm}) of the induced culture was monitored for 7-8 h. Induced *M. haemolytica* cultures were centrifuged at 3,000 × g for 20 min at 4°C and filtered through 0.22 μm Steriflip-GP filter units (Millipore, Billerica, MA) to remove bacterial debris. Bacteriophage filtrates were stored at 4°C until further characterized.

Enrofloxacin hypothesis

A total of 66 *M. haemolytica* isolates were used in a chi-square test to determine statistical association between enrofloxacin susceptibility of *M. haemolytica* isolates and the presence of inducible bacteriophages. Two groups (n = 33) of *M. haemolytica* isolates with either intermediate susceptibility (inhibition zone diameter 24-26 mm) or high susceptibility (inhibition zone diameter 35-45mm) were examined. Enrofloxacin susceptibility was determined by disk diffusion assay (Klima et al., 2011). A chi-square

value was generated by the FREQ of SAS program (SAS Institute Inc., Cary, NC). Associations were considered statistically significant if $P \leq 0.05$.

Host range analysis

A plaque assay (spot test) was used to determine host range of the induced bacteriophages (Davies and Lee, 2006). Lawns of *M. haemolytica* indicator strains (consisted of 10 individual reference strains and 70 individual field strains listed in Table 3.1) were prepared by mixing 1 mL of overnight liquid cultures with 2 mL of molten agar (0.7% agarose in BHIB; BD Canada, Inc., Mississauga, ON) supplemented with 2 mM $CaCl_2$, and poured onto regular BHI agar plates. After the top agar solidified, 10 µL of each phage filtrate was spotted and absorbed onto prepared agar plates. Plates were incubated upright overnight at 37°C and examined for clearing zones. Eight selected phage filtrates and eighty *M. haemolytica* indicator strains were used for this analysis. In order to confirm the zones of clearing were not due to growth inhibitory effect of bacteriocins, 10 µL of *M. haemolytica* cultures (identical strains used for phage induction) were spotted onto lawns of *M. haemolytica* indicator strains and examined for zones of growth inhibition.

Electron microscopy

Morphological examination of bacteriophage using transmission electron microscope (TEM) was performed as described by Carlson (2005). Briefly, 30 mL of phage filtrate was centrifuged in a fixed-angle rotor at 25,000 × g for 90 min to sediment

phage particles. Supernatants were carefully removed and phage pellets were resuspended in sterile 0.1M ammonium acetate buffer (pH 7.0). This washing process was repeated a second time to remove proteins, sugars, and salt. Final phage pellets were resuspended in 250 µL of 0.1M ammonium acetate buffer. One drop of the phage suspension was absorbed onto a carbon-coated 200 mesh copper grid (Canemco, Inc., Canton de Gore, QC). Phage samples were negatively stained with 5% uranyl acetate solution (pH 4.0) for approximately 1 min. Excess stain was removed with filter paper and grids were examined by TEM (Hitachi H7100: Hitachi High-Technologies Canada, Inc., Toronto, ON) at 75.0 kV and a magnification range of 20,000× to 100,000×.

Restriction fragment length polymorphism (RFLP) analysis

Genomic bacteriophage DNA was extracted and purified from concentrated phage suspensions using a Phage DNA Isolation kit (product # 26100, Norgen Biotek Corp., Thorold, ON) according to the manufacturer's instructions. Proteinase K was included in the extraction in an effort to increase DNA yields. Extracted DNA was quantified fluorometrically using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Burlington, ON) and NanoDrop 3300 fluorospectrometer (Fisher Scientific Limited, Nepean, ON). Isolated phage genomic DNA was digested using the same restriction enzymes HindIII and ClaI (New England Biolabs Inc., Pickering, ON) as employed by Davies and Lee (2006). Extracted DNA was incubated with restriction enzymes for 4 h at 37°C. Incubation time was determined experimentally to ensure complete digestion of phage DNA and non-specific degradation of DNA bands did not occur. Digested phage DNA fragments were separated by agarose gel (0.7% w/v) electrophoresis and visualized

with ethidium bromide staining. Gels were soaked in 1 mM $MgSO_4$ solution to remove background fluorescence as necessary. RFLP profiles were analyzed with BioNumerics software (Applied Maths, Inc., Austin, TX).

Sequencing

Eight unique *M. haemolytica* temperate bacteriophages were selected for genome sequence analysis based on several criteria. Phages were chosen to represent different virion morphologies, RFLP profiles, and host attributes. Host attributes, including PFGE types of *M. haemolytica* isolates, isolate serotypes and origin of the sample were considered (Table 3.1). Phage genomic DNA was prepared from φMH535A, φMH587A, φMH622A, φMH1127A, φMH1152A, φMH2256A, φMH3927A, and φMHG8. In order to prepare the larger amount of bacteriophage DNA required for whole genome sequencing, selected *M. haemolytica* isolates were induced in quadruplicate. Induction procedures were similar to those previously outlined, but additional concentration and purification steps by precipitation with polyethylene glycol (PEG) were implemented to maximize phage yield and purity (Carlson, 2005). Briefly, 1 µL of RNase A (7,000 U/mL) was included at the end of phage induction to remove residual bacterial RNA. Sodium chloride was added to achieve a final concentration of 0.57 M and samples were mixed on ice for 1 h to improve phage precipitation. Phage samples were centrifuged and filtered to remove bacterial debris. Phage filtrate was mixed with PEG 8000 (Sigma-Aldrich Canada Ltd., Oakville, ON) to achieve a concentration of 10% (v/v) and mixed on ice for 1 h and samples were stored overnight at 4°C. Phage suspensions were centrifuged at 11,000 × g for 20 min at 4°C and the supernatant was carefully removed.

Phage pellets were resuspended in 250 μL of SM buffer. Phage DNA was extracted and DNA concentration determined as described previously. Subsequent DNA quality control assurance and amplification of the eight bacteriophage samples were conducted by Eurofins MWG Operon prior to sequencing by GS FLX Titanium series chemistry (Roche 454) (Eurofins MWG Operon, Huntsville, AL). Whole genome sequencing yielded excellent coverage of all samples ranging from 34.8× to 67.5×. Sequencing data was assembled by Celera Assembler (Version 5.3) and critical gaps of selected samples were identified and closed by conventional Sanger sequencing technology.

Genome annotation

The φMH1152A consensus sequence was used for gene identification and annotation. Initial genome annotation was done using myRAST program (Aziz et al., 2008). SeqBuilder application (DNASTAR, Inc., Madison, WI) was used to visually scan the sequence for potential genes. All translated proteins were scanned for homologues using BLASTP and PSI-BLAST (Altschul et al., 1990 and 1997). GenBank nr database (Benson et al., 2011) and Conserved Domain Database (Marchler-Bauer et al., 2011) were used to compare protein coding sequences (CDS). Ribosomal binding sites (RBS) were identified using RBSfinder program online and rho-independent terminators were identified using MFold (Zuker, 2003) and TransTerm (Ermolaeva et al., 2000). Promoters were identified by neural network promoter prediction (Reese, 2001) with visual inspection. Transfer RNA (tRNA) genes were screened using Aragorn (Laslett and Canback, 2004) and tRNAScan (Lowe and Eddy, 1997). Sequence manipulation and graphical representation of phage genome were made using the Geneious v5.4 program

(Biomatters Ltd., Auckland, New Zealand). The φMH1152A consensus sequence was deposited into GenBank database with an accession number JN255163.

Statistical analysis

RFLP and PFGE-related analyses were performed using BioNumerics software (Applied Maths, Inc., Austin, TX). Dendrograms were generated with UPGMA clustering of Dice coefficient values with 1.0% optimization and 1.5% position tolerance settings.

3.3 Results

Isolated bacteriophages were characterized by TEM for phage morphology, RFLP analysis for genetic diversity, and host range analysis for lysis and plaque-forming capability. The correlation between host antimicrobial resistance and the presence of inducible prophages was also investigated with regard to phages that originated from *M. haemolytica* that exhibited different levels of resistance to enrofloxacin. Eight distinct bacteriophages were identified and subsequently sequenced using next generation 454™ sequencing technology to gain insights into the genomic makeup of the bacteriophages isolated in this study. The sequence information was further evaluated against previously published *M. haemolytica* bacteriophage and bacterial genomes.

Preliminary studies on phage induction were performed with reference strain BAA410. The published genome of *M. haemolytica* BAA410 was found to contain a complete, inducible prophage (Highlander et al., 2006). The presence of temperate bacteriophages in *M. haemolytica* strain BAA410 was confirmed initially by TEM (Table

3.1). This strain was consistently used as a control in subsequent bacteriophage induction. Overall, a total of 74 *M. haemolytica* isolates (two reference plus 72 field strains) were induced and screened for temperate bacteriophages. Among these, 56 *M. haemolytica* isolates were confirmed positive for temperate bacteriophages (Table 3.1). This accounted for close to 76% of all isolates examined. Each phage was designated as a φ[host strain used for phage induction] for identification. For instance, φMHBAA410 was induced and isolated from BAA410 strain of *M. haemolytica* (MH). Based on TEM images, two major phage morphologies were observed including *Myoviridae* (Fig. 3.1a and Fig. 3.1c) and *Siphoviridae* (Fig. 3.1b). All phages examined in this study belonged to the tailed phages, order *Caudovirales*. Typical tailed phages contained icosahedral heads, but capsids were often shrunken due to sample preparation or the staining procedure (Ackermann, 2009), occasionally making morphological identification difficult. A total of 29 *M. haemolytica* isolates produced phages displaying *Myoviridae* morphology. A typical *Myoviridae-like* phage from φMH755A is presented in Fig. 3.1a, showing an icosahedral head and a contractile tail that consists of a central tube with sheath located below the capsid. Six *Myoviridae*-like phages exhibited shorter tails, φMH1719A being one of these (Fig. 3.1c). A further 21 *M. haemolytica* isolates produced *Siphoviridae*-like phages upon induction. A typical *Siphoviridae*-like phage from φMHG8 is shown in Fig. 3.1b, showing a long, non-contractile tail with no sheath. Multiple phage morphologies were apparent in single preparations of six of the isolates analyzed, with virions resembling both *Myoviridae* and *Siphoviridae* phages (Table 3.1).

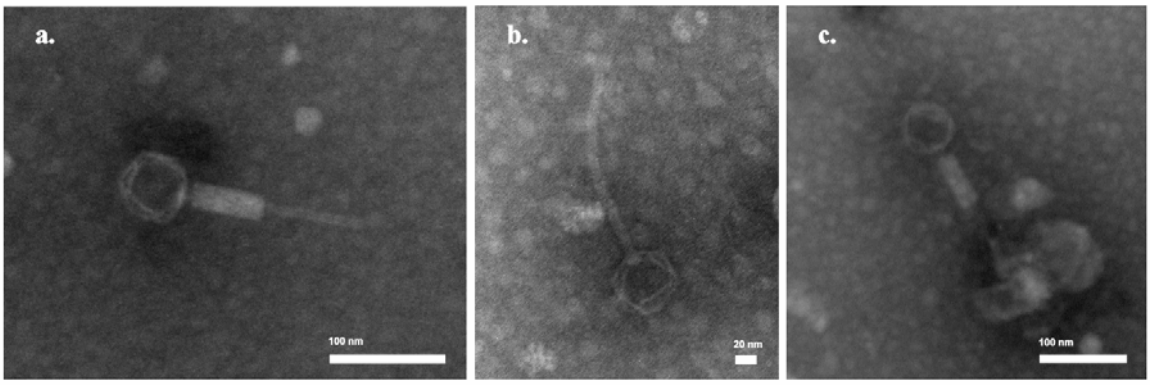


Fig. 3.1. Representative TEM micrographs of negatively stained virions. φMH755A, *Myoviridae*-like phage (a); φMHG8, *Siphoviridae*-like phage (b); φMH1719A, *Myoviridae*-like phage (c). Scale bars are 100nm in (a, c), 20nm in (b).

Results from TEM identification of temperate bacteriophages in *M. haemolytica* isolates were used to determine the validity of our enrofloxacin hypothesis. Intermediate enrofloxacin susceptible and enrofloxacin susceptible isolates were identified as either phage positive or phage negative, but there was no evidence of a statistical correlation (P = 0.415).

Restriction fragment length polymorphic analysis of phage DNA was performed to determine genetic variability of induced bacteriophages from *M. haemolytica* isolates. The resulting patterns were used to characterize and differentiate phages in this study. Phage DNA was extracted from 56 *M. haemolytica* isolates that were identified positive for phage by TEM. Profiles were obtained successfully for 40 of these phage isolates while DNA yields of the other isolates were too low to generate a viable RFLP pattern. Representative RFLP profiles for eight of the phage isolates are depicted in Fig. 3.2. Considerable variability was observed for fragment banding patterns between individual profiles. Fragment analysis of phage RFLP profiles were performed using BioNumerics program. Isolates with multiple phage morphologies were excluded in this analysis to

avoid ambiguity. A dendrogram was generated with 14 distinct clusters being identified with a relatedness of ≥70% (Fig. 3.3). The cluster analysis showed substantial diversity, and phages belonging to each cluster group were designated RFLP types 1-14 (Table 3.1).

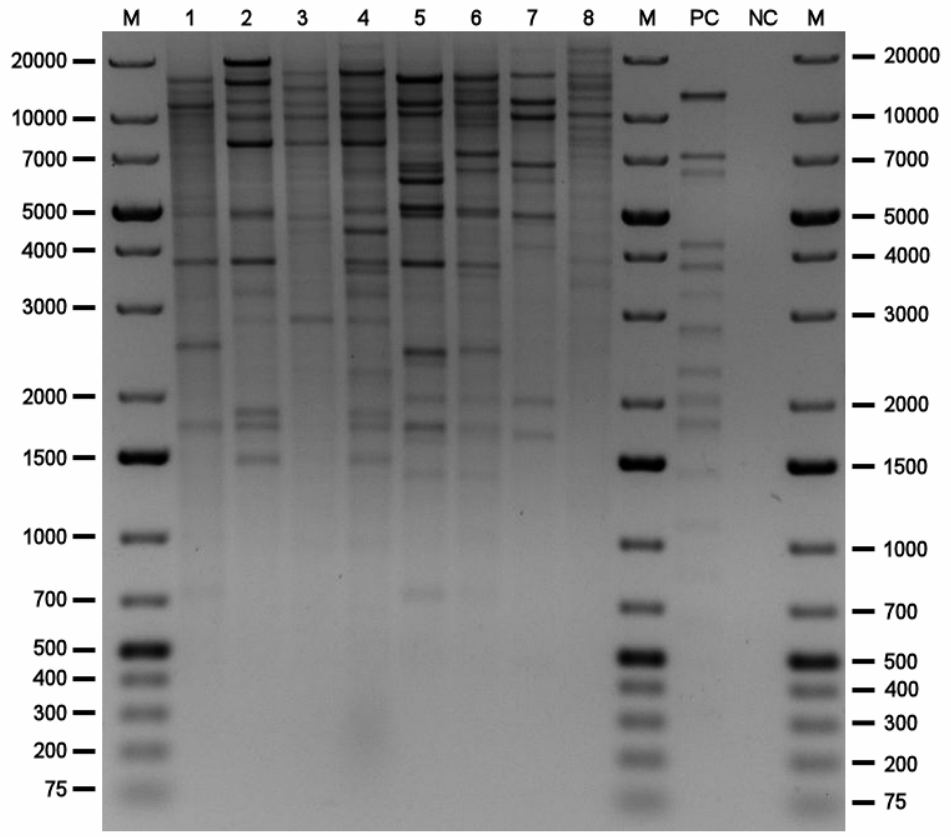
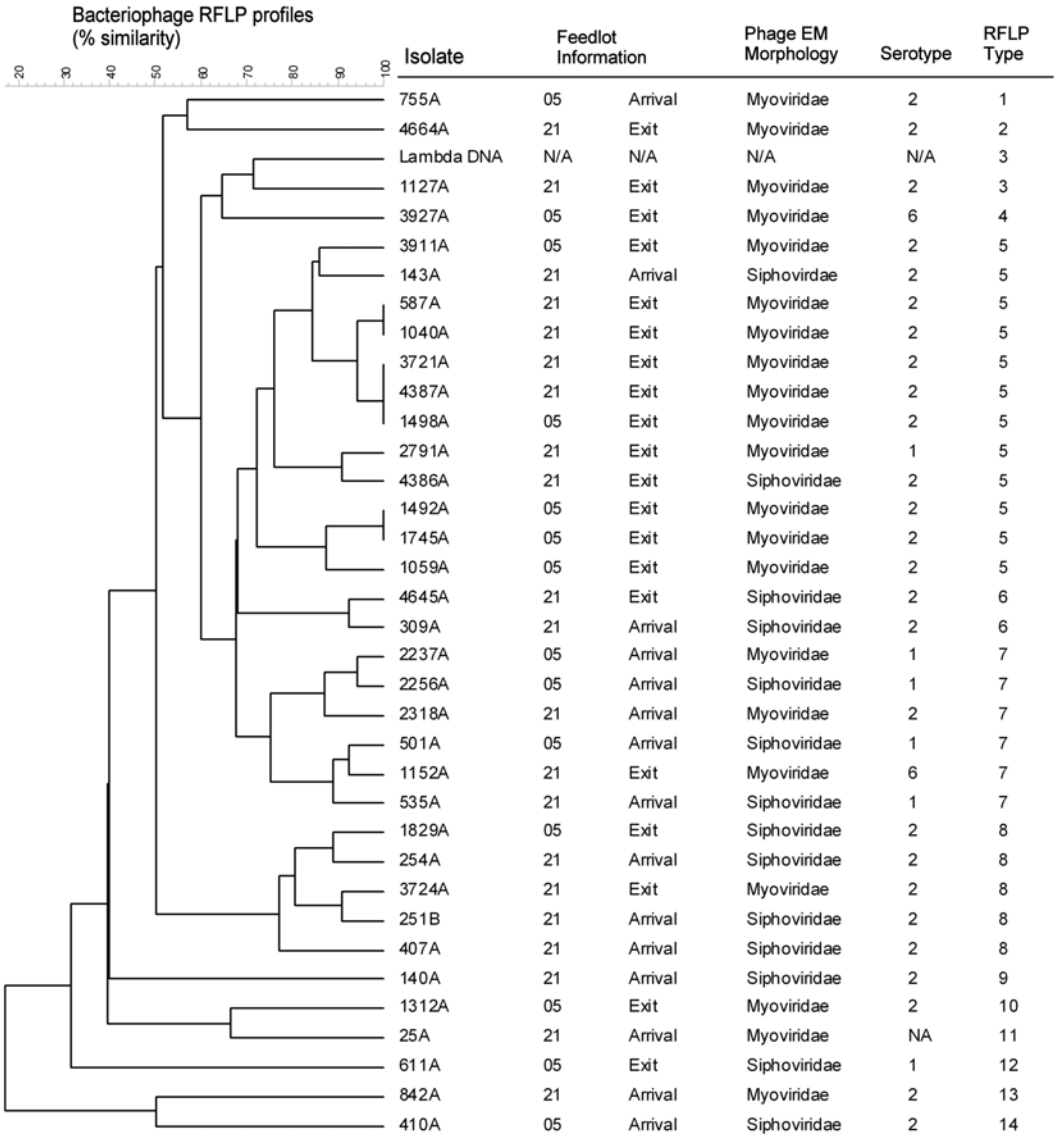


Fig. 3.2. Representative RFLP profiles of eight *M. haemolytica* phage DNA digested with HindIII and ClaI (lanes 1-8). Lane 1, φMH535A; lane 2, φMH587A; lane 3, φMH622A; lane 4; φMH1127A; lane 5, φMH1152A; lane 6, φMH2256A; lane 7, φMH3927A; lane 8, φMHG8. PC, positive control, 100ng lambda DNA digested with HindIII and ClaI. NC, negative control, contained water instead of DNA template. M, 1kb plus DNA ladder.



Isolate	Feedlot Information		Phage EM Morphology	Serotype	RFLP Type
755A	05	Arrival	Myoviridae	2	1
4664A	21	Exit	Myoviridae	2	2
Lambda DNA	N/A	N/A	N/A	N/A	3
1127A	21	Exit	Myoviridae	2	3
3927A	05	Exit	Myoviridae	6	4
3911A	05	Exit	Myoviridae	2	5
143A	21	Arrival	Siphovirdae	2	5
587A	21	Exit	Myoviridae	2	5
1040A	21	Exit	Myoviridae	2	5
3721A	21	Exit	Myoviridae	2	5
4387A	21	Exit	Myoviridae	2	5
1498A	05	Exit	Myoviridae	2	5
2791A	21	Exit	Myoviridae	1	5
4386A	21	Exit	Siphoviridae	2	5
1492A	05	Exit	Myoviridae	2	5
1745A	05	Exit	Myoviridae	2	5
1059A	05	Exit	Myoviridae	2	5
4645A	21	Exit	Siphoviridae	2	6
309A	21	Arrival	Siphoviridae	2	6
2237A	05	Arrival	Myoviridae	1	7
2256A	05	Arrival	Siphoviridae	1	7
2318A	21	Arrival	Myoviridae	2	7
501A	05	Arrival	Siphoviridae	1	7
1152A	21	Exit	Myoviridae	6	7
535A	21	Arrival	Siphoviridae	1	7
1829A	05	Exit	Siphoviridae	2	8
254A	21	Arrival	Siphoviridae	2	8
3724A	21	Exit	Myoviridae	2	8
251B	21	Arrival	Siphoviridae	2	8
407A	21	Arrival	Siphoviridae	2	8
140A	21	Arrival	Siphoviridae	2	9
1312A	05	Exit	Myoviridae	2	10
25A	21	Arrival	Myoviridae	NA	11
611A	05	Exit	Siphoviridae	1	12
842A	21	Arrival	Myoviridae	2	13
410A	05	Arrival	Siphoviridae	2	14

Fig. 3.3. Dendrogram of RFLP profiles from 35 *M. haemolytica* phages and a lambda DNA (control). Isolate origin (feedlot information), phage morphology, isolate serotype, and phage RFLP type data included. A total of 14 phage RFLP types were identified based on ≥70% similarity.

Pulsed-field gel electrophoresis (PFGE) is a highly reproducible and discriminatory tool for molecular typing of bacteria and has been used to characterize *M. haemolytica* genomes (Klima et al., 2011). Corresponding PFGE profiles of *M. haemolytica* isolates used in this study were selected and analysed using BioNumerics. The associations between temperate bacteriophage genomes (RFLP) and host genomes (PFGE) were examined in 35 *M. haemolytica* isolates. Clusters of PFGE profiles were generated and compared with *M. haemolytica* phage RFLP types (Fig. 3.4). Additional information such as phage morphology and isolate serotype were included to determine correlations among these properties. Isolates with RFLP type 7 were clustered together during this analysis and they exhibited >74% relatedness between each other based on their corresponding PFGE profiles. Serotypes were closely associated with bacterial PFGE profiles (Klima et al., 2011), which were also reflected in phage RFLP type 7. Phage morphology did not appear to be correlated with isolate serotype, phage RFLP type, or bacterial PFGE profile.

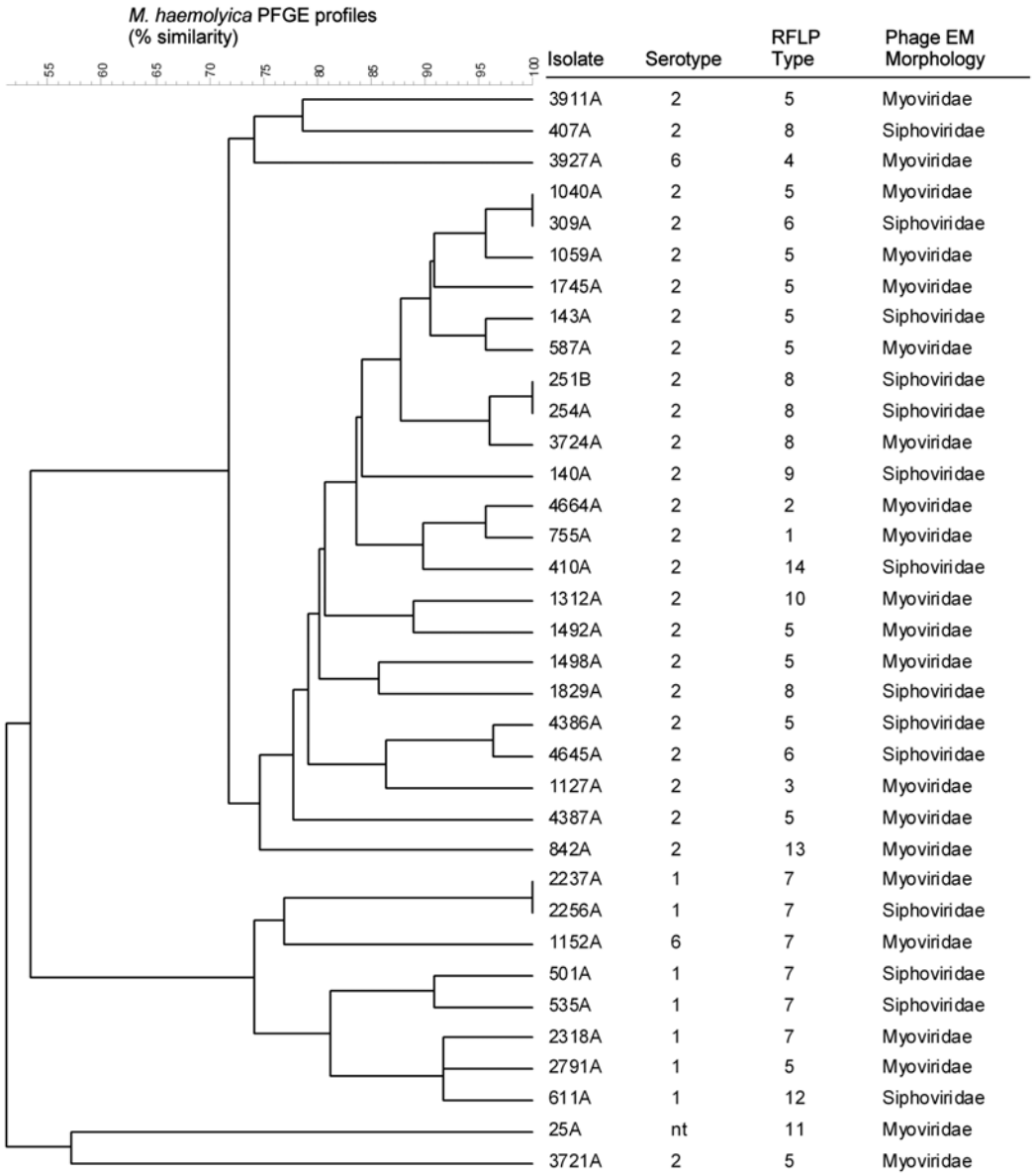


Fig. 3.4. Dendrogram of PFGE profiles from selected *M. haemolytica* isolates. Phage RFLP type, isolate serotype and phage morphology data included.

Host range specificities of eight selected bacteriophages were determined by plaque assay against a total of 80 *M. haemolytica* indicator strains consisting of 10 reference strains of various serotypes (serotype 1, 2, 5, 6, 7, 8, 9, 12, 14) and 70 field strains (serotypes 1, 2, and 6) (Table 3.1). A typical plaque assay with *M. haemolytica* indicator strain 413A is shown in Fig. 3.5. Results from host range analysis are summarized in Table 3.2. Zones of lysis ranging from slightly visible to turbid were recorded and expressed as number of incidences (frequency) where plaques were detected for indicator strains of each serotype. Phage φMH587A generated more plaques than other phages, while φMHG8 was the only phage unable to form plaques on any of the indicator strains tested. Apart from serotype 2 indicator strains, few plaques were formed with either serotype 1 or serotype 8 indicator strains. Most plaques on serotype 1 indicator strains were formed by φMH1152A. Plaques were absent on serotype 5, 6, 7, 9, 12, and 14 indicator strains.

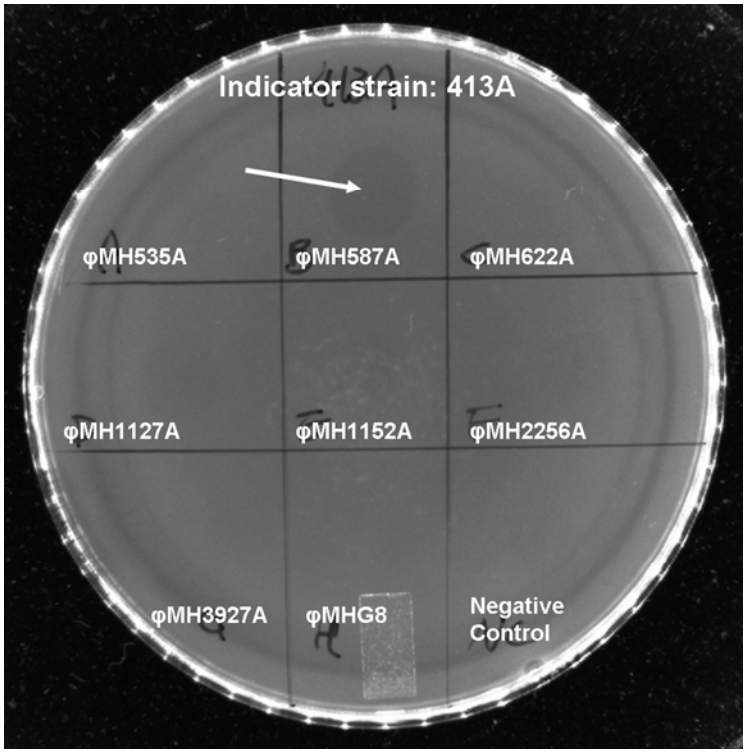


Fig. 3.5. Plaque assay of eight phage filtrates spotted on a bacterial lawn of *M. haemolytica* 413A as indicator strain. Negative control contained sterile water instead of phage filtrate. A turbid zone of lysis (plaque) is shown with an arrow.

Table 3.2. Host range summary of selected eight *M. haemolytica* temperate bacteriophages against *M. haemolytica* indicator strains of various serotypes.

			Frequency of plaques formed by phage							
			φMH 535A	φMH 587A	φMH 622A	φMH 1127A	φMH 1152A	φMH 2256A	φMH 3927A	φMH G8
	serotype	n	A	B	B	B	C	A	C	D
Indicator strain	1	11	1	0	1	1	9	1	0	0
	2	56	8	50	7	27	11	14	1	0
	5	1	0	0	0	0	0	0	0	0
	6	6	0	0	0	0	0	0	0	0
	7	1	0	0	0	0	0	0	0	0
	8	1	1	1	1	1	1	1	1	0
	9	1	0	0	0	0	0	0	0	0
	12	1	0	0	0	0	0	0	0	0
	14	1	0	0	0	0	0	0	0	0
Total number of plaques			10	51	9	29	21	16	2	0

A: Phage induced from serotype 1 host.
B: Phage induced from serotype 2 host.
C: Phage induced from serotype 6 host.
D: Phage induced from serotype 8 host.

A summary of sequencing data and assembly result of eight selected phage samples is shown in Table 3.3. Preliminary sequence analysis of assembled large contigs from each of these phage samples was accomplished by BLAST search using NCBI's nucleotide database. Contigs from φMH535A, φMH587A, φMH1127A, φMH1152A, and φMH2256A showed high degree of nucleotide similarity to regions of a published complete genome sequence of bacteriophage φMhaA1-BAA410 and bacteriophage φMhaA1-PHL101 induced from *M. haemolytica* serotype A1 (Highlander et al., 2006). Partial sequence similarity to bacteriophage φMhaA1-BAA410 also existed in φMHG8 large contigs, but to a lesser extent. Large contigs from φMH622A exhibited no significant alignment with other sequences in the database. Contigs from φMH3927A, and φMHG8 also showed limited sequence similarity to small regions of *Actinobacillus pleuropneumoniae* and *Actinobacillus succinogenes*, while contigs from other samples exhibited little similarity to *Actinobacillus*-related sequences.

Table 3.3. A summary of eight *M. haemolytica* temperate bacteriophage sequences.

	Sample							
	φMH 535A	φMH 587A	φMH 622A	φMH 1127A	φMH 1152A	φMH 2256A	φMH 3927A	φMH G8
Total Number of Reads	14738	12306	9563	9263	11121	14155	8862	24910
Total Number of Bases	4881131	3992786	3002471	2756307	3502947	4657467	2731940	8283565
Average Read Length	331	324	313	297	314	329	308	332
Number of reads assembled	12827	10723	8062	7542	9696	12307	7631	22730
Number of reads too short (<64)	324	273	245	291	272	327	196	550
Number of Contigs	1459	913	516	928	729	1294	549	1998
Largest Contig	27323	34755	4106	25180	34896	31471	46040	15414
Total number of bases in Contigs	645263	382622	208012	363916	313744	552010	260541	1011590
Coverage (all reads)	39.49	63.91	67.48	45.72	60.5	55.63	58.54	34.83
%GC	41.1	41.4	35.7	41.4	41.6	41.2	41.4	41.5

Among eight phage samples, contigs from φMH1152A demonstrated the highest sequence similarity and coverage to the genomes of bacteriophage φMhaA1-BAA410 and φMhaA1-PHL101. To confirm the assembly, PCR reactions were designed and PCR-generated sequences were integrated. After manual editing, a 34,719 bp consensus sequence of φMH1152A genome was constructed. A total of 54 open reading frames were predicted from this consensus sequence (Fig. 3.6 and Table 3.4). Predicted attachment (*att*) sites, ribosomal binding sites, promoters, and terminators are also shown in Fig. 3.6. Genes encoding tRNA were found to be absent in φMH1152A genome. A summary of identified genes with predicted protein function is listed in Table 3.4. The φMH1152A consensus sequence was pairwise-aligned to the previously described genome of bacteriophage φMhaA1-PHL101. A graphical alignment map is depicted in Fig. 3.7, which shows 99.2% sequence similarity between these two genomes. Comparisons of φMH1152A consensus sequence to genome sequences of *M. haemolytica* PHL213 (NZ_AASA00000000), *M. haemolytica* serotype A2 str. OVINE (NZ_ACZX00000000), and *M. haemolytica* serotype A2 str. BOVINE (NZ_ACZY00000000) showed various degrees of similarity. Contigs 38, 39, 129, 159 of *M. haemolytica* PHL213 genome contained 99%-100% identical sequences to different regions of our φMH1152A consensus sequence and together they roughly covered the entire phage sequence with minimal overlaps and gaps. Contigs 00021, 00030, 00073, 00134 of *M. haemolytica* serotype A2 str. OVINE genome contained regions that were 93%-99% identical to regions of φMH1152A sequence, but several gaps were present. Regions of contig 00033 of *M. haemolytica* serotype A2 str. BOVINE genome exhibited 97% identical sequences and cover 75% of φMH1152A sequence.

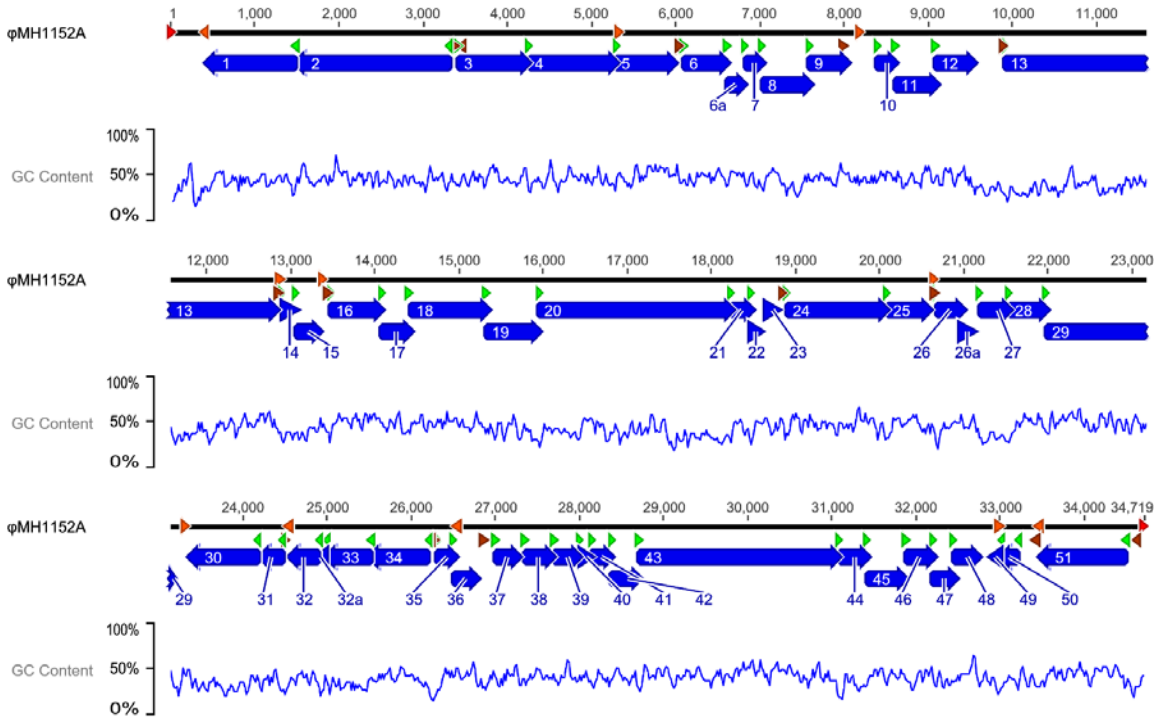


Fig 3.6. Genome map of bacteriophage φMH1152A. Predicted genes are numbered from 1 to 51. Percentage GC content is shown below the gene with a sliding window size of 50 nucleotides. Predicted attachment (*att*) sites, ribosomal binding sites (RBS), promotors, and terminators are shown with red, green, brown, and orange colored triangles.

Table 3.4. Bacteriophage φMH1152A ORF analysis and gene prediction.

ORF	Range (bp)	Siza (aa)	Predicted function	Best BLASTP match (organism)	BLASTP e value	Identities (%)
1	428-1468	346	capsid portal protein Q	YP_655469.1|capsid portal protein Q [Mannheimia phage phiMHaA1]	0	100
2	1477-3294	605	phage terminase, ATPase subunit	YP_655470.1|terminase ATPase subunit [Mannheimia phage phiMHaA1]	0	99
3	3429-4256	275	capsid scaffolding protein O	YP_655471.1|capsid scaffolding protein O [Mannheimia phage phiMHaA1]	0	100
4	4270-5298	342	major capsid protein N	YP_655472.1|major capsid protein N [Mannheimia phage phiMHaA1]	0	100
5	5308-5997	229	terminase, small subunit	YP_655473.1|terminase small subunit [Mannheimia phage phiMHaA1]	2E-167	100
6	6109-6624	171	head completion protein L	YP_655474.1|head completion protein L [Mannheimia phage phiMHaA1]	9E-124	100
6a	6621-6830	69	tail synthesis protein X	YP_655475.1|tail synthesis protein X [Mannheimia phage phiMHaA1]	1E-45	100
7	6839-7045	68	possible holin	YP_655476.1|possible holin [Mannheimia phage phiMHaA1]	1E-42	100
8	7038-7604	188	endolysin	YP_655477.1|endolysin [Mannheimia phage phiMHaA1]	3E-138	100
9	7601-8056	151	conserved hypothetical phage protein	YP_655478.1|hypothetical bacteriophage protein [Mannheimia phage phiMHaA1]	2E-106	100
10	8405-8626	73	hypothetical Zinc-finger containing protein	YP_655479.1|hypothetical protein MhaA1p11 [Mannheimia phage phiMHaA1]	3E-47	100
11	8623-9108	161	tail completion protein R	YP_655480.1|tail completion protein R [Mannheimia phage phiMHaA1]	4E-115	100
12	9101-9559	152	tail synthesis protein S	YP_655481.1|tail synthesis protein S [Mannheimia phage phiMHaA1]	2E-107	100
13	9927-12839	970	tail protein T	YP_655483.1|tail protein T [Mannheimia phage phiMHaA1]	0	100

14	12904-13080	58	conserved hypothetical protein	YP_655484.1|hypotheticalprotein MhaA1p16 [Mannheimia phage phiMHaA1]	1E-34	100
15	13073-13360	95	conserved hypothetical protein	YP_655485.1|hypotheticalprotein MhaA1p17 [Mannheimia phage phiMHaA1]	1E-62	100
16	13489-14094	201	baseplate assembly protein V	YP_655486.1|baseplate assembly protein V [Mannheimia phage phiMHaA1]	6E-145	100
17	14094-14429	111	baseplate assembly protein W	YP_655487.1|baseplate assembly protein W [Mannheimia phage phiMHaA1]	2E-78	100
18	14426-15343	305	baseplate assembly protein J	YP_655488.1|baseplate assembly protein J [Mannheimia phage phiMHaA1]	0	100
19	15330-15962	210	tail formation protein I	YP_655489.1|tail formation protein I [Mannheimia phage phiMHaA1]	1E-153	100
20	15965-18244	759	variable tail fiber protein H	YP_655490.1|variable tail fiber protein H [Mannheimia phage phiMHaA1]	0	99
21	18245-18487	80	conserved hypothetical protein	YP_655491.1|hypothetical protein MhaA1p23 [Mannheimia phage phiMHaA1]	3E-54	99
22	18477-18596	39	conserved hypothetical protein	ABD90652.1|hypothetical protein [Mannheimia phage phiMhaA1-BAA410]	1E-13	100
23	18652-18798	48	conserved hypothetical protein	ABD90625.1|hypothetical protein [Mannheimia phage phiMhaA1-BAA410]	1E-28	100
24	18905-20086	393	phage tail sheath protein	YP_655492.1|tail sheath protein FI [Mannheimia phage phiMHaA1]	0	100
25	20095-20601	168	major tail tube protein	YP_655493.1|tail tube protein FII [Mannheimia phage phiMHaA1]	3E-120	100
26	20680-20994	104	phage tail protein E	YP_655494.1|tail protein E [Mannheimia phage phiMHaA1]	1E-68	100
26a	20970-21134	54	possible of extension protein E' of E	ZP_05850311.1|P2 GpE family phage tail protein [Haemophilus influenzae NT127]	4E-12	60
27	21198-21545	115	conserved hypothetical protein	YP_655495.1|hypotheticalprotein MhaA1p28 [Mannheimia phagephiMHaA1]	1E-75	100

28	21547-21984	145	phage tail protein U	YP_655496.1|tail proteinU [Mannheimia phage phiMHaA1]	4E-103	100
29	21984-23180	398	phage tail protein D	YP_655497.1|tail proteinD [Mannheimia phage phiMHaA1]	0	97
30	23363-24169	268	conserved hypothetical phage protein	ZP_04976993.1|hypothetical bacteriophage protein [Mannheimia haemolytica PHL213]	0	100
31	24204-24464	86	conserved hypothetical protein	YP_655499.1|hypothetical protein MhaA1p32 [Mannheimia phage phiMHaA1]	4E-56	100
32	24564-24893	109	conserved hypothetical protein	YP_655500.1|hypothetical protein MhaA1p33 [Mannheimia phage phiMHaA1]	9E-74	100
32a	24894-24974	26	conserved hypothetical protein	ZP_05991374.1|hypothetical protein COI_0691 [Mannheimia haemolytica serotype A2 str. OVINE]	4E-9	96
33	24992-25510	172	conserved hypothetical protein	YP_655501.1|hypothetical protein MhaA1p34 [Mannheimia phage phiMHaA1]	2E-123	100
34	25514-26200	228	CI repressor	YP_655502.1|CI repressor [Mannheimia phage phiMHaA1]	3E-169	100
35	26324-26536	70	Cro family repressor	YP_655503.1|Cro repressor [Mannheimia phage phiMHaA1]	9E-43	100
36	26520-26801	93	conserved hypothetical protein	YP_655504.1|hypotheticalprotein MhaA1p37 [Mannheimia phage phiMHaA1]	2E-62	100
37	27013-27285	90	conserved hypothetical protein	YP_655505.1|hypotheticalprotein MhaA1p38 [Mannheimia phage phiMHaA1]	2E-61	100
38	27368-27700	110	conserved hypothetical protein	YP_655506.1|hypotheticalprotein MhaA1p39 [Mannheimia phage phiMHaA1]	3E-71	100
39	27713-28006	97	conserved hypothetical protein	YP_655507.1|hypotheticalprotein MhaA1p40 [Mannheimia phage phiMHaA1]	3E-68	100
40	28018-28146	42	conserved hypothetical protein	ZP_04976983.1|hypothetical protein MHA_0402 [Mannheimia haemolytica PHL21]	1E-23	100
41	28158-28400	80	conserved hypothetical protein	YP_655508.1|hypotheticalprotein MhaA1p41 [Mannheimia phage	4E-53	100

				phiMHaA1]		
42	28397-28729	110	conserved hypothetical protein	YP_655509.1|hypotheticalprotein MhaA1p42 [Mannheimia phage phiMHaA1]	2E-78	100
43	28726-31086	786	replication protein A	YP_655510.1|replication protein A [Mannheimia phage phiMHaA1]	0	100
44	31099-31431	110	conserved hypothetical protein	YP_655511.1|hypotheticalprotein MhaA1p44 [Mannheimia phage phiMHaA1]	6E-74	100
45	31431-31883	150	single-stranded DNA-binding protein	YP_655512.1|single-stranded DNA binding protein [Mannheimia phage phiMHaA1]	7E-108	100
46	31894-32226	110	conserved hypothetical phage protein	ZP_04976977.1|hypothetical bacteriophage protein [Mannheimia haemolytica PHL213]	2E-78	100
47	32216-32485	89	conserved hypothetical protein	YP_655514.1|hypotheticalprotein MhaA1p47 [Mannheimia phage phiMHaA1]	4E-59	100
48	32460-32750	96	conserved hypothetical protein	YP_655515.1|hypotheticalprotein MhaA1p48 [Mannheimia phage phiMHaA1]	7E-66	100
49	32885-33001	38	conserved hypothetical protein	none		
50	33020-33202	60	conserved hypothetical protein	YP_655516.1|hypothetical protein MhaA1p49 [Mannheimia phage phiMHaA1]	1E-33	100
51	33480-34478	332	integrase	YP_655517.1|integrase [Mannheimia phage phiMHaA1]	0	100

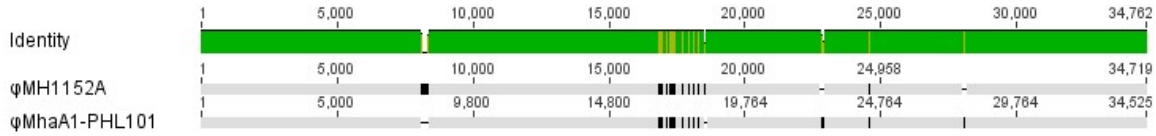


Fig. 3.7. Alignment map showing regions of nucleotide similarity between published sequence of bacteriophage φMhaA1-PHL101 and consensus sequence of φMH1152A. Sequence disagreements are shown in black.

3.4 Discussion

Unknown phages have traditionally been identified by electron microscopy, which has become a crucial feature for comparative virology and phage classification (Ackermann, 2009). The bacteriophage morphologies observed here were relatively diverse within the tailed phage group, which is consistent with previous studies that identified three major tail morphologies from *M. haemolytica* temperate bacteriophages belonging to *Myoviridae*, *Siphoviridae*, and *Podoviridae* (Davies and Lee, 2006). Similar morphologies were also observed in bacteriophages of a related organism, *Pasteurella multocida* (Ackermann and Karaivanov, 1984). Lysogeny is a common feature in many bacterial populations (Weinbauer, 2004), including *M. haemolytica*. Previous studies have shown that bacteriophage particles were widely distributed in *M. haemolytica* with 75% of 32 *M. haemolytica* isolates examined containing inducible phages (Davies and Lee, 2006). The prevalence of phages was similar in this study, where 76% of 74 *M. haemolytica* isolates examined contained inducible bacteriophages.

Uniquely, in this study multiple phage types were recovered from single *M. haemolytica* isolates. This was observed on several occasions where two different phage morphologies (*Myoviridae* and *Siphoviridae*) were present in single isolate preparations. Although it is common to find multiple prophages in the genomes of *E. coli* O157:H7, *Streptococcus pyogenes*, *Bacillus subtilis*, and many other bacterial species (Canchaya et

al., 2003b; Casjens, 2003), and the induction of multiple prophages has been demonstrated in a marine bacterium *Silicibacter* sp. strain TM1040 (Chen et al., 2006), previous studies have not reported multiple prophage induction from *M. haemolytica*. The *M. haemolytica* strain BAA410 contained at least two intact prophages in its genome (Gioia et al., 2006) with a P2-like phage, two Mu-like phages, and λ-like fragments being detected in the genome. Typically P2-like and Mu-like phages are myoviruses, while λ is a siphovirus (Guttman et al., 2005). The combination of myovirus and siphovirus sequences in *M. haemolytica* genome might explain our observation of multiple phage morphologies from strain BAA410 and other phage preparations (Table 3.1), assuming that these prophage sequences were complete and inducible. Prophages can exhibit different levels of inducibility and some prophage-like elements in the genome can be defective or non-mitomycin C-inducible (Casjens, 2003), such as certain prophages identified in *Silicibacter* sp. strain TM1040 (Chen et al., 2006).

Phage RFLP analysis provided a rapid assessment of genetic diversity of the induced bacteriophages (Carlson, 2005; Pullinger et al., 2004). Successful restriction enzyme digestion also confirmed that phage genome composition was indeed double-stranded DNA. Results were used to characterize and differentiate phages in this study. Among 35 phage RFLP profiles obtained, substantial genetic variation was evident from the patterns generated with double enzyme digestions. At ≥70% RFLP relatedness, three groups of phages (587A and 1040A; 3721A, 4387A, and 1498A; 1492A and 1745A) showed identical phage RFLP profiles often exhibiting the same phage morphology and same host attributes including isolate serotype, sampling location, or sampling time (arrival or exit) (Fig. 3.3). This was expected as prophages can constitute a sizable part of

the host genome and account for biological differences between isolates of the same species (Canchaya et al., 2003b; Casjens, 2003), including changes in host restriction system, sensitivity to antimicrobials, and host immunity from phage re-infection (Casjens, 2003; Clokie and Kropinski, 2009; Willey et al., 2008). Interestingly, field isolates that contained multiple phages were all collected from the same feedlot. This suggests multiple phage induction usually occurs in a particular group of *M. haemolytica* isolates and that these phages are geographically dispersed. Cluster analysis of host PFGE profiles showed a slight correlation with phage RFLP type 7 cluster (Fig. 3.4). Results from phage RFLP analysis suggest prophages or prophage-like sequences are important contributors to bacterial diversity and bacterial evolution (Brüssow et al., 2004; Casjens, 2003; Hendrix et al., 1999), and the exchange of genetic information between bacteria may be carried out by prophages through horizontal transfer (Banks et al., 2002; Briani et al., 2001; Ohnishi et al., 2001).

Host range analysis was used to test the efficiency of a phage's ability to infect and develop in a particular host. Plaque formation indicates phages are capable of infecting and lysing the bacterial strains examined. This approach may be used as a discriminatory tool that compliments RFLP analysis, as host range analysis of the eight selected bacteriophages showed distinct lysis patterns on a collection of indicators strains (Table 3.2). Overall, phages exhibited a limited host range that was serotype specific, an observation similar to previous findings (Davies and Lee, 2006). Plaques were observed on serotype 1, serotype 2, and serotype 8 indicator strains. No plaques were formed on any other serotypes examined. Typically, temperate phages provide the host immunity from re-infection by the same phage or other phages of the same immunity group

(Casjens, 2003; Clokie and Kropinski, 2009; Gill and Hyman, 2010; Guttman et al., 2005; Willey et al., 2008). Since the majority of our indicator strains are known to harbour prophages, their presence might have prevented infections of related phages. None of the phages induced were able to form clear plaques such as one would see from a strictly lytic bacteriophage. This was probably due to lysogenization of some bacteria within the plaque, analogous to typical turbid plaques made by phage λ or by temperate phages in general (Gill and Hyman, 2010; Guttman et al., 2005).

A variety of antimicrobials are used for treating animals infected with BRD (Fulton, 2009). Previous studies demonstrated that the presence of prophage increased the sensitivity of *M. haemolytica* to a broad-spectrum fluoroquinolone, danofloxacin, and exposure to this antimicrobial induced prophage in *M. haemolytica* (Froshauer et al., 1996). Here we set out to determine if a similar effect occurred for enrofloxacin, another fluoroquinolone antimicrobial compound. Since no enrofloxacin-resistant isolates were detected in our collection of *M. haemolytica* field isolates (Klima et al., 2011), 33 *M. haemolytica* field isolates with high enrofloxacin susceptibility and 33 field isolates with low enrofloxacin susceptibility were selected and the presence of phage in each isolate was determined. Our results showed there was no significant correlation ($P = 0.415$) between presence of inducible phage and enrofloxacin susceptibility in *M. haemolytica*. Both danofloxacin and enrofloxacin inhibit bacterial DNA gyrase (Elkholy et al., 2009; Froshauer et al., 1996), which result in DNA damage during replication. When bacteria activate their SOS system in response to DNA damage, RecA protein is activated, which in turn degrades one of the repressors in the phage lytic pathway and promotes phage induction (Clokie and Kropinski, 2009; Froshauer et al., 1996). Even though the mode of

action is similar for these two antimicrobial compounds, we did not find increased enrofloxacin sensitivity from lysogenic *M. haemolytica* isolates. Further work will be necessary to investigate the relationship of antimicrobial sensitivity and phage induction.

Prophages are prevalent in many widely diverse bacterial species. They can contribute important biological properties to bacterial hosts. For instance, lysogenic conversion of the host can offer protection against further phage infection and increase the virulence of a pathogenic host (Casjens, 2003). To study the evolution of both bacteriophages and their hosts, nucleotide sequence analysis is essential. Complete sequences of fully functional double stranded DNA tailed phage genomes have already been determined from various bacteria (Casjens, 2003). This includes some well-studied *E. coli* phages, such as temperate phage λ and T-series of obligately lytic phages (Guttman et al., 2005). It is often difficult to detect homologies in newly sequenced phage genomes due to the extreme diversity among bacteriophage (Hendrix et al., 1999). Therefore, a newly discovered phage is expected to have novel genes as compared to closely related phages (Casjens, 2003), and approximately half of the sequenced phage genes can be found to be novel during genomic analyses (Hambly and Suttle, 2005). This is not completely in agreement with the phage sequences we obtained, though none of them were identical, some were extremely similar. The sequence similarity can be contributed by extensive genome mosaicism, which is common in closely related phages. This genome mosaicism is characterized by patches of high sequence similarity separated by non-homologous regions (Casjens, 2003; Skurnik et al., 2007), which was evident among the five phage sequences we obtained that closely aligned with bacteriophage φMhaA1-PHL101. Patches of similar phage sequences seem to be prominent and

widespread in our collection of *M. haemolytica*, even though they were induced from different *M. haemolytica* strains with different serotypes and sampling locations. φMH1152A consensus sequence exhibited extremely high sequence identity to φMhaA1-PHL101. It also aligned well with contigs of all three complete *M. haemolytica* genomes currently available in the database. This is expected as homologs between coding regions in *M. haemolytica* PHL213 and *M. haemolytica* serotype A2 ovine and bovine strains share 95% to 99% nucleotide identity (Lawrence et al., 2010a). Although φMH1152A showed patches of very high sequence identity to these *M. haemolytica* genomes, alignments were not always found on the same contig. This was probably due to large-scale inversions and rearrangements that were identified among these *M. haemolytica* genomes (Lawrence et al., 2010a). Overall, the prevalence and sequence similarity from φMH1152A consensus sequence suggests widespread horizontal transfer of genetic material among phages and bacteria (Brüssow and Hendrix, 2002; Casjens, 2003, Hendrix et al., 1999; Skurnik et al., 2007), and similar phages are widely distributed geographically (Hatfull et al., 2006).

In conclusion, the diversity of temperate bacteriophages has been successfully measured and examined from a specific group of *M. haemolytica* strains that are broadly representative of our entire *M. haemolytica* collection. These phages were characterized extensively in terms of morphology, host specificity, genomic diversity, and comparative genomics. Lysogeny is known to be widespread in bacteria and a broad range of temperate bacteriophages was found in a significant portion of *M. haemolytica* strains used in this study. Whole genome sequencing was carried out with eight selected phages and one consensus sequence was constructed which showed high degree of sequence

homology to a previously characterized φMhaA1-PHL101 genome and regions of three *M. haemolytica* bacterial genomes.

CHAPTER FOUR

4.1 Conclusions

The attempt to isolate a strictly lytic bacteriophage specific to *M. haemolytica* in this study was unsuccessful in spite of an intensive and comprehensive screening with various methodological approaches. Several explanations have been suggested earlier as to why this task was difficult to accomplish. Among these, CRISPR-mediated phage resistance seems to be the most probable cause since it has been proven experimentally in other bacterial species and CRISPR sequences have been identified in the *M. haemolytica* genome (Lawrence et al., 2010b). The techniques and knowledge acquired during the lytic bacteriophage work were proven valuable for subsequent temperate bacteriophage characterization. Optimal bacterial growth and plating conditions were crucial in temperate bacteriophage induction and analysis, which were determined by experimenting with different phage isolation and detection methods. Utilization of these techniques in temperate bacteriophage work also confirmed that these procedures were indeed capable of identifying potential lytic bacteriophages from the environment. In addition, these results will provide insight in future experimental design and sampling methods when similar lytic bacteriophage studies are to be conducted. In this study, collected samples constituted a limited geographic distribution, the exact nutrient requirement of the lytic bacteriophage was unclear, and variables such as transport medium, transport time, and processing of raw sample may negatively impact bacteriophage integrity and infectivity. Factors such as these can all have significant influences on successful phage isolation, which should be carefully considered.

Temperate bacteriophages were successfully induced and isolated from a group of

M. haemolytica strains. These temperate phages were extensively characterized, including sequencing of eight selected phages. Although this study only summarizes preliminary sequencing results, further in-depth bioinformatic and genomic analysis will reveal the significance and variations between these temperate bacteriophage genomes, and the molecular biology of temperate bacteriophages in general. These phage genome sequences will provide better understanding of contribution of phage to bacterial pathogenesis and their potential for treating bacterial infections (McGrath et al., 2004). These objectives can be accomplished by identifying sequences of prophage-encoded virulence factors in pathogenic bacteria, and sequences of specific phage-encoded enzymes that can be incorporated in a targeted phage-derived therapeutic (McGrath et al., 2004). The evolutionary history and lifestyle of these phages can be elucidated by performing a detailed comparative genomic analysis (Gill and Hyman, 2010; McGrath et al., 2004), which will also assist in a more consistent taxonomic classification of phages. Additionally, the sequencing information may improve future prophage recognition, annotation, and studies of different types of non-homologous (convergent) gene modules that are used for a particular function by some temperate phages (Casjens, 2003). Accumulation of novel phage sequence information will assist in recognition of conserved phage morphogenetic proteins such as portal proteins or terminases. This will facilitate detection of similar phages from the environment and global analysis of prophage sequences using molecular tools such as DNA array analysis or PCR amplification (Casjens, 2003).

Typically, temperate phages are unfavorable candidates for therapeutic applications due to their abilities to carry out lysogenic conversion and potential

exchange of virulence genes with the target bacterium by generalized transduction (Gill and Hyman, 2010; Johnson et al., 2008; Kutateladze and Adamia, 2010; Skurnik et al., 2007). Nevertheless, temperate phages are often more easily isolated so various alternative strategies have been derived. One approach is to genetically modify temperate phages to become permanently lytic (Goodridge, 2010). An example of this method was accomplished by generating *vir* gene mutants of temperate bacteriophages that were repressed for lysogeny and capable of infecting *Propionibacterium acnes* lysogens (Rapson et al., 2003). Alternatively, temperate phages can be engineered to express bacteria-toxic genes or reduce bacterial pathogenicity during lysogeny (Gill and Hyman, 2010; Schaak, 2003). It should be noted that these virulent derivatives of temperate phage are still considered members of their phage families (Carlson, 2005). The effectiveness of using temperate phages as a therapeutic was determined in two studies where lysogenic phages were isolated from induced cultures of *S. aureus* and successfully used in treatment of *S. aureus* infections in mice (Capparelli et al., 2007; Matsuzaki et al., 2003). In addition, the use of cocktails containing phages of different host ranges may avoid the problems caused by lysogeny, where a lysogen formed by one phage will remain susceptible to a second phage within the cocktail (Gill and Hyman, 2010). As phage genome sequences became available, one can confirm the absence of known toxin and drug-resistance genes or determine the genes encoding lytic enzymes. Genes of interest can then be cloned to create recombinant proteins exhibiting lytic activities, which may be an effective solution to control bacterial infections (Fischetti, 2005; Loessner et al., 1998). Alternatively, identification of potential phage-encoded phage resistance genes in prophage genome sequences of different bacterial species can be used to develop

recombinant phage resistance system against harmful bacteriophages that infect economically important bacteria such as members of the lactic acid bacteria used in fermentation processes (McGrath et al., 2004).

BRD is the leading cause of morbidity and mortality in commercial feedlot cattle and results in substantial financial losses from reduced feed efficiency and carcass quality (Duff and Galyean, 2007). *M. haemolytica* has been implicated as the predominant bacterial pathogen associated with BRD (Duff and Galyean, 2007; Gioia et al., 2006; Griffin et al., 2010). More efficacious methods to control and prevent infection by *M. haemolytica* would have a positive impact on beef feedlot industry in Canada. One possibility lies on the prospect of phage therapy. This study investigated the presence of strictly lytic bacteriophage(s) specific to *M. haemolytica* from nasopharyngeal and water samples, and the diversity of temperate bacteriophages induced from *M. haemolytica*. Even though it is less favourable to utilize temperate phages in phage therapy and as an alternative mitigation strategy for BRD infection, the knowledge acquired from temperate phage research is imperative in understanding interactions between bacteriophage and its host. A wealth of information can be retrieved from the sequenced temperate phage genomes, which will serve as a foundation for future studies such as utilizing lytic components of temperate phage to control bacterial infection.

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